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THE NOVEL APPLICATION OF CHITOSAN FOR THE INTRANASAL DELIVERY OF INSULIN

By Michael Hinchcliffe, BSc (Hons)

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy, December 1996

Abstract

The findings of this project have added to the pool of information reported in the literature regarding the application of the nasal route for the delivery of insulin and other peptide drugs. The preliminary studies reported in this project were apparently the first studies performed to investigate the potential use of chitosan in nasal delivery systems. Nasal delivery systems were investigated in rat and sheep models. The efficacy of chitosan as a nasal absorption enhancer for insulin was compared to that of several other compounds which had been reported in the literature to enhance nasasl drug absorption. Erythrocyte haemolysis studies were also performed to evaluate the membrane damaging effects of the various compounds tested.

The grade of chitosan predominantly used was a medium viscosity glutamate salt (MVCSN) which was 82% deacetylated and had a molecular weight of about 162,000. Other grades of chitosan of similar degree of deacetylation were also investigated for comparison with MVCSN (low viscosity grades of chitosan glutamate (LVCSN) and lactate (CSN lactate), medium viscosity chitosan hydrochloride (CSN HCl) and high viscosity chitosan base (HVCSN)).

The efficacy of chitosan in enhancing the nasal absorption of both insulin and salmon calcitonin, used an an alternative peptide, was demonstrated in rat and sheep models. Nasal insulin delivery systems were extensively investigated in rat and sheep models. In the rat model, insulin / LVCSN formulations at pH ~4 were more effective than formulations at pH ~7 in enhancing intranasal insulin absorption which was assessed indirectly from the degree of hypoglycaemia following dose administration. The reduced absorption in the latter formulation which was in the form of a suspension was attributed to complex formation between insulin and LVCSN. In the rat model, the absorption enhancing efficacy of MVCSN was second only to that of LPC. This was encouraging in view of the severe membrane damaging effects that LPC solutions have been shown to cause. In contrast, chitosan solutions have been shown to be relatively non-toxic to biomembranes. In the sheep model, a formulation incorporating MVCSN was much more effective than a formulation containing LPC in promoting nasal insulin absorption. These differences were attributed to the animal models used to investigate nasal absorption.

The degree of nasal absorption enhancement was improved by increasing the solution concentration of MVCSN until and optimal concentration was attained (approximately 0.5% and 0.35% in rat and sheep models, respectively). Further evaluation of nasal insulin / chitosan formulations in sheep, suggested that the formulation concentration of chitosan was important for its absorption enhancing efficacy and at optimal chitosan concentration nasal insulin absorption was limited by the dose concentration of insulin. In both rat and sheep models, the nasal administration of hypotonic or isotonic formulations of insulin with chitosan did not influence the degree of nasal absorption enhancement attained. However, in rats, a hypertonic formulation was shown to further

improve nasal insulin absorption which was attributed to the combined effects of the chitosan and the increased tonicity of the formulation on the nasal membrane.

The grade of chitosan used in the nasal absorption studies appeared to influence the degree of absorption enhancement obtained. In the rat model there was no difference in the absorption enhancing efficacy of CSN lactate and MVCSN although the performace of HVCSN was marginally reduced. In contrast, in the sheep model, MVCSN was more effective than LVCSN and CSN lacate in enhancing nasal insulin absorption although there was no difference in the performance of MVCSN and CSN HCL.

In studies in the rat, MVCSN was shown to have a transient effect on the permeability of the nasal mucosa to insulin which lasted about 30 minutes. This supports the claims that chitosan is non-damaging to the nasal mucosa. Erythrocyte haemolysis studies showed that MVCSN was non-damaging to rat erythrocyte membranes at concentrations which were higher than the concentrations used in nasal absorption studies. This was encouraging since the other compounds investigated for comparison with chitosan in this project were shown to be potent haemolytic agents at concentrations which were much lower than the concentrations which were effective for nasal absorption enhancement. MVCSN was less damaging to erythrocyte membranes than the other grades of chitosan tested.

This project demonstrated that chitosan enhanced the nasal absorption of insulin in rat and sheep models. In the sheep model the bioavailability of nasal insulin, relative to the subcutaneous route, was generally less than 5%. However, the hypoglycaemia which followed nasal insulin / chitosan dose administration was encouraging and a similar degree of efficacy in humans could be feasible for the therapeutic application of nasal insulin.

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CHAPTER 1

INTRODUCTION

1.1 Background

The dramatic increase in the use of peptides and proteins as therapeutic agents is not only due to biotechnological advancement but also to the greater understanding of their role in physiology and pathology (Banga and Chien, 1988). Many types of peptides and proteins find therapeutic application and several examples are given below:

1) Hormones such as insulin for the treatment of diabetes mellitus, calcitonin for the treatment of osteoporosis and erythropoietin to stimulate erythropoiesis in patients with renal disease.

2) Enzymes, including those of thrombolytic or fibrinolytic activity, such as tissue plasminogen activator and urokinase.

3) Coagulation factors, such as factor VIII used in the treatment of haemophilia A.

4) Lymphokines and cytokines such as interferons, interleukins, tumour necrosis factor and granulocyte colony stimulating factors are used as immunomodulators in cancer therapy and autoimmune diseases.

5) Immunotherapeutic agents for example immunoglobulins and antitoxins used to infer passive immunisation in the treatment of cancer and vaccines such as Hepatitis B and influenza surface antigen preparations used for active immunisation.

(Gesellchen and Santerre, 1991, Kompella and Lee, 1991, Davis, 1992, Smith et al., 1993).

Peptides and proteins may be produced by chemical synthesis but perhaps the greatest advancement has been in genetic engineering or recombinant DNA technology. Recombinant DNA technology not only allows the production of natural peptides and proteins to be optimised and produced on a large scale but also via gene manipulation or chemical modification methods there is the potential to develop and produce drugs having improved potency and bioavailability with reduced immunogenicity (Gesellchen and Santerre, 1991, Smith et al., 1993).

Although the oral route is generally preferred for the administration of drugs, particularly those required in chronic therapies (Lee and Yamamoto, 1990, Smith et al., 1992, Davis, 1992), it is largely non effective for the systemic delivery of most natural peptide and protein drugs (Lee, 1991). The oral bioavailability of peptide and proteins is generally low due to a number of physical and physiological factors which limit their absorption from the gastrointestinal tract. Orally administered drugs must be able to withstand harsh chemical and enzymatic degradation in the gastrointestinal tract. Thus, the enzymatic barrier is one of the main barriers limiting the gastrointestinal absorption of peptides and proteins. Peptide and protein drugs tend to be large molecular weight hydrophilic compounds having a low permeability across the gastrointestinal mucosa and those that are absorbed must avoid the 'first pass' metabolism and clearance predominantly by the liver but also by the gut mucosa (Humphrey, 1986, Lee et al., 1991).

Due to the unsuitability of the oral route, most peptide and protein therapeutic agents must be administered parenterally i.e. by the injection of the dosage form into the body. The main routes for parenteral administration are by intravenous (i.v.), intramuscular (i.m.) or subcutaneous (s.c.) injection (Banerjee et al., 1991). To maintain therapeutic efficacy, peptides and proteins must generally be administered frequently because of their short biological half lives. For acute clinical applications, requiring limited injections, parenteral administration is acceptable. However, for chronic therapy alternative non-parenteral routes which are easily accessible as well as controlled delivery systems would be preferable (Kimura, 1984, Banga and Chien, 1988, Eppstein and Longenecker, 1988, Illum, 1992). Furthermore, chronic injective therapy requires some patient skill for effective self medication and because of the pain of injection, inconvenience and possible infection or immunogenicity associated with the use of some drugs, patient compliance tends to be low with treatments interrupted or irregular (Pontiroli et al., 1989). There are a number of non-parenteral routes other than the oral route which can be exploited for the systemic administration of peptide and protein drugs such as transdermal, ocular, buccal, rectal, vaginal, pulmonary and nasal routes (Siddiqui and Chien, 1987, Banga and Chien, 1988). Of these, the intranasal route is perhaps the most viable and favourable for chronic systemic medication using peptides and polypeptides (Chien and Chang, 1985, Davis, 1986, Longenecker, 1986). Intranasal delivery systems for a number of compounds have been investigated and some of these compounds will be discussed later. The emphasis of the project is the development of a nasal delivery system for insulin which would be a major break-through in the treatment of diabetes mellitus.

1.2 Insulin and Insulin therapy

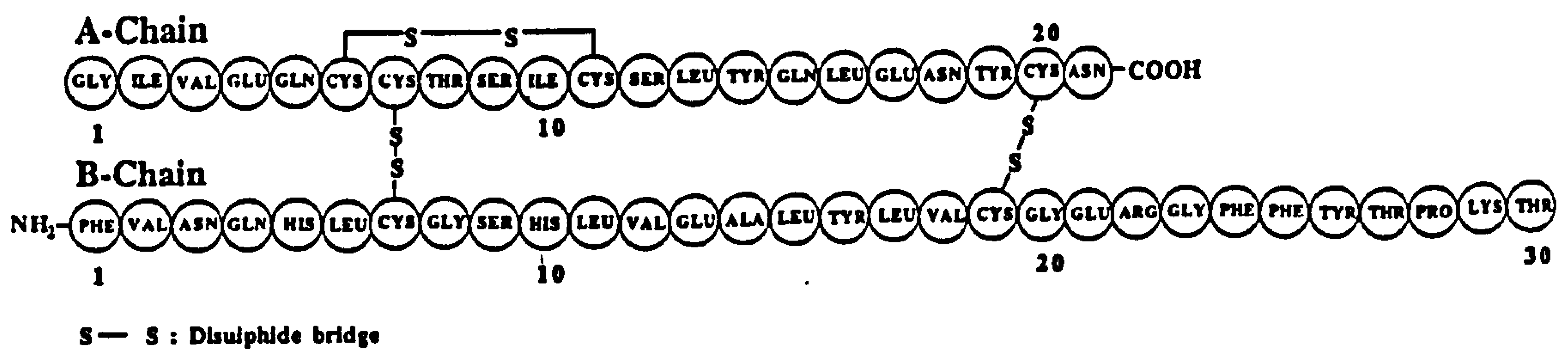
Insulin is the main drug used for the treatment of diabetes mellitus. Current insulin therapy requires that the drug is parenterally injected and hence attention has been focused on alternative routes for the administration of insulin (Gizurarson and Bechgaard, 1991). A brief review of insulin and insulin therapy will be given and reference has been made to publications by: Hardy (1981), Karam et al. (1986), Kelleher (1988), Pickup (1988 and 1991), Espinal (1989), Kahn and Shechter (1990), Gizurarson and Bechgaard (1991), Kennedy (1991), Owens et al. (1991) and Illum and Davis (1992). Additional information regarding insulin therapy is given in Chapter 7.

1.2.1 Chemistry of insulin

Human insulin is a small protein molecule of molecular weight about 5810. It consists of two peptide chains, an A chain containing 21 amino acid residues and a B chain containing 30 amino acid residues, which are covalently linked via disulphide bridges. A disulphide linkage is also present in the A chain. The primary structure of human insulin is given in Figure 1.1. There is a species difference in the exact A and B chain sequence of amino acids. Human and porcine insulin differ by only one amino acid residue. In porcine insulin (molecular weight about 5780) alanine (ALA) replaces threonine (THR) at position 30 of the B chain. Human and bovine insulin differ by three amino acids. In bovine insulin (molecular weight about 5730) ALA and valine (VAL) replace THR and isoleucine (ILE), respectively, at positions 8 and 10 of the A chain and ALA replaces

THR at position 30 of the B chain. Insulin exists as a compact three dimensional structure in which the aliphatic side chains of the A and B chains create a non polar core and hydrophilic residues are exposed on the surface of the molecule in addition to a number of hydrophobic residues. The basic structure of insulin must be preserved in order that it retains its full biological activity and modifications to the basic residues will lead to full or partial loss of biological activity. In solution, insulin exists as a monomer, dimer or hexamer depending on its concentration, pH and the ionic strength of the solution.

Figure 1.1 The primary structure of human insulin



1.2.2 The biosynthesis and secretion of insulin

The biosynthesis of insulin begins when the insulin gene codes for the synthesis of the rapid turnover single chain peptide preproinsulin in the ribosomes of the rough endoplasmic reticulum. Human preproinsulin, of molecular weight about 11,500 contains 110 amino acids. Pancreatic microsomal peptidases rapidly cleave preproinsulin, in the rough endoplasmic reticulum, to the precursor peptide proinsulin. Proinsulin, of molecular weight about 9000, is a single chain peptide containing 86 amino acids. It consists of the insulin A and B chains linked by a 35 amino acid connecting or C-peptide. The proinsulin molecules are transported to and packed within secretory granules in the golgi apparatus. In these granules proinsulin is enzymatically cleaved into insulin and the C-peptide. Proinsulin has a low physiological (insulin-like) activity whilst the C-peptide has no physiological activity. Insulin and the C-peptide are secreted together in equimolar amounts and small amounts of proinsulin and cleaved intermediate peptides will also be present.

Insulin is secreted in the endocrine pancreas by the B cells of the islets of Langerhans. The cells of the islets of Langerhans, are scattered between the acinar cells of the exocrine pancreas, from which they are separated by a thin membrane. The secretions of the pancreatic acinar cells play a major role in digestion. The islets of Langerhans are highly vascularised compared to the acini and are richly innervated by autonomic nerves. Four main cell types can be identified in the islets of Langerhans: A (α) cells comprise about 20% of the islet cell mass and mainly secrete glucagon and proglucagon; B (β) cells, which secrete insulin, C-peptide and proinsulin, make up about 75% of the islet cell

mass; D (δ) cells comprise 3-5% of the islet cell mass and secrete somatostatin; and F cells secrete pancreatic polypeptide and account for less than 2% of the islet cell mass. Insulin is stored, in crystal form, within granules in the islet B cells. Each crystal also contains zinc atoms. The release of insulin from the cell occurs by exocytosis. In normal adults the pancreas contains about 7 mg of insulin and about 2 mg of insulin are secreted daily.

Insulin secretion is central to the regulation of glucose homeostasis. Many nutrient and neurohormonal factors are involved in the regulation of insulin secretion. Secretagogues can directly stimulate the pancreatic islets or potentiate its response to other stimulants. In addition, substrates can inhibit insulin secretion. Secretagogues include glucose, mannose, ribose, leucine, arginine, glucagon, gastrin inhibitory polypeptide, cholecystokinin and drugs such as sulphanylureas. Blood glucose concentration is the main secretagogue stimulating the release of insulin. However, the mechanisms by which the insulin secretory response is activated are unclear although they appear to depend on the intracellular concentration of calcium ions. Insulin is degraded predominantly in the liver, kidney and muscle. About 50% of insulin reaching the liver from the portal vein is degraded before reaching the general circulation. The half-life of circulating insulin in normal subjects is about 3-5 minutes.

1.2.3 The physiological role of insulin

The main endocrine function of insulin is to promote the storage of ingested nutrients by controlling carbohydrate, lipid and protein metabolism. It will indirectly affect every tissue in the body although principally it acts in the tissues which are specialised for energy storage: the liver, muscle and adipose tissue. Insulin and the other pancreatic hormones may also have a paracrine regulatory effect on the surrounding endocrine cells of the pancreas. An example of this paracrine effect is the inhibition of glucagon secretion by insulin, whilst glucagon stimulates insulin secretion.

In the liver insulin regulates a number of metabolic pathways. It promotes anabolism by increasing glucose utilisation via the glycolytic pathway and increasing the synthesis and storage of glycogen from glucose (glycogenesis). Liver glycogen stores are the main source of post absorptive glucose. The synthesis of protein, triglycerides and the storage of very low density proteins is also increased. Insulin also has a number of anti-catabolic effects such as inhibiting the breakdown of stored glycogen (glycogenolysis) and decreasing the catabolism of fatty acids, amino acid, proteins and urea. In muscle, insulin promotes the synthesis of protein and glycogen. However, muscle glycogen stores cannot be used in glucose homeostasis due to the lack of the enzyme glucose-6-phosphatase in muscle tissue. The storage of fat, as triglycerides, in the cytoplasm of adipose cells is a highly efficient means of energy storage compared to carbohydrates or proteins. Insulin promotes the storage of triglycerides in adipose tissue and prevents the lipolysis of stored triglycerides.

1.2.4 Mechanisms of action of insulin

Insulin elucidates its effects via a sequence of reactions which start when insulin binds to

a specific cell surface receptor. The insulin receptor is a membrane bound glycoprotein consisting of two subunits, one which binds insulin and the second which transduces the signals which lead to insulin action. However, the events following the binding of insulin to the cell surface receptors are unclear and discussion of these is beyond the scope of this text. Many of the effects of insulin action, such as nutrient transport, stimulation or inhibition of enzymes, protein phosphorylation or RNA synthesis, occur rapidly, within seconds or minutes. Here it is thought that insulin binds to a specific cell surface receptor. Other effects of insulin may take many hours. These include the synthesis of protein, DNA, or cell growth. These events may be due to insulin binding to insulin-like growth factor receptors.

1.2.5 Commercial sources of insulin

Commercially, insulin is extracted mainly from porcine pancreas and purified by crystallisation. Extractions of insulin from the bovine pancreas are also available but their use commercially has declined in the advent of the large scale production of human sequence insulins which have an amino-acid sequence identical to that of human insulin. Semisynthetic human insulin is produced by the enzyme modification of porcine insulin. Biosynthetic human insulins are produced by recombinant DNA technology in fermentation processes. There are three main methods by which this can be achieved. Human insulin type crb was the first method to be used commercially. Certain strains of bacteria are genetically modified by recombinant DNA technology. The human insulin nucleotides are inserted into the DNA of the bacteria and separate insulin A and B chains are obtained and combined, via disulphide bonding, to produce insulin. Human insulin type prb is produced by the enzymic cleavage of proinsulin which has been obtained from bacteria genetically modified by recombinant DNA technology. Human insulin type pyr is produced from a precursor molecule which has been obtained from a yeast which has been genetically modified by recombinant DNA technology.

Differences in the amino-acid sequence of animal insulins may lead to the formation of insulin antibodies when these preparations are administered to humans. Porcine insulin tends to be much less immunogenic than bovine insulin due to fewer differences from the primary structure of human insulin. Human insulins should be theoretically non-immunogenic although problems may arise due to the presence of contaminants. Unpurified insulin preparations may contain a number of contaminants including large molecular weight material, proinsulin, partially cleaved extraction or insulin bioproducts, insulin derivatives and other pancreatic islet hormones. Although many of these are biologically inert, relative to insulin, they may add to the immunogenicity of the insulin preparation. Insulin preparations can be partly purified by crystallisation methods. Repeated recrystallisation will improve the purity of the insulin. Gel filtration on sephadex gel columns will reduce or limit the contamination with materials of molecular weight higher than that of insulin. Chromatographic methods such as ion exchange chromatography will further reduce contamination. Insulin preparations will usually contain less than 20 parts per million (ppm) proinsulin.

1.2.6 Diabetes mellitus

Diabetes mellitus describes the clinical syndrome characterised by inappropriate elevated blood glucose levels (hyperglycaemia). These arise from a number of disorders rather than from a single disease entity. Hyperglycaemia, thus a disruption of the normal metabolic feedback mechanisms, occurs due to a deficiency in the secretion of the pancreatic hormone insulin, or due to reduced biological effectiveness of the insulin produced or a combination of the two. In the western world diabetes mellitus can be classified into two main types: Type I or insulin-dependent diabetes mellitus (IDDM); and Type II or non-insulin dependent diabetes mellitus (NIDDM). Other forms exist such as malnutrition related diabetes mellitus (MRDM) and those associated with certain other conditions and syndromes.

The onset of IDDM is most commonly in childhood. Links between the disease and genetic factors are not as marked as with NIDDM. Environmental factors in the genetically predisposed individual are thought to play a major role in the development of the disease. These environmental factors include certain viral infections such as mumps, rubella and coxsackie virus B4 in addition to certain toxins which result in the destruction of pancreatic B cells. There is also a strong associations between the disease and an auto-immune reaction in which antibodies are produced to components of the pancreatic B cells, leading to 'self-destruction' of the B cells.

NIDDM is generally a less severe form of diabetes mellitus which predominantly affects middle-aged adults. The majority of patients who develop the disease are obese and this criterion is used to sub-classify NIDDM into obese and non-obese types. In NIDDM patients, there is usually no significant destruction of pancreatic B cells and often normal levels of insulin are secreted. However, NIDDM is usually associated with insulin resistance whereby there is a decrease in the response of peripheral tissues to insulin. The pancreatic B cells appear to lose the ability to respond to the hyperglycaemia resulting from resistance to insulin and hence NIDDM is also associated with insulin deficiency.

In the untreated state, insulin insufficiency is associated with the acute effects of both hyperglycaemia, such as thirst, polyuria and lethargy, and hypoglycaemia, such as sweating, palpitations, nausea, confusion and drowsiness, which may lead to eventual coma and death. In IDDM, insulin administration is essential to maintain normal blood glucose concentrations whereas in NIDDM, the majority of patients can control the disease by regulating their diet or a combination of dietary regulation and oral hypoglycaemic agents such as sulphonylureas which stimulate the release of insulin from pancreatic B cells. Poor diabetic control may lead to the development of chronic complications such as retinopathy, neuropathy and nephropathy.

1.2.7 Current insulin therapy

Insulin may be administered by intramuscular, intravenous or intraperitoneal injection although most commonly it is administered by subcutaneous injection either as a single or as multiple daily injections. Ideally, insulin therapy should mimic the normal pulsatile

patterns of physiological insulin production (Mattews et al., 1987), i.e. provide a constant basal concentration of insulin between meals and a boost of insulin after meals (prandial insulin). Close control of insulin concentrations in the diabetic patient will require a multiple injection regimen and close monitoring of blood glucose concentrations and a strict control of diet. A number of types of insulin are available for subcutaneous administration: short acting, intermediate acting and long acting preparations. The insulin regimen will usually be prescribed depending on the individuals requirements. However, there are many problems associated with subcutaneous insulin treatment such as poor compliance, adverse insulin pharmacokinetics, variable and unpredictable subcutaneous insulin absorption and lack of adequate feed-back control. Thus, the difficulties in achieving a normal physiological profile of insulin by subcutaneous therapy has lead to the investigation of new approaches for insulin delivery such as sustained release implants and non parenteral methods of insulin delivery including nasal administration. Several examples of studies investigating intranasal insulin absorption are given later in the text. The development of safe and effective nasal delivery systems for insulin, or indeed other drugs which must be given parenterally, is an exciting and demanding challenge. Although nasal systems may not totally replace injection regimens, they may lower the frequency of injections, allow greater patient control of blood glucose concentrations, thus preventing chronic complications, and help to improve the lives of millions of diabetics.

1.3 Intranasal administration of peptide and protein drugs

Drugs have often been administered nasally for their local or topical action on the nasal mucosa (Parr, 1983). These include compounds administered for the treatment of nasal allergies, nasal decongestants and anti-infective preparations. Some of the compounds administered for topical treatment have resulted in side-effects due to their absorption into the systemic circulation.

The intranasal route for the systemic delivery of drugs has been shown to be effective for a large number of compounds. Extensive reviews of the many compounds administered intranasally to man and animals, including peptides and polypeptides, have been given by Chien and Chang (1985 and 1987), Chien et al. (1989) and Fisher (1990 and 1994). Examples of some of the compounds which have been administered nasally will be given later in the text. The nasal route has also been implicated as a potential site for the delivery of drugs to the brain (Gopinath et al., 1978).

The nasal route is an ideal alternative to parenteral or oral administration for chronic systemic delivery of therapeutic drugs. The accessibility of the nasal route facilitates self medication thus improving patient compliance compared to parenteral routes (Pontiroli et al., 1989). The nasal cavity has a relatively large absorptive surface area and the high vascularity of the nasal mucosa ensures that absorbed compounds are rapidly removed. Drugs absorbed into the rich network of blood vessels pass directly into the systemic circulation and thus avoid hepato-gastrointestinal first-pass metabolism (Chang and Chien, 1984, Chien and Chang, 1985). Thus, the nasal route offers potential advantage

for the systemic delivery of peptide and polypeptide drugs which are susceptible to degradation by the chemical and enzymatic systems of the gastrointestinal tract or first-pass clearance in the liver. The nasal mucosa has a high permeability compared to other mucosal surfaces (Davis, 1992). The plasma pharmacokinetic profile following intranasal absorption is similar to that obtained by bolus iv injection for many compounds (Illum, 1992). This has implications particularly in drug replacement therapy in that there is the opportunity to design drug dosing regimens to mimic the normal pulsatile secretion pattern of peptides and polypeptides. The efficacy of pulsatile delivery compared to continuous delivery has been demonstrated for a number of peptide and polypeptide hormones such as insulin, human growth hormone and luteinising hormone-releasing hormone (LHRH). Parenteral administration by s.c and i.m. routes, often results in the formation of a drug depot at the site of injection and with slow absorption from the depot tends to result in sustained delivery rather than pulsatile delivery (Eppstein and Longenecker, 1988). In addition to non-parenteral routes for drug delivery, the development of parenteral controlled delivery systems for pulsatile delivery, biofeedback or self-regulated delivery and site-specific or targeted delivery is another expanding field of biomedical research (Banerjee et al., 1991).

Despite the potential of the nasal route, there are a number of factors which limit the intranasal absorption of peptide and protein drugs for systemic delivery. Thus, the bioavailability achieved following intranasal dose administration tends to be uneconomically low compared to parenteral routes and poorly reproducible (Eppstein and Longenecker, 1988, Lee and Yamamoto, 1990). This is reflected in the fact that very few intranasal peptide and protein preparations are currently available for systemic medication. The normal physiology of the nasal cavity presents a number of barriers to peptide and protein drug absorption. These barriers include the physical removal from the site of deposition in the nasal cavity by the mucociliary clearance mechanisms, enzymatic degradation in the mucus layer and nasal epithelium and the selective permeability of the nasal epithelium (Chien and Chang, 1987, Banga and Chang, 1988, Eppstein and Longenecker, 1988, Lee and Yamamoto, 1990, Illum, 1992). The development of nasal drug delivery systems requires an understanding of the structure and function of the nasal cavity, the intranasal barriers which limit peptide and protein absorption and the strategies by which these barriers may be overcome.

The nasal route has also been implicated as a potential site for the delivery of drugs to the brain (Gopinath et al., 1978). The significance of the intranasal delivery of drugs to the brain will be briefly discussed since it will not be considered elsewhere in this text. The free movement of many substances from blood to the cells in the central nervous system is limited by the blood brain barrier. The blood brain barrier is formed by the 'high-resistance tight junctions' between the cells in the brain capillary endothelium (Rubin and Porter, 1989). The blood brain barrier severely restricts the delivery of many therapeutic drugs to the brain, particularly hydrophilic drugs such as peptides and polypeptides. The problems of drug delivery to the brain may possibly be overcome by utilisation of the nasal route. This also has implications as a portal of entry of pathogenic bacteria and

viruses to the central nervous system (Gopinath et al., 1978). It has been suggested that compounds delivered to the brain may be absorbed from the nasal olfactory mucosa into the cerebrospinal fluid (CSF) occupying the perineural space. The CSF in the perineural space is continuous with that in the subarachnoid space surrounding the brain and the cerebral ventricles. The CSF in the subarachnoid space is separated from the brain by a fragile membrane, the *pia mater*, which unlike the blood brain barrier does not constitute a significant barrier to absorption. Absorption into the CSF may occur through the perineural sheath, a connective tissue sheath which follows the path of the olfactory nerve fibres from the perineural space to the olfactory submucosa. It has also been suggested that absorption from the olfactory mucosa to the brain may be via olfactory neurones which enter the olfactory bulb in the cranial cavity (Faber, 1937, Yoffey and Courtice, 1956, Gopinath et al., 1978, Sakane et al., 1994).

The potential of the nasal route for the delivery of compounds to the brain has been demonstrated using a number of compounds such as an aqueous solution of potassium ferrocyanide / iron ammonium citrate (Faber, 1937), colloidal gold particles (Gopinath et al., 1978), progesterone (Kumar et al., 1982), taurine (Lindquist et al., 1983), hydrophilic and lipophilic muramyl peptides (Fogler et al., 1985), wheat germ agglutinin conjugated to horseradish peroxidase (Shipley, 1985), vasotocin (Pavel, 1986), aluminium lactate (Perl and Good, 1987), 3,3-bis(4-pyridylmethyl)-1-phenylindolin-2-one (Hussain et al, 1989) and sulphisomidine (Sakane et al., 1994). Sakane et al. (1995) demonstrated a relationship between drug molecular weight and its direct absorption from the nasal cavity to the CSF, in rats, using fluorescein isothiocyanate-labelled dextran. For dextrans up to a molecular weight of at least 20000 Da, intranasal absorption into the CSF was shown to decrease as molecular weight increased. Nasal absorption of compounds, such as dyes, proteins and vaccinia virus, into the lymphatic system via the respiratory and olfactory nasal mucosa has also been reported (Yoffey and Drinker, 1938a, Yoffey et al., 1938b, Yoffey and Sullivan, 1939, Yoffey and Courtice, 1956). The lymphatic system eventually drains into the venous system and thus offers an indirect route for the entry compounds into the systemic circulation. However, it is the respiratory region of the nasal cavity which is important for the systemic delivery of drugs administered intranasally.

1.4 Structure and function of the adult human nasal cavity

The nasal cavity is the main airway by which ambient air enters the body. Nasal breathing is of extreme importance since it functions to condition inspired air before passing to the delicate alveolar tissue in the lungs, a function which will be lost or impaired if totally reliant on breathing via the mouth. Inspired air is conditioned in the nasal cavity by cleaning, warming and humidifying and the structure of the nasal cavity is well adapted to these functions. Many sources of reference have been used during this review of the structure and function of the adult human nasal cavity including: Proctor et al. (1973), Mygind (1978 and 1993), Berglund and Lindvall (1982), Cole (1982), Proctor (1982 and 1985), Tos (1982), Widdicome and Wells (1982), Proctor and Chang (1983), Schreider (1986), Olsson and Bende (1986), Chien and Chang, (1987), Sleigh et

al. (1988), Chien et al. (1989), Su, (1991), O'Donoghue et al. (1992), Fawcett and Raviola (1994). Additional reference sources are given in the text.

1.4.1 The gross anatomy of the adult human nasal cavity

The nasal cavity is located mainly within the skull although projects, in-part, anteriorly into the external nose which consists of a bone and cartilaginous skeleton covered by connective tissue and skin. The nasal septum, composed of cartilage and bone, divides the nasal cavity into two approximately equal halves. Each half of the cavity begins anteriorly at the nares or nostrils of the external nose and extends, approximately 10-14 cm, posteriorly to the nasopharynx. At the nasopharynx, the septum ends and two halves of the airway join together. Each half of the nasal cavity has a medial wall, formed by the nasal septum, a roof, a floor, which is parallel with the hard palate, and a lateral (outer side) wall which is formed by the bones and cartilage skeleton of the skull. A midline- and cross-section of the adult human nasal cavity is shown in Figures 1.2 and 1.3, respectively.

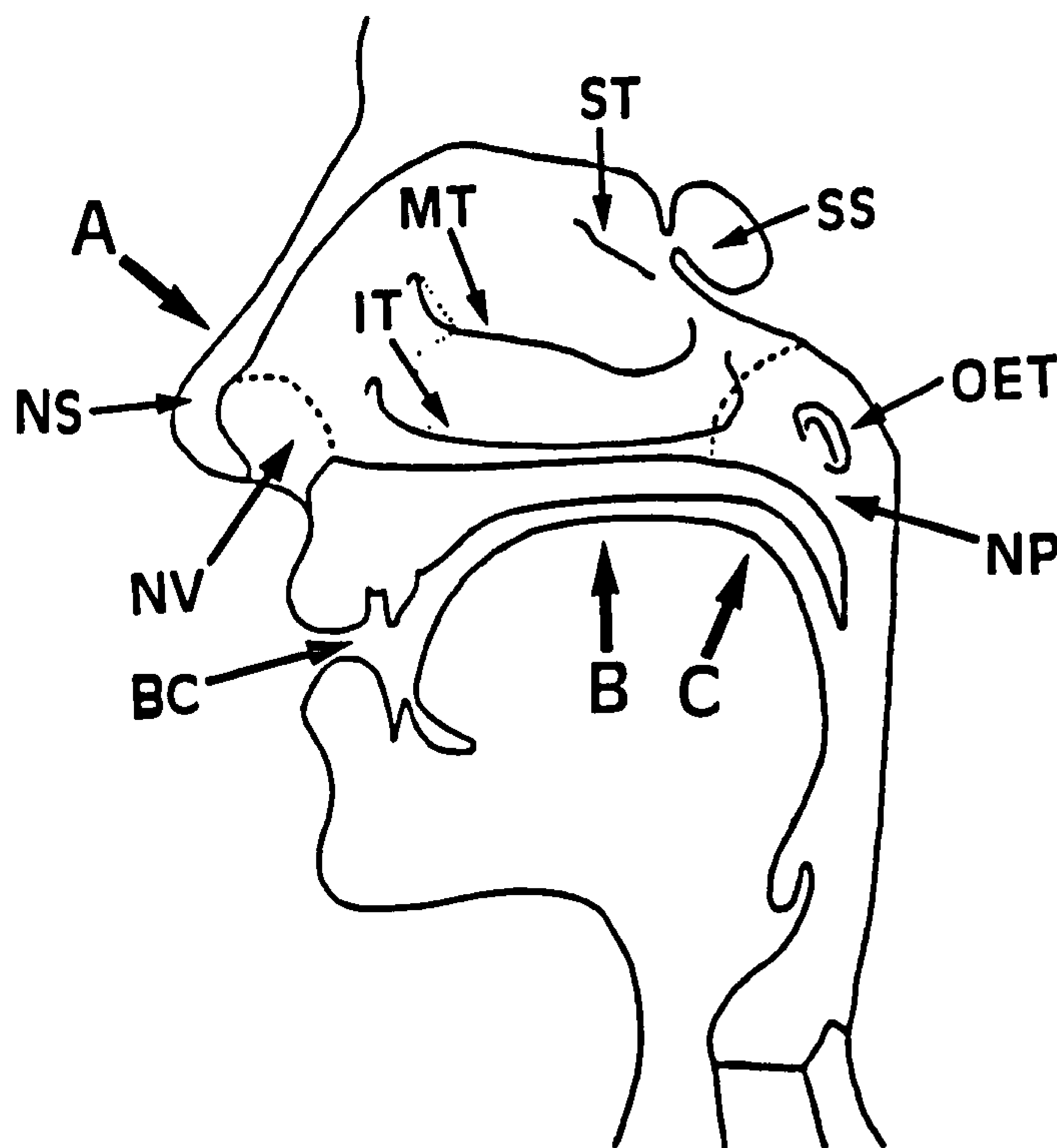
The nasal cavity can be divided into three functional regions: the vestibular-, the respiratory- and the olfactory regions. The anterior part of the vestibular region begins just inside each of the nares and terminates at a ridge of cartilage called the internal ostium or nasal valve approximately 1.5-2 cm from the nares. Beyond the internal ostium lies the main part of the nasal cavity in which the respiratory and olfactory regions are located. The total volume of the nasal cavity is approximately 15-20 cm^3 with a total surface area of about 150 cm^2. The vestibule has a surface area of about 20 cm^2 and a cross-sectional area which narrows from about 0.9 cm^2 to 0.3 cm^2 at the internal ostium which is the narrowest part of the nasal cavity. Each side of the main nasal cavity extends about 5 cm from the floor to the roof and approximately 10 cm from the internal ostium to the nasopharynx. The width of the cavity varies between 0.1-0.3 cm, due to the presence of the turbinates which are described below, and has a cross-sectional area of about 0.14 cm^2. The surface area of the main nasal cavity is about 130 cm^2 of which about 10-20 cm^2 is occupied by the olfactory region and the remainder is occupied by the respiratory region.

In the main nasal cavity, bony scroll-like inward projections in the lateral wall form the turbinates or conchae. Each of the turbinates is attached at the upper end to the lateral wall and projects downwards in the cavity. The turbinates, which are covered in soft spongy tissue, increase the surface area of the cavity. It is because of the turbinates that the lateral wall is often described as folded or uneven although the remainder of the wall is fairly flat. The scroll-like passages or spaces formed by the overhanging turbinates, between the turbinate and the lateral wall, are called meatuses. From the main nasal airway, the meatuses extend laterally and upwards and then medially to form a simple scroll. There are three turbinates termed the inferior-, middle- and superior turbinates each having a corresponding meatus. The inferior turbinate, the largest of the three turbinates, lies just above the nasal floor and begins at the internal ostium. The middle

turbinate is situated further into the nasal cavity and the superior turbinate is located deep in the nasal cavity.

The main nasal airway extends upwards from the floor of the nasal cavity to the middle turbinate and from the nasal ostium to the posterior of the turbinates. The passage above the middle turbinate in which the olfactory region is located and the meatuses are generally free of inspirational air flow. Air filled cavities within the bones of the skull, referred to as the paranasal sinuses, open into the nasal cavity. The paranasal sinuses are evaginations in the bones of the skull surrounding the nasal cavity which are lined with mucosa which is continuous and identical with that lining the nasal cavity. This can have important implications during nasal infections which may spread to the sinuses resulting in localised pain or headache. The sinuses consist of paired maxillary and frontal sinuses, a labyrinth of about 20 ethmoidal sinuses and a number of sphenoidal sinuses which open directly or indirectly into the nasal cavity. In addition a palatine sinus may also be present. The nasolacrimal duct (tear duct) opens into the anterior of the inferior meatus.

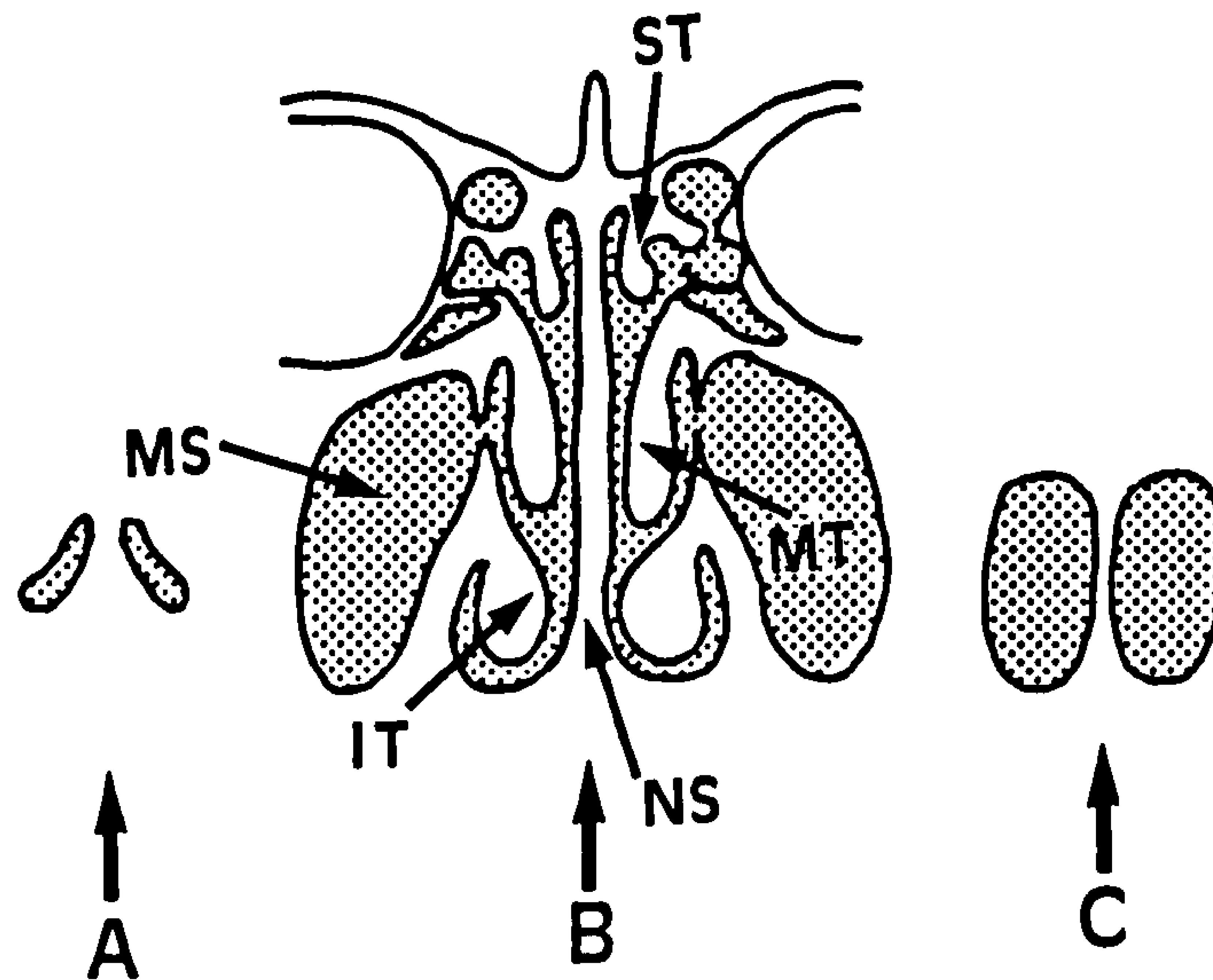
Figure 1.2. Midline-section (lateral wall) of the adult human nasal cavity
Adapted from Mygind (1978) and Proctor (1982)



BC. Buccal cavity, NV. nasal vestibule, NS. nasal septum, IT. inferior turbinate, MT. middle turbinate, ST. superior turbinate, SS. sphenoidal sinus, OET. orifice of eustacean tube, NP. nasopharynx. Cross-sections through the nasal cavity at positions A, B and C are shown in Figure 1.3

Figure 1.3. Cross-section of the adult human nasal cavity

Adapted from Mygind (1978)



NS. nasal septum, IT. inferior turbinate, MT. middle turbinate, ST. superior turbinate, MS. maxillary sinus. Cross-sections through the nasal cavity: A. Internal osteum, B. Middle of nasal cavity, C. Posterior of nasal cavity

The anatomy of the nasal cavity is well adapted to the function of conditioning inspired air. The slit-like nasal airway (1-5 mm wide) is narrowest at the internal osteum and widest at the nasopharynx. At the internal osteum the high resistance to airflow and the relatively high linear velocity of the air stream (12-18 m/sec compared to about 1 m/sec in the trachea) combined with the bending of the air stream (almost a 90° bend) and turbulence facilitates the impaction of the majority of particles carried in the inspired air stream in the anterior of the nasal cavity from where they are mainly removed by mucociliary clearance. In the main part of the nasal cavity the cross-sectional area of the nasal cavity increases and the linear velocity of the air stream is reduced. The narrowness of the airway, the increased surface area combined with the reduced air velocity optimise the contact between the inspired air and the nasal mucosa where air is warmed and humidified and gases and vapours are absorbed. In the posterior of the nasal cavity, the air stream bends about 90° as it passes to the nasopharynx and the air stream increases in velocity resulting in the impaction of particles in the posterior of the nasal cavity from where they are removed by mucociliary clearance.

1.4.2 Nasal mucosa

The nasal vestibule is lined with stratified squamous epithelia which is continuous with the facial skin and is in-part keratinised particularly close to the nares. Long stiff hairs or vibrissae, sebaceous glands and sweat glands are abundant in the anterior portion of the vestibule. The vibrissae play a defensive role by filtering large particles from inspired air. Posteriorly, the vibrissae and sebaceous / sweat glands become sparse and a

transitional band of non-ciliated cuboidal or columnar epithelium replaces the stratified squamous epithelium and extends into the anterior of the main nasal cavity. Ducts from anterior serous cells located in the respiratory mucous open in the upper part of the vestibule and release watery or serous fluid secretions. The main nasal cavity is lined with mucous membrane which is continuous with that of the paranasal sinuses, nasopharynx and nasolacrimal ducts. The mucous membrane is composed of two layers, an epithelium and a tunica propria separated by a basement membrane, and is anchored to the underlying bony or cartilagenous skeleton. The respiratory and olfactory regions of the main nasal cavity have respiratory and olfactory mucosa, respectively.

1.4.2.1 Olfactory mucosa

The olfactory mucosa is a specialised region of the nasal cavity which contains the receptors for the sense of smell. The olfactory mucosa is confined to the roof of the nasal cavity and the upper part of the nasal septum. The epithelial cells are columnar pseudostratified which tend to be taller than those of the respiratory mucosa and of which there are three main types: olfactory cells, sustentacular cells and basal cells. The olfactory cells are the receptor cells for the sense of smell. These cells are bipolar neurones which are distributed between the sustentacular or supporting cells. The apex of the bipolar neurone is tapered into a dendritic process which extends to the epithelial surface and terminates in a bulbous expansion called the olfactory bulb or knob which lies above the apical epithelial surface. The olfactory bulb contains numerous basal bodies from which non-motile cilia extend and lie parallel to the apical epithelial surface. These cilia, except at the base, do not have the typical 9 + 2 axonemal structure and tend to be much narrower and longer than typical cilia. These modified cilia increase the surface area of the receptor cell. Pinocytotic vesicles are present on the apical surface of the olfactory cells. The base of the olfactory cell tapers to form an un-myelinated nerve axon which extends just below the basal lamina into the connective tissue and joins with neighbouring axons to form small bundles of nerve fibres which become surrounded by Schwann cells. These ensheathed bundles, known as *fila olfactoria*, join with other bundles to form the olfactory nerves which penetrate the bones of the skull in the roof of the nasal cavity before passing into and synapsing in the olfactory bulb of the brain (Gopinath et al., 1978).

The sustentacular cells are rich in organelles and are thought to provide both metabolic and physical support to the olfactory cells. At the apical surface of the sustentacular cells, microvilli project into the mucus and pinocytotic vesicles are present. Basal cells lie close to the basal lamina between the olfactory and sustentacular cells into which basal cells are are able to differentiate. Processes from the basal cells sheath the olfactory cell axons for a small portion of its length. Brush cells having large microvilli on the apical surface may also be present in small numbers. These may be involved in sensory reception and in-particular the sneeze reflex.

Underlying the olfactory mucosa and separated by a basal lamina, the lamina propria consists of connective tissue with subepithelial pigment and lymphoid cells, glands,

vascular and nerve tissue. The branched tubuloalveolar olfactory glands of Bowman continually secrete a serous fluid on to the epithelial surface via ducts. Air-borne odorants dissolve in this serous fluid prior to detection by the epithelial receptor cells. There is a rich sub-epithelial capillary plexus and a deeper lying venous plexus and lymphatic system although the vasculature of the olfactory mucosa is not as rich as in the respiratory mucosa. Trigeminal nerve fibres are present along with the olfactory nerves.

1.4.2.2 Respiratory mucosa

The respiratory mucosa lines most of the nasal cavity and resembles that of the trachea and bronchi. The respiratory epithelium is mainly a ciliated columnar pseudostratified epithelium but stratified cuboidal or simple cuboidal epithelia may also be present particularly over the more exposed regions such as parts of the turbinates. The respiratory epithelium is composed of five main cell types: ciliated columnar cells, non-ciliated columnar cells, goblet cells, basal cells and brush cells. Ciliated columnar cells are the most abundant cell type and occur in groups which are interspersed with non-ciliated cells. Goblet cells which secrete mucus glycoproteins, are abundantly but unevenly distributed in the respiratory epithelium. The ratio of ciliated cells to goblet cells is approximately 5:1. The density of goblet cells is lower on the nasal septum than the turbinates and the distribution is lower on the middle turbinate than the inferior turbinate. The distribution in the mucosa of the paranasal sinuses is similar to that on the septum. The density of goblet cells tend to increase in an anterior to posterior direction on the septum and turbinates. Basal cells are undifferentiated intermediate cells which can mature into columnar or goblet cell types. Unlike the other cell types, these cells do not reach the apical surface of the epithelium. Brush cells have a brush border of microvilli up to 2 μm long and may have an absorptive function in the regulation of the fluid balance in the mucus layer. Microvilli are abundant on the apical surface of both ciliated and non-ciliated cells. Up to 500 microvilli each about 1-2 μm long and 0.1 μm in diameter may be found. Macrophages, leucocytes and clusters of lymphocytes may also migrate throughout the epithelium.

1.4.3 Junctional complexes between epithelial cells

Epithelial cells in the nasal mucosa are generally in close apposition and adjacent cells are extremely adherent. Cohesion at the apices of epithelial cells occurs in specialised regions collectively referred to as the junctional complex. The junctional complex consists of three regions: zonula occludens, zonula adherens and macula adherens and under light microscopy appears as a dark band or terminal bar near the apex of the epithelial cells.

The zonula occludens is often referred to as the tight junction and is the region of the junctional complex which is closest to the apical cell surface. The zonula occludens is a zone or band which circumferentially extends round the lateral cell membrane. The lateral membrane between adjacent cells appear to converge and fuse at close intervals. The molecular nature of these bands is not fully understood and the literature tends to be controversial. The zonula occludens controls the diffusion of ions and molecules between cells and constitutes a barrier to the movement of macromolecules across the

epithelium by this route. The number of sites of fusion in the tight junction determines the permeability of the epithelium by the paracellular route. Leaky epithelia, present in some kidney tubules, contain few sites of contact whereas intestinal epithelia contains many sites and is highly impermeable. The human nasal epithelia has been described as non-uniform by Inagaki et al. (1985) with connections of goblet-goblet and goblet-ciliated cells being weak, thus the epithelium is relatively 'leaky', compared to the connections of ciliated-ciliated cells.

In contrast to the zonula occludens, the zonula adherens and the macula adherens do not constitute a barrier to the movement of molecules between cells and are both cell adhesion devices. The zonula adherens lie just below the zonula occludens and consist of a circumferential, adhesive filled, space between adjacent lateral cell membranes. The macula adherens is typically located just below the zonula adherens and forms a discontinuous band of disc shaped plaques around the lateral membrane. The intercellular space is filled with an adhesive material. Filaments from both the zonula adherens and the macula adherens extend into the cytoplasm and anchor the cytoskeleton to the cell surface. An additional junctional specialisation between lateral cell membranes is the gap junction where the intercellular space is reduced to narrow channels through which metabolites and ions can diffuse between cells and in this way function in the electrical coupling of cells throughout the epithelium

1.4.4 The basal lamina and lamina propria

The basal lamina (basement membrane) separates the epithelium from the underlying lamina propria and consists of two layers; a low density lamina lucida adjacent to the epithelium and an underlying and more dense lamina densa. The basal lamina consists of collagen fibres, glycoproteins and proteoglycans. The collagen in the lamina densa is cross-linked forming a layer which is flexible but has high tensile strength. Collagen fibres also anchor the lamina densa to the collagen fibres of the lamina propria. The basal lamina supports the epithelial layer. Large molecules crossing the epithelium may be passively filtered on the basis of size, shape (mainly due to the network of collagen fibres) and electrostatic charge (mainly due to the proteoglycan heparan sulphate which carries a strong anionic charge).

The lamina propria (tunica propria) underlying the basal lamina anchors the mucosa to the underlying skeletal structures and in addition provides support to the mucosa. The lamina propria consists of a loose type connective tissue of extracellular ground substance with moderately abundant and loosely interwoven collagen fibres and proteoglycan and related substances. The ground substance is a hydrated gel consisting mainly of water, proteoglycans (chondroitin sulphates, heparin sulphate, heparin, keratan sulphate and hyaluronic acid) and electrolytes. Embedded in the connective tissue are glands, subepithelial cells, vascular and nervous tissue. There are numerous branched and tubuloalveolar glands regularly distributed throughout the connective tissue layer of the respiratory mucosa. These discharge their mucus or serous secretions onto the surface of the nasal vestibule (anterior serous nasal gland) or respiratory epithelium (seromucous

glands) via short ducts. The paranasal sinuses contain much fewer glands which are mainly confined round the orifices of the sinus. The main mucosa of the paranasal sinuses is almost devoid of glands. Subepithelial cells consist of fibroblasts and fibrocytes, which secrete extracellular material (fibrils and ground substance), histiocytes which contain very active substances such as hydrolytic enzymes in cytoplasmic vesicles (lysosomes). Macrophages, leucocytes, lymphocytes, plasma cells and mast cells have also been identified in the connective tissue of the nasal mucosa.

1.4.5 Vasculature of the nasal cavity

The nasal mucosa, particularly that of the respiratory region, is highly vascular, having an extensive blood supply and lymphatic drainage system. The main blood supply is via branches of the maxillary artery and facial artery arising from the external carotid artery and the ophthalmic artery derived from the internal carotid artery. Branches of the major nasal arteries anastomose in the basal regions of the lamina propria forming a deep network or plexus. Branches from the arterial plexus ascend through the mucosa giving rise to arterioles which terminating in a subepithelial capillary plexus just below the epithelial layer or supplies the subepithelial glands and tissues. Blood drains from the respiratory mucosa into venous plexuses. Fine networks of small vessels tend to be located in the superficial regions of the lamina propria whereas networks of larger vessels tend to be located in the deeper layers. The venous plexuses drain into larger veins in the anterior and posterior of the nasal cavity. Rich superficial venous or cavernous plexuses consisting of tortuous networks of large anastomosing veins are abundant over the regions of the inferior and middle turbinates and over part of the septum. The venous plexuses, especially the cavernous plexuses and others which are located in the regions of main inspirational air flow, also receive blood directly from the arteries via arteriovenous anastamoses. Arteriovenous anastamoses enable blood to be shunted to the venous system thus by-passing the capillary network and therefore are extremely important for the regional regulation of blood flow due to mechanical, thermal, psychological or chemical stimulation. The plexus has the ability to engorge with blood resulting from vasoconstriction of the deeper veins and vasodilation of arterioles which supply the plexus via the capillary networks. The engorgement of the plexus with blood may lead to a swelling of the mucosa which can temporarily occlude the airway and make the tissue appear erectile. This occlusion of the airways is thought to occur cyclically and alternately between the two sides of the nasal cavity as a function to prevent the drying-out of the mucous membrane on any one side. Nasal veins do not have valves and thus there is a postural variation in blood pressure and flow dynamics.

Relatively large capillaries adjacent to the basal lamina and smaller capillaries around the glands contain pores or fenestrations, each closed by a very thin pore diaphragm, between the peripheral endothelial cells. The fenestrations facilitate rapid fluid transport processes in the nasal mucosa. Other small non-fenestrated capillaries are also present in the lamina propria and these capillaries tend not to be associated with the epithelial surface or glands. The superficial capillary network and those supplying the glands join to form venules which drain into larger superficial veins. The endothelial basal lamina of the

superficial nasal blood vessels is characteristically porous. This renders the arterioles and venules particularly susceptible to vasoactive agents carried in the blood since they lack an internal elastic membrane and thus the endothelial basal lamina is continuous with the basal lamina of the underlying smooth muscle cells. The superficial fenestrated capillaries supply the mucous layer covering the epithelium with moisture for the humidification of inspired air. The superficial venous networks especially the erectile cavernous plexuses in the mucosa enables inspired air to be heated before passing to the delicate tissues of the lower respiratory tract. Blood tends to flow from the rear of the cavity countering the flow of inspired air and thus provides rapid and efficient heat exchange. The regional blood flow in the cavernous sinusoids can be controlled in terms of flow speed and volume to provide efficient temperature conditioning of inspired air.

The lymph vessels also form a rich plexus in the respiratory mucosa. The lymph vessels of the respiratory region of the nasal cavity predominantly drain into upper deep cervical lymphatic vessels and into upper deep cervical and retropharyngeal lymph nodes before entering veins at the base of the neck. The lymph vessels absorb and thus drain away the tissue fluid which passes through the capillary walls and which collects in the tissue spaces of the nasal mucosa. The removal of tissue fluid ensures that the airway is kept clear.

1.4.6 Nervous innervation of the respiratory mucosa

The nerve supply to the respiratory mucosa is via an integration of sensory and motor nerves of the somatic and autonomic branches of the peripheral nervous system. The trigeminal nerve is the major sensory nerve of the facial region and branches of its fibres are distributed throughout the mucous membranes of the nasal cavity and the paranasal sinuses. The trigeminal nerve is composed of mainly somatic sensory fibres with a general somatosensory function (receive sensory information from the mucosa of the nasal cavity and paranasal sinuses). Autonomic parasympathetic and sympathetic fibres provide motor innervation of the nasal mucosa. The integration of sensory, secretomotor and vasomotor functions enables the main functions of the nasal cavity, i.e. conditioning inspired air by warming, humidifying and cleaning, to be performed.

Various reflexes are associated with the nasal mucosa. Since man is generally regarded as an obligate nasal breather then some of these reflexes function to protect the lower respiratory tract from irritants. Stimulation of sensory receptors, usually nerve endings, in the nasal mucosa give rise to sensory sensations which via the central nervous system elicits a response reaction in the nasal mucosa. This may involve sneezing or allergic reactions to sensory irritants for example pollutant gases such as sulphur dioxide and inert dust which may cause localised pain or discomfort sensations. However, the respiratory mucosa in humans has limited somatosensory function and has poor capacity for the localisation and discrimination of sensory stimuli. The respiratory mucosa will respond to mechanical and chemical stimuli but lacks thermoreceptors so does not respond to hot or cold air. Sensory stimulation as a result of breathing unconditioned air, resulting in secretomotor and vasomotor reflexes via parasympathetic and sympathetic

pathways, is suggested to be via thermoreceptors in the nasal vestibule (Cauna, 1982). Exercise also initiate a nasal reflex by decreasing nasal resistance to air flow thus facilitating nasal breathing to meet the demand for increased respiration. This occurs by graded vasoconstriction of nasal blood vessels.

The nasal blood vasculature receives vasomotor innervation from both sympathetic and parasympathetic nerves. The nerves form a plexus of nonmyelinated nerve fibres in the lamina propria. Sympathetic nervous innervation causes vasoconstriction whereas parasympathetic stimulation results in vasodilation. Normally, vessels of the cavernous plexuses are kept partially constricted by constant sympathetic stimulation. In contrast, vessels are not continually innervated by parasympathetic stimulation. As previously mentioned, the vasomotor tone can result in rapid changes not only in the volume and speed of blood flow in the nasal mucosa but also airflow through the nasal cavity. When the venus plexuses are engorged with blood then airflow through the nasal cavity is reduced. Secretomotor innervation of nasal glands is via parasympathetic nerves and there is no direct sympathetic innervation. However, glandular secretion is influenced by their blood supply and thus indirectly affected by sympathetic fibres.

1.4.7 Nasal mucus secretion

A thin layer of clear nasal fluid or mucus forms a continuous cover over the surface of the nasal mucosa. Mucus is a mixture of secretions derived from a number of sources. These include mucous secretions from goblet cells and subepithelial mucous glands, serous secretions from subepithelial serous glands, secretions from lacrimal glands (tears), tissue fluid transudate and condensed water from expired air. Normal mucus is hypertonic compared to plasma and has a pH between about 5.5-6.5. Mucus is composed of 95-97 % water with 2.5-3 % mucus glycoproteins or mucins, and the remainder consists of electrolytes (up to about 2%), proteins and other macromolecules. Sloughed cells and micro-organisms may also be present as well as lipids and DNA which may be derived from damaged cells. Water movement across the nasal epithelium into the nasal lumen is thought to occur by passive diffusion as a result of the osmotic gradient set-up by active ion transport. The hypertonicity of mucus is probably due to active ion transport in combination with water evaporation from the mucosal surface.

Mucus glycoproteins constitute 70-80% of the dry-weight of mucus and are composed of a single polypeptide chain to which are attached sugar side chains. Mucus glycoproteins may be acidic or neutral in nature. Acid glycoproteins are predominate in human mucus and contain sialic acid and/or sulphate groups. Neutral glycoproteins contain large amounts of fucose but may also contain small amounts of sialic acid. The glycoproteins give mucus characteristic rheological (visco-elastic) properties which are related to its function of providing a protecting coating to the nasal epithelium and facilitating mucociliary clearance of materials deposited in the nasal cavity.

The protein content of mucus is in the form of immunoglobulins, albumin, lactoferrin, lysozyme and other enzymes and protease inhibitors. IgA is the major immunoglobulin

in nasal mucus and accounts for up to 50% of the total protein content. The immunoglobulins and nasal immunology are mentioned later. Lactoferrin, an iron binding protein, and the enzyme lysozyme (muraminidase) may have antimicrobial activity against many airborne microbes (Rodwell, 1988). Lysozyme has optimal activity at slightly acidic pH and thus is suited to the normal pH of the nasal cavity (Tachibana et al., 1986). However, lysozyme activity may be reduced during cases of acute common cold, acute rhinitis or acute sinusitis where nasal pH is often alkaline. Lysozyme and proteolytic enzymes such as acid hydrolases and peroxidases present predominantly in the lysosomal granules of macrophages and neutrophils are able to digest phagocytosed foreign antigenic material. In addition, these and other cells may discharge the contents of their granules outside of the cell and directly into mucus and these may act in breaking down materials for example inflammatory products which are not ingested and in the activation of chemical mediators and proenzymes (Hamaguchi et al., 1986, Roitt et al., 1987). Hochstrasser (1983) suggests that proteolytic mucus lysosome enzymes are inactivated and compensated by the antiproteolytic capacity of normal nasal mucus. It was reported that in cases of common cold, the number of granulocytes, mainly neutrophils infiltrating nasal secretions was increased and there was a corresponding increase in the amount of neutrophilic lysosomal enzymes present in mucus (Hochstrasser, 1983). However, the trypsin-like protease activity of these enzymes did not increase due to the increase in serum antiproteases such as α_1-antityrpsin. This was thought to be a protective mechanism to prevent irreversible, protease induced damage to the nasal mucosa (Hamaguchi et al., 1986). Amylase, an enzyme which hydrolyses starch and glycogen, has been demonstrated in the secretory granules of submucosal serous glands and has been speculated to be involved in controlling secretion fluidity (Tachibana et al., 1986b).

1.4.8 Nasal enzymes

The nasal mucosa contains a variety of enzymes which are present in nasal secretions, on the surface of or in the cytosol of epithelial cells, where they may be free or membrane bound, and in the lamina propria where they are particularly associated with glandular tissue. Much of the literature relates to studies in animals although Sarkar (1992) considered that despite inter-species variations, the profile of nasal drug metabolising enzymes in humans will be similar. The enzymes identified in nasal mucosa include monooxygenases, reductases, transferases, esterases and proteases (Irwin et al., 1995).

Monooxygenase enzymes include cytochrome P-450-dependent monooxygenases which are particularly abundant in the olfactory region and may function in the biotransformation and removal of odorant compounds thus ensuring that the acuity of olfaction does not decrease due to the build-up of a high background odour (Dahl et al., 1982). The cytochrome P-450-dependent monooxygenases are thought to be important as a first line defensive mechanism against inhaled xenobiotics, particularly environmental pollutants, volatile chemicals and odorous chemicals, which are biotransformed into nontoxic compounds which may then be excreted (Hadley and Dahl, 1983). However, biotransformations of some inhaled compounds into chemically

reactive metabolites have been implicated as a cause of nasal lesions or tumours in animal species (Bond, 1986, Brittebo et al., 1986). Mucociliary clearance and swallowing of chemically reactive metabolites in mucus may also have implications in the induction of tumours in the alimentary tract as well as in the upper respiratory tract (Dahl et al., 1985). Studies by Bond et al. (1988) indicate that there is an uneven distribution of xenobiotic metabolising enzymes along the respiratory tract and presumes that the balance of activating and inactivating enzymes will determine whether a compound will be carcinogenic in a particular tissue.

Various types of proteolytic enzymes are present in nasal mucosal membranes and are also a constituent of nasal secretions and are mentioned later in the text. However, these have not been isolated and characterised in the nasal cavity and their presence is based on studies with model peptide drugs such as enkephalins, substance P, insulin and proinsulin (Lee and Yamamoto, 1990). Proteolytic enzymes may exhibit exopeptidase or endopeptidase activity. Exopeptidases cleave the terminal N or C bonds in peptide chains and include aminopeptidases and angiotensin-converting enzyme (dipeptidyl peptidase and dipeptidyl carboxypeptidase). Endopeptidases such as endopeptidase-24.11 (neutral endopeptidase) cleave the internal peptide bonds (Kashi and Lee, 1986, Lee and Yamamoto, 1990). Aminopeptidases are the principal proteolytic enzymes in the nasal cavity (Audus and Tavakoli-Saberi, 1991). Stratford and Lee (1986) identified a number of aminopeptidases in nasal mucosa homogenates in the albino rabbit. Aminopeptidase N and A were shown to be membrane bound whereas aminopeptidase B was a cytosolic enzyme. Leucine aminopeptidase and lysosomal aminopeptidase were also identified. Compared to various other mucosae (buccal, rectal and vaginal) the overall subcellular distribution of aminopeptidases in the nasal mucosa are the most similar to those in ileal mucosa. Membrane bound aminopeptidase activity accounted for almost half of the total enzyme activity compared to 20% in ileal mucosa and 80% in buccal, rectal and vaginal mucosa (Lee and Yamamoto, 1990, Illum, 1992). However, the subcellular distribution of aminopeptidases in different mucosae will vary depending on the peptide substrate used in the aminopeptidase assay (Irwin et al., 1995). The evolutionary development of proteolytic enzymes in the nasal cavity is probably a protective mechanism against the invasion of foreign peptidagenous and proteinaceous materials (Hussain et al., 1990).

1.4.9 Nasal immunology

Large numbers of microorganisms enter the nasal cavity either with inspired air or via direct finger contact with the nostril. Rhinoviruses, which are responsible for the common cold may also reach the nasal cavity via the nasolacrimal duct (Mygind and Winther, 1987). The relatively thin mucosal lining covering most of the nasal cavity constitutes a weak mechanical barrier to the entry of potentially pathogenic microbes and protection against most infectious agents is provided by a number of innate and adaptive immune mechanisms (Weir, 1977, Brandtzaeg, 1988). Innate immune mechanisms are non-specific to the microbial antigen and in most instances will be sufficient to prevent manifestation of the disease. These include the physical barrier of the nasal mucosa, mucociliary clearance, anti-microbial agents (e.g. lysozyme, protease enzymes) and

phagocytic cells (predominantly macrophages and neutrophils). A number of soluble factors such as complement and interferon are also involved (Weir, 1977, Mygind and Winther, 1987). If innate immune mechanisms are breached then an antigen specific adaptive immune response such as immunoglobulin (antibody) production and cell-mediated immune mechanisms via activated lymphocytes will be initiated (Mygind and Winther, 1987). Nasal associated lymphoreticular tissue (NALT) is located in the submucosa in the nasopharyngeal area (tonsils) and is ideally positioned to encounter environmental antigens (McGhee and Kiyono; 1993). NALT represents an inductive site for the adaptive mucosal immune system in the upper respiratory tract and hence possibilities exist to exploit NALT in the development of vaccines. Studies in rats indicate that of the airway associated lymphoid tissue, NALT may be the major IgA inductive site.

IgA is the major immunoglobulin in nasal mucus. There are two types of IgA, one an IgA monomer (7S monomer) passes directly into nasal secretions from the blood and the second and predominant type, secretory IgA (mainly an 11S dimer containing a joining or J chain), is secreted locally in the nasal mucosa. The IgA's can bind to and neutralise viral antigens thus preventing their colonisation of the nasal mucosa. IgG may also be present in nasal mucus the source of which appears to be from the blood although subepithelial cells containing IgG have been demonstrated. IgD, IgE and IgM have been detected in mucus as a result of transudation or local production. IgE producing cells and mucosal mast cells containing IgE may be present in allergic individuals.

1.4.10 Mucociliary transport

Mucociliary transport is the movement of mucus by cilia. Cilia are fine, contractile projections on the apical surface of the columnar epithelial cells and there are approximately 200-300 cilia per cell each cilium being between 5-10 μm long and 0.1-0.3 μm wide. The respiratory cilia have the characteristic 9 plus 2 axonemal structure. The density of ciliated cells increases posteriorly in the nasal cavity. Mucus consists of two fluid layers: a viscid gel layer (mucus or epiphase) floats on a less viscid sol layer (periciliary fluid layer or hypophase) immediately adjacent to the epithelial surface. Mucus is not present as a uniform sheet but forms droplets, flakes and plaques. Mucus is transported by the symmetric beating action of cilia. During the cyclic beating of the cilia, contact of the ciliary tips with the viscid gel layer is sufficient to generate a propulsive force on the mucus. During mucus transport the gel layer is propelled whereas the sol layer remains stationary. The synchronous beating of cilia in the nasal mucosa generates metachronal waves over the surface of the nasal mucosa. In patches or fields of mucosa, the cilia beat frequency and direction of beat may vary, although the cilia in each patch will display metachrony. Ciliated regions of mucosa are also interspersed will non-ciliated cells. Mucus is transported by a combination of several metachronal waves which overcomes any irregularities or variations in the beat frequency or direction between the patches of cilia and allows the continued transport of mucus over non-ciliated regions. Although the overall transport of mucus is generally towards the nasopharynx, the pattern of transport of mucus droplets, flakes or plaques across the

surface of the nasal mucosa may exhibit numerous directional changes due to regional variations in the frequency and direction of each metachronal field.

The nasal cilia beat frequency (CBF) is about 10 Hz (range 4.5 to 11.9 Hz), based on *in vitro* studies using nasal biopsies obtained from healthy volunteers (Duchateau et al., 1985). Measurements were performed at 30°C. However, in the same study the average temperature in the inferior turbinate of 6 healthy subjects was found to range between 29.2-34.2°C and thus CBF may increase posteriorly in the nasal cavity with increasing temperature of the mucosal surface in this direction. Mucus is continually transported, at about 5 mm per minute, over the surface of the respiratory mucosa. The depth of the sol layer must be regulated to ensure efficient mucus transport and transport rates will be reduced if the sol layer becomes too deep or too shallow. Microvilli, on the epithelial cell surface, may contribute to the overall fluid regulation in the mucus layers via transport mechanisms. In the nasal cavity, the mucus layer is generally renewed every 15 minutes by transport posteriorly towards the nasopharynx, from where it is removed into the stomach by swallowing. In addition, in the anterior regions of the inferior turbinates, mucus may also be transported in an anterior direction towards the nares and removed as it drains into the vestibular region of the nasal cavity by blowing or wiping the nose.

Mucociliary transport is an important physiological defence mechanism referred to as mucociliary clearance. The majority of particles and gases which are inhaled via the nose are removed before passing to other parts of the respiratory system. Any particles or gases which adhere to or dissolve in the mucus layer will be cleared from the nasal cavity by mucociliary transport. However, less soluble gases and small particles may evade the mucociliary clearance mechanism and penetrate further into the respiratory tract.

1.5 Animal models for intranasal drug absorption studies

A prerequisite for the development of nasal delivery systems, for testing in human clinical trials is that they are first evaluated and optimised in animal models (Ritschel, 1987). Ideally, nasal drug absorption in the animal model will correlate with nasal absorption in humans. Thus, results from animal studies can be reliably extrapolated to humans. However, the results obtained from absorption studies will often be influenced by the animal model employed and extrapolation to absorption in humans should be approached with caution. Nevertheless, animal models are invaluable in pharmaceutical research, not only for preclinical evaluation of new drug compounds but also for optimising the various formulation factors which will influence drug absorption. Various animal models have been used in intranasal drug absorption studies using species such as mice, rats, guinea pigs, rabbits, dogs, sheep and monkeys (Chien et al., 1989, Gizurarson, 1990, Illum, 1995). Attention will be focused on the rat and sheep nasal models which have been extensively utilised throughout this project. However, comparative characteristics of the nasal cavities of man and several animal species are given in Table 1.1. Reviews of the animal models used in intranasal drug absorption studies, to which reference has been made in the following text, have been given by Chien et al (1989), Gizurarson (1990) and Illum (1995).

1.5.1 The rat model

The rat is widely used in intranasal drug delivery studies. Rats are relatively inexpensive animals which are easy to maintain, handle and use experimentally. They breed easily and reach maturity quickly and hence tend to be readily available commercially. Several strains of rat are available and they are usually used in the weight range 200-300 g (4-5 months old). The structure of the rat nasal cavity will be briefly discussed before describing the various experimental rat models which have been employed for nasal drug delivery studies.

1.5.1.1 Structure of the rat nasal cavity

The description of the rat nasal cavity is based upon Hebel and Stromberg (1976), Schreider and Raabe (1980), Gross et al., (1982), Proctor and Chang (1983), Schreider (1986), Young (1986), Popp et al., (1986), Fisher (1990) and Uraih and Maronpot (1990). A midline section of the rat nasal cavity is shown in Figure 1.4. The rat nasal cavity is incompletely separated by the nasal septum, in the posterior ventral region, allowing the two halves of the nasal cavity to join at the beginning of the nasopharyngeal duct leading to the nasopharynx.

In the main part of the nasal cavity two pairs of turbinates project from the lateral wall of the nasal cavity. The nasoturbinate projects ventrally from the roof of the nasal cavity leaving the dorsal meatus between the roof and nasoturbinate which extends the length of the cavity. The maxilloturbinate projects dorsally in the ventral region of the nasal cavity. Between the floor of the nasal cavity and the maxilloturbinate is the ventral meatus which is the main passage for inspired air and extends to the nasopharyngeal duct. A middle meatus is positioned between the naso- and maxilloturbinates. The turbinates, particularly the nasoturbinate, have a hook-like arrangement with a laterally pointing curve. In the posterior of the nasal cavity is the ethmoid recess, lying ventral to the posterior of the dorsal meatus, which contains a complex arrangement of turbinates referred to as endoturbinates (positioned near to the nasal septum) and ectoturbinates (positioned near to the lateral wall). The dorsal, middle and ventral meatus communicate medially at the common meatus which extends along the length of the nasal cavity between the nasal septum and the turbinate bones. Folds of accessory cartilage, referred to as the atrioturbinates, project from the vestibule to the naso- and maxilloturbinate and these separate the common meatus from the ventral, middle and dorsal meatus in the anterior of the respiratory region although there is communication between the two via a slit between the cartilages which diminishes posteriorly. Paired paranasal sinuses are present in the rat although the literature contains conflicting reports of their number and size. Ethmoid, frontal, sphenoid and maxillary sinuses have been reported. A feature not found in humans is the vomeronasal organ of Jacobson consisting of paired blind epithelial tubes running along the ventral region of the nasal septum from the vestibule, to which it communicates via a short lateral duct, to the nasopalatine duct (incisive canal) which connects the nasal and oral cavities. The function of the vomeronasal organ is not clear although it may be involved in olfaction.

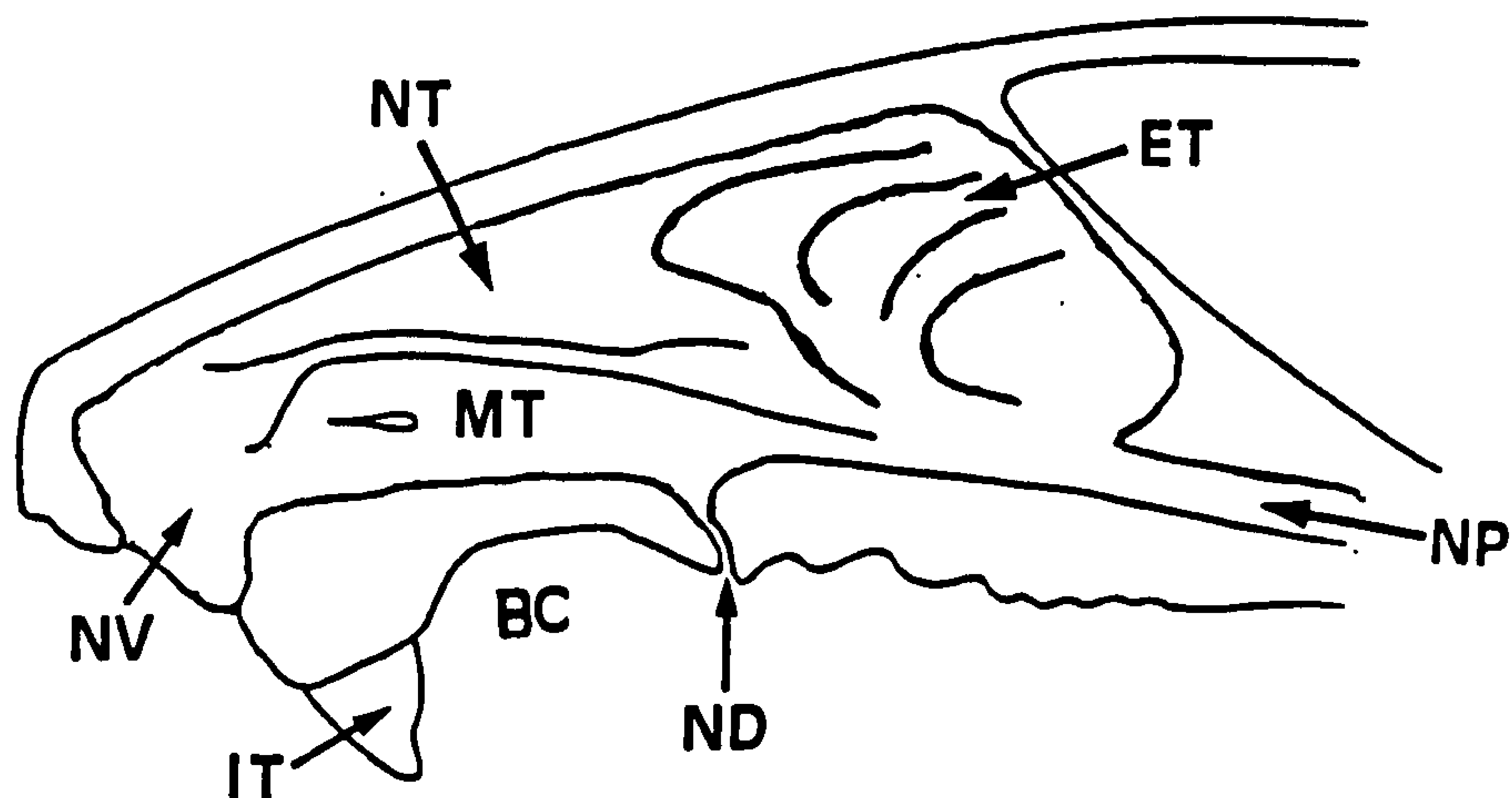
The mucosa lining the rat nasal cavity contains three distinct types of epithelium: stratified squamous, respiratory and olfactory. The proportion of the total nasal surface area occupied by the various epithelia types was determined by Gross et al. (1982) using two groups of rats, weighing 115 g and 288 g. The percentage composition of the epithelial types were 3-4 % stratified squamous epithelium, 44-47 % respiratory and 50-52 % olfactory. Simple squamous stratified epithelium, which is in-part keratinised, lines the vestibule, the anterior of the naso- and maxilloturbinate regions and the floor of the ventral meatus up to the nasopalatine duct. Some vibrissae may be present originating from hair follicles in the underlying lamina propria. Generally, in contrast to humans, the lamina propria consisting of a simple collagenous matrix is devoid of hair follicles and glands. However, ducts originating from glands underlying the anterior respiratory epithelium, particularly the nasal septum, discharge serous secretions into the vestibule.

Respiratory epithelium occupies much of the turbinates, nasal septum, the anterior part of the ethmoid recess and the maxillary sinus. Exceptions are the upper posterior region of the dorsal meatus. The lateral walls of the vomeronasal organ are also lined with respiratory mucosa. Five types of cell are found in the respiratory epithelium: ciliated, non-ciliated, goblet, basal and brush cells. The lamina propria of the respiratory region contains a rich vascular system, abundant tubuloalveolar serous and mucous glands and nerve endings originating from the trigeminal nerve which regulate vascular tone and glandular secretions. The lateral wall between the naso- and maxilloturbinates contains large venous sinuses referred to as swell bodies. Smaller venous sinuses are also found in the region of the maxilloturbinates and the ventral region of the nasal septum. The distension and collapse of the swell bodies give the nasal mucosa erectile properties and regulates the pattern of air-flow in the cavity depending on factors such as temperature, humidity and carbon dioxide concentration in inspired air. A cyclical alteration in air conductivity in the rat nasal passages (nasal cycle), similar to that in the human nasal passage, has been demonstrated by Bolsen-Møller and Fahrenkrug (1971). Olfactory mucosa lines most of the ethmoid recess including the exo- and endoturbinates and the upper posterior region of the dorsal meatus. A small isolated patch of specialised olfactory mucosa, referred to as the Septal organ of Rodolfo-Masera, is located in the ventral region of the nasal septum posterior to the vomeronasal organ. This is thought to be part of a chemosensitive alert system and may also function in monitoring inspired air for odours. The vomeronasal organ is also lined medially with specialised olfactory epithelia. The olfactory epithlium contains bipolar olfactory cells, columnar sustentacular cells and basal cells.

The structure of the rat nasal cavity is generally more complex than that of the human nasal cavity due to the complexity of the nasal turbinates. However, the epithelial structure is similar in the human and the rat nasal cavity although the proportion of respiratory and olfactory epithelia differ in the two species. In humans, the olfactory epithelium occupies about 10% of the total surface area of the nasal cavity compared to about 50% in rats. These differences in structure and function of the rat nasal cavity reflects the greater importance of olfaction in the rat. The absolute dimensions of the rat

nasal cavity are much smaller than in humans which is obvious from the size difference between the two species. However, if the surface area of the nasal cavity is corrected for body weight or total body area, the nasal surface area in the rat is between about 10-40 or 3-7 times larger, respectively, than the surface area of the human nasal cavity. Again, these dimensions reflect the greater importance of olfaction in the rat.

Figure 1.4. Midline-section (lateral wall) of the rat nasal cavity
Adapted from Fisher (1990)



NV. nasal vestibule, NT. nasoturbinate, MT. maxilloturbinate, ET. ethmoidturbinates, NP. nasopharynx, ND. nasopalatine duct, BC. buccal cavity, IT. incisor tooth.

1.5.1.2 Rat models for intranasal drug delivery studies

Two main types of experimental nasal rat model are generally described in the literature, the *in situ* (nasal perfusion) model, the *in vivo* (surgical) model.

The *in situ*, nasal perfusion, model was described by Hirai et al. (1981a). The technique is performed under terminal anaesthesia with the rat usually positioned ventral surface up. The trachea is cannulated, using plastic tubing, to maintain the patency of the airway during the experimental period. A second cannula is inserted into the oesophagus and positioned at the posterior of the nasal cavity. The nasopalatine duct is sealed, usually using a cyanoacrylate adhesive, to prevent drainage of the drug solution from the nasal cavity into the oral cavity. The oesophageal cannula is connected to a reservoir containing the drug solution and is continually stirred. The drug solution will usually be maintained at 37°C. A peristaltic pump is used to circulate drug solution from the reservoir and through the nasal cavity. The perfused drug solution passes out of the nostrils and is recollected in the reservoir via a funnel. The drug solution will be continually recirculated through the rat nasal cavity and a sample of the perfusate will be periodically taken from the reservoir and analysed for drug content. The amount of drug absorbed is evaluated by measuring the disappearance of drug from the perfusate. However, the disappearance of drug from the perfusate does not necessarily indicate nasal absorption since drug instability, e.g. degradation of peptide and protein drugs by

nasal proteolytic enzymes, and aggregation of the drug compound may reduce the concentrations of drug in the perfusate. Perfusate drug concentrations may also decrease due to systematic losses such as adsorption to cannula tubing (Gibson and Olanoff, 1987). Evaporation from the drug reservoir may also be problematic with this technique. Use of the *in situ* model allows the evaluation of drug absorption as a function of time. However, both the rate of perfusion and the perfusate volume have been shown to influence the rate of drug absorption and so must be carefully controlled (Hussain et al.,1985, Huang et al., 1985). The rate of absorption of phenobarbital was shown to increase as the perfusion rate increased (between 1.0-2.0 ml/min) although was independent of perfusion rate above 2 ml/min. A linear relationship was shown to exist between the *in situ* rate constants of absorption and the reciprocal of the volume of perfusate and the authors showed that the rate constants agreed well with rate constants calculated from *in vivo* absorption studies.

The best indication of drug absorption into the systemic circulation is from the direct measurement of drug concentrations in the blood requiring periodic blood sampling. Alternatively, the appropriate biological response evoked by the drug may be monitored for example hypoglycaemia or hypocalcaemia following insulin and calcitonin absorption, respectively. In addition, the measurement of drug concentrations in samples other than blood, such as urine, may be used as an indication of absorption. Measurement of the biological response may be useful for screening the efficacy of absorption from nasal delivery systems if an assay method for the drug is not available or is complicated,time consuming and expensive. The *in situ* model has also been used to investigate the degradation of drugs by nasal enzymatic systems. Examples of the use of the *in situ* model will be given later in the text.

The *in vivo* surgical model, also performed under terminal anaesthesia, was also described by Hirai et al. (1981a). Rats are positioned ventral surface up, the trachea and oesophagus are cannulated and the nasopalatine duct is sealed as previously described for the preparation of the *in situ* model. The oesophageal cannula was then sealed using cotton and adhesive to prevent the drainage of the nasal dose into the oesophagus and stomach. Dose solution is usually administered to the nasal cavity via one nostril, using a micropipette, and the nostril sealed with adhesive to prevent dose drainage from the nasal cavity. The *in vivo* method of dose administration is more comparable to dose administration in humans in contrast to the perfusion technique. Intranasal absorption is usually assessed by analysing blood samples collected periodically from the caudal (tail) vein, femoral artery / vein, carotid artery or the dorsal aorta.

Fisher et al. (1985) simplified the *in vivo* model and found that the results obtained were comparable to those obtained using the model described by Hirai et al. (1981a). In the simplified model, the oesophagus was not cannulated but was occluded by tying it to the tracheal cannula and neither the nasopalatine duct nor the nostrils were sealed. However, considerable drainage of the dose into the trachea was observed if the nasopalatine duct and nostrils were not sealed and the animals were positioned ventral surface down. A

simplified rat model has also been reported by Lau-Cam et al. (1991) which did not require surgery and hence was suggested to be a less stressful and more physiological means of investigating intranasal drug absorption. The absolute bioavailability of propranolol administered via the nasal route (100 μl volume) was shown to be very similar in the model to that obtained following surgical preparation of animals as described by Hirai et al. (1981a) and Fisher et al. (1985). However, as rats are obligate nasal breathers then the air flow through the nasal cavity may influence dose distribution and therefore the area for absorption (Chandler, 1994). This may have important implications when drugs are used which are absorbed to a lesser extent than propranolol or smaller dose volumes are administered. Mayor and Illum (1994) have shown that the use of anaesthetic agents, particularly long acting anaesthetics, such as pentobarbitone sodium, resulted in the increased nasal absorption of insulin in rats. This was suggested to be mainly due to impaired mucociliary clearance in anaesthetised rats. Drainage and mechanical removal of dose from the nasal cavity will also be prevented by anaesthesia. A reduction in the rate of mucociliary clearance in the rat trachea by anaesthesia has previously been demonstrated (Patrick and Stirling, 1977). Anaesthesia has also been shown to decrease the mucociliary clearance rate in sheep trachea (Landa et al., 1975).

Dose volume is an important consideration in nasal absorption studies and most often dose volumes between 20-50 μl will be administered. Chandler et al. (1991a) investigated the effect of dose volume on dose distribution in the rat nasal cavity using histological techniques. Doses of 20, 50 or 100 μl of 1% polyoxyethylene-9-lauryl ether (Laureth-9) solution, an agent known to cause damage to epithelial membranes, were administered to one nostril and tissues were fixed for histological examination after 5, 20 or 60 minutes. The tissue damaging effects of a 100 μl dose of Laureth-9 were observed on both sides of the nasal cavity after 5 minutes although the damage was more severe on the dosed side. Tissue damage, especially on the undosed side, was increased with increasing contact time. Following contact for 5 minutes, the 50 μl dose severely damaged the dosed side of the nasal cavity but there was only slight damage around the septum on the undosed side. However, damage to the undosed side of the nasal cavity increased with increased contact time. In contrast, damage to the nasal tissue induced by a 20 μl dose was confined to the dosed side of the nasal cavity irrespective of contact time. Thus, dose volume should be carefully controlled during nasal absorption experiments particularly when comparing formulations. Investigation of nasal absorption from powder delivery systems is difficult in the rat, compared to larger species, due to the narrowness of the nares and the difficulty in accurately administering small quantities of powder. However, Bjork and Edman (1988) investigated the nasal absorption of insulin from lyophilised powder formulations in the rat administering about 1 mg of powder.

1.5.2 The sheep model

In biomedical research, the sheep is commonly used as an animal model although relatively few studies employing the sheep as an animal model for intranasal absorption studies have been reported. An important feature of the sheep model for intranasal

absorption studies is that the animals are conscious, although sheep may be sedated during dose administration, and hence normal patterns of mucociliary clearance should not be disrupted. Thus, compared to small animal models, nasal absorption in the sheep model will allow better prediction of nasal absorption in humans. The manageable size of the sheep combined with their passive and tolerant nature enable them to be easily handled in nasal absorption studies. They are generally readily obtainable throughout the year at the specific weights required. The large nostril size of the sheep, enables both liquid and powder nasal delivery systems to be evaluated and doses comparable to those administered to humans can be given to sheep. Chien et al. (1989) suggests that the sheep model is suitable and practical for evaluating parameters involved in the nasal delivery of drug from a more sophisticated formulation. The paired external jugular veins which pass down the neck on either side of the trachea are easy to locate, and can be readily cannulated by non surgical procedures for the purpose of serial blood sampling. Sheep can be repeatedly used in drug absorption studies provided an adequate wash-out period is allowed between successive dose administrations. The washout period should take into account the pharmacokinetic profile of the drug and replenishment of blood volumes in sheep

1.5.2.1 Structure of the sheep nasal cavity

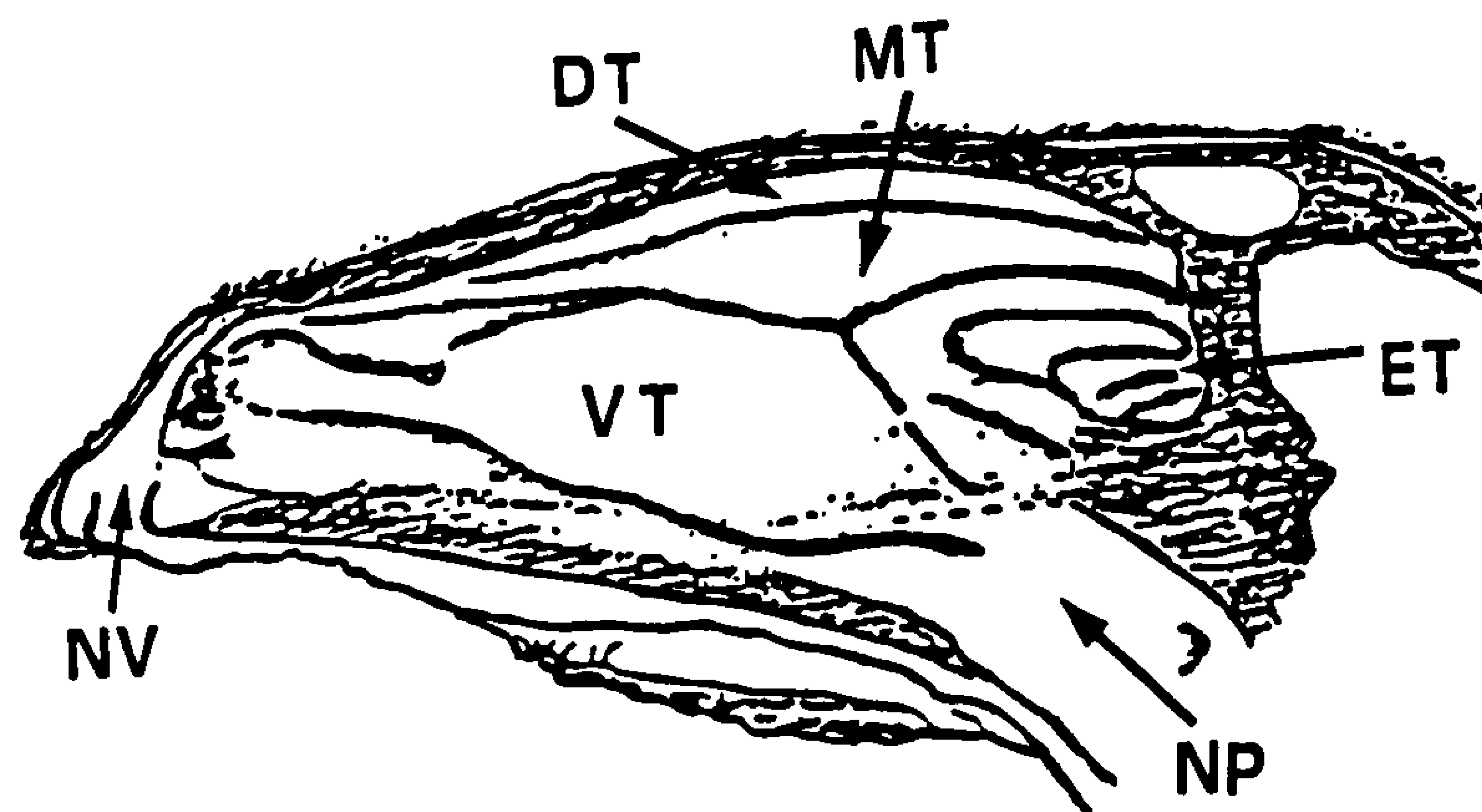
The description of the sheep nasal cavity is based upon May (1964) and Hare (1975). A midline section of the sheep nasal cavity is shown in Figure 1.5. The nasal cavity of the sheep is pyramidal shape with the apex located in the anterior of the cavity. The nasal septum incompletely separates the nasal cavity in the posterior, ventral region of the cavity, although the separation may be completed by a thick fold of the mucosal lining, extending from a plate of the septal cartilage, which contains a rich submucosal venous plexus. If the separation is incomplete, the two sides of the cavity will join to form a common, nasopharyngeal meatus at the floor of the nasal cavity close to the nasopharynx. Two major scroll-like turbinate bones, the dorsal turbinate and the more complex ventral turbinate, project inwards from the lateral wall and extend, antero-posteriorly, across most of the nasal cavity. The ventral turbinate bone occupies most of the lateral wall of the cavity and is separated into upper and lower regions (ventral and dorsal plates), which are arranged in coils. A series of smaller ethmo-turbinates are located in the superior part of the nasal cavity and the largest of these, at the posterior of the superior region, is the middle turbinate.

There are three major meatuses in the nasal cavity created by the turbinates. The dorsal meatus, between the dorsal turbinate and the cavity roof, is long and narrow and extends the whole length of the nasal bone. The ventral meatus, between the cavity floor and the ventral turbinate. Between the dorsal and ventral turbinates is the middle meatus which is divided, posteriorly into dorsal (olfactory) and ventral (respiratory) parts by the middle turbinate. The ventral meatus is the largest and least restricted of the passages providing a direct communication between the nares and the nasopharynx. Ethmoidal meatuses, small, narrow, passages between the ethmoid bones in the postero-ventral part of the nasal cavity, are also found. The three meatuses communicate medially at the common

meatus, a narrow slit-like passage extending between the floor and roof and between the septum and turbinates. The common meatus is narrow dorsally and widens ventrally. The vomeronasal organ consisting of two blind-tubes, about 7 cm long and 0.3-0.4 cm wide, are positioned on either side of the nasal septum, at the level of the nasal floor, and enter the oral cavity. There are five paranasal sinuses in sheep (frontal, maxillary, palatine, sphenoid and ethmoidal) which are embedded in the various bones of the skull. Each sinus is connected, either directly or indirectly, to the nasal cavity. The mucosa of these sinuses is continuous with that of the nasal mucosa.

Stratified squamous epithelia containing numerous serous glands lines the vestibule just inside the nares. In the main part of the nasal cavity there is a transition from stratified squamous epithelia to pseudostratified columnar epithelium. The reddish coloured mucosa of the respiratory region, containing an abundance of mucus secreting goblet cells and numerous subepithelial tubuloalveolar glands which are mainly serous in nature, occupies the majority of the nasal cavity. The postero-superior regions of the nasal cavity, the ethmoturbinates, dorsal turbinate and adjacent region of the nasal septum are lined with the yellow coloured mucosa of the olfactory region containing olfactory cells (bipolar neurones), sustentacular cells and basal cells. There are numerous serous secreting tubuloalveolar glands and also sensory nerve endings. The nasal mucosa, particularly that of the respiratory region, has a rich vascular system with abundant small arteries, veins, capillary plexuses and venous plexuses.

<u>Figure 1.5. Midline-section (lateral wall) of the sheep nasal cavity</u>
Adapted from Gizurarson (1990)



NV. nasal vestibule, NT. dorsal turbinate, MT. middle turbinate, ET. ethmoidal turbinates, VT. ventral turbinate, NP. nasopharynx,

The nasal cavity of the sheep is more complex than that of humans due to the complexity of the nasal turbinates. However, as in the rat, the epithelial structure of the sheep nasal cavity is similar to that in humans. The absolute dimensions of the sheep nasal cavity are much larger than in humans which is probably related to a function of the nasal cavity in

sheep for the dissipation of heat through through water vaporisation.

1.5.2.2 The sheep model for intranasal absorption studies

Sheep models have been used and described by Longecker et al. (1987) and Illum et al. (1989). Sheep are usually used in the conscious state although a sedative may be administered immediately prior to dose administration to facilitate the handling of the sheep and to prevent or limit sneezing during dose administration. A low dose of ketamine, administered intravenously, has been used to induce sedation and typically the sedative effects of the ketamine last about three minutes. Although long acting anaesthetics have been shown to reduce mucociliary clearance in sheep (Landa et al., 1975) sedation should have very little overall effect on mucociliary clearance.

Cannulation of the jugular vein allows serial blood samples to be collected (Hecker, 1983) and intravenous ketamine for induction of anaesthesia to be administered. Since the jugular veins are bilateral, intravenous drug doses, for reference purposes, may be administered into one jugular vein and blood samples collected from the contralateral jugular vein. Jugular vein cannulae may remain indwelling in sheep for several weeks provided that their patency is maintained by regularly flushing in with an isotonic solution containing anticoagulant (typically heparinised normal saline). This will allow cross-over studies to be performed. Intranasal doses may be administered using various devices which should be inserted to a depth of about 7-10 cm into the sheep nostril to allow doses to be administered to the main part of the nasal cavity. The sheep provides a convenient model to investigate nasal delivery systems prior to their evaluation in human clinical trials.

Table 1.1. Comparative characteristics of the nasal cavity of man and animal species

Species [Weight (kg)]	Nasal volume (ml)	Surface area (cm^2)	Length (cm)	Bend to nasopharynx	Turbinate complexity	Clearance half-life (min)	Equivalent nasal volume (µl) *
Man [70]	20	160	7.5	90°	Single scroll	15	150
Rat [0.25]	0.4	14	2.3	15°	Double scroll	5	13
Sheep [60]	114	327	18	30°	Double scroll	42	307
Dog [10]	20	221	10	30°	Branching (complex)	20	207
Monkey [7]	8	62	5.3	80°	Single scroll	10	58
Rabbit [3]	6	61	5.2	50°	Branching (complex)	10	58
Guinea pig [0.6]	0.9	27	3.4	30°	Double scroll	7	25
Mouse [0.03]	0.03	2.8	0.5	15°	Double scroll	1	3

Adapted from Gizurarson (1990) and Illum (1995).

1.6 Intranasal absorption of peptide drugs

It is recognised that the intranasal route has great potential for the delivery of peptide and protein drugs for systemic medication. However, a number of factors affect intranasal drug absorption. These are often referred to as the barriers to drug absorption. These will be described below along with the approaches which may be used to overcome these barriers.

1.6.1 Barriers to peptide and polypeptide drug absorption

The most significant barriers to intranasal peptide and protein drug absorption are:

i) Deposition and clearance from the nasal cavity
ii) Penetration of the mucus layer and the epithelial membrane
iii) Enzymatic degradation

1.6.1.1 Deposition and clearance of drugs from the nasal cavity

Particles which are deposited in the nasal cavity will tend to be rapidly cleared by mucociliary mechanisms with a half life of clearance of about 15-30 minutes (Illum et al., 1987). Thus, for drugs administered intranasally, mucociliary clearance will tend to limit the time available for drug formulations deposited in the nasal cavity to be absorbed across the nasal mucosa. This may be an important factor for peptide and protein drugs which do not tend to exhibit optimal absorption characteristics (Schipper et al., 1991, Illum; 1992). The site of deposition and clearance of drug formulations administered to the nasal cavity will depend largely on the delivery system and the type of administration device used (Chien and Chang; 1987, O'Hagan and Illum; 1990). The type of administration device will influence the size of the particles administered to the nasal cavity since it will determine droplet size if aerosol devices are involved. Particle size is an important determinant for the site of deposition of inhaled compounds in the nasal cavity (Phalen, 1984). For non-spherical particles this will depend on the equivalent aerodynamic diameter of the particles which relates the tendency of the particles to settle to that of spherical particles of unit density. Inhaled aerosolized particles of aerodynamic diameter less than 1 µm will tend to pass through the nasal cavity to the lower respiratory tract. Particles of diameter greater than about 10 µm will tend to be entirely deposited in the nasal cavity and will be trapped in the mucus layer. The proportion of particles between 1-10 µm deposited in the nasal cavity will be influenced by several other factors such as the velocity of the air stream and the turbulence of air flow. Larger particles tend to deposit more anteriorly and smaller particles more posteriorly in the nasal cavity.

The fate of particles administered intranasally from a delivery device will be influenced by a number of factors such as particle size, size distribution, density, shape and hygroscopicity, respiratory patterns, nasal pathology and the delivery device employed. The nasal deposition of particles administered from a delivery device such as a nasal spray will be mainly by inertial impact with sedimentation and diffusion being of less importance (Chien and Chang; 1987). Several studies have evaluated the deposition of compounds from nasal delivery systems and are detailed below. In most of these studies, tracer compounds radiolabelled with ^{99m}technetium were used allowing the fate of the compounds administered to the nasal cavity to be easily followed by gamma

scintigraphy.

Aoki and Crawley (1976), studied the nasal distribution and clearance of radiolabelled human serum albumin (HSA), administered as spray or drops, in human volunteers. Drops were shown to be deposited over the main part of the nasal cavity 2 minutes after administration and changes in the volume or concentration of the HSA solution did not affect the pattern of distribution. Spray was shown to be mainly deposited beyond the nasal cavity in the pharynx and although the size of the spray droplets were not given it is likely that most were considerably less than 10 μm in diameter otherwise a significant proportion of droplets would be expected to deposit in the nasal cavity. Mygind and Vesterhauge (1978) used a plastic cast of the human nose to investigate the intranasal distribution of toluidine blue dye administered as an aerosol or as drops. Aerosol administered from a metered dose pump proved to be the best administration device delivering a constant dose which was evenly distributed over both the septal and lateral walls of the nasal cavity and covered a large surface area. A metered dose aerosol tended to distribute the dye as a narrow band predominantly on the anterior septal wall with little distributed on the lateral wall. Administered as two actuations of the metered dose aerosol per 'nostril', one directed into the upper part of the nose and one in the lower part of the nose, improved the distribution of dye. Spray delivered from a plastic bottle nebulizer was variable with fairly even distribution obtained provided that the bottle was squeezed vigorously and hence this device would be unsuitable for the delivery of accurate drug doses. Drops, administered from a drop-bottle, were poorly distributed tending to deposit on a very small surface area of the nasal cast predominantly in the anterior third of the nasal cavity.

Hardy et al. (1985) administered radiolabelled albumin solutions as drops or a spray to human volunteers. Spray was deposited mainly in localised regions in the anterior of the nasal cavity and was slowly cleared to the pharynx predominantly along the inferior meatus. This slow clearance was attributed to the dose predominantly depositing in the non-ciliated regions of the nasal cavity. Administration of drops resulted in more extensive spread in the nasal cavity, due to mucociliary transport, which was more pronounced when three drops (90 μl volume) were administered rather than one drop (30 μl). Three drops were sufficient to cover most of the walls of the nasal cavity. Approximately 40% of the total dose, following administration as spray or drops, was cleared within about 10 minutes although overall drops were cleared much faster than spray since drops tended to deposit in the ciliated regions. In a study in human volunteers, approximately 80% of the total dose of insoluble radiolabelled teflon particles (median aerodynamic diameter of 3.2 μm) were deposited in the nasal cavity with much of the rest remaining in the dose actuator (Newman et al.,1987). The majority of the particles were distributed on a single area in the anterior of the nasal cavity and failed to penetrate much beyond the internal osteum. Aerosol distribution was not improved by administering two actuations of the metered dose aerosol per 'nostril' as proposed by Mygind and Vesterhauge (1978) following studies using a human nasal cast and this was attributed to the wider nasal passages of the nasal cast compared to the human nose *in*

vivo. Liversidge et al. (1988) found that the dosage form (either spray or drop) and site of administration (either dorsal or ventral surface of the nostril) influenced the nasal distribution and clearance of radiolabelled diethylenetriamine pentacetic acid (DTPA) in dogs. Greatest deposition on the turbinates and the slowest clearance was achieved by administration of drops to the dorsal surface, just inside the nostril, during inhalation. This is of significance since the turbinate mucosa having a high surface area and vascularity should theoretically provide the optimal surface for drug absorption. A drop of DTPA administered to the ventral surface resulted in low turbinate deposition and rapid clearance from the nasal cavity. Administration of a spray to both surfaces resulted in turbinate deposition which was intermediate between that of drops administered to the ventral and dorsal surfaces and was cleared fairly rapidly.

Harris et al. (1986) investigated the relationship between intranasal deposition, clearance, *in vivo* absorption and biological response in human volunteers. Solutions containing desmopressin, a synthetic derivative of the posterior pituitary hormone arginine vasopressin, and radiolabelled HSA were administered from metered-dose pumps (dosing 50 or 100 µl per actuation) or as drops using either a plastic catheter tube or a single dose pipette each administering 200 µl. The doses of desmopressin administered from each delivery device was 300 µg. Deposition and clearance were followed by gamma scintigraphic detection of radiolabelled HSA and the intranasal absorption of desmopressin was assessed directly from plasma desmopressin concentrations or indirectly from its biological response (coagulating activity). Drops from both devices tended to be deposited in the posterior of the nasal cavity close to the nasopharynx and were cleared rapidly (50% of the dose cleared within about 20 minutes). Sprays tended to be deposited in the anterior of the nasal cavity and spray droplets were cleared at a relatively slower rate (50% clearance of 100 µl (2 x 50 µl) or 200 µl (2 x 100 µl) spray dose was 240 minutes or 120 minutes, respectively). The faster clearance of drops compared to sprays supports the findings of Hardy et al. (1986). Desmopressin was absorbed to a greater extent following the administration of sprays and the 100 µl dose performed better than the 200 µl dose. The studies indicated a relationship between deposition, clearance and intranasal absorption and the importance of nasal spray volume and concentration. In a later study in humans, the effects of concentration and dose volume on the nasal bioavailability and biological response of desmopressin (300 µg doses), administered from a metered dose spray, were evaluated (Harris et al., 1988). Bioavailability and enhanced biological response were significantly greater after administering 2 x 50 µl compared with 1 x 50 µl and 1 x 100 µl doses. Based on these studies, Harris et al. suggested that the bioavailability of drugs administered to the nasal cavity may be increased by increasing their residence time in the nasal cavity. Subsequently, the effect of viscosity on patterns of deposition and clearance of nasally administered solutions containing desmopressin and radiolabelled HSA methylcellulose, as a viscosity enhancing agent, were investigated in humans (Harris et al., 1988b). Methylcellulose was assessed as a spray from a metered dose pump at concentrations of 0, 0.25 and 0.50% (mean diameters of the spray droplets were 51.3 µm, 81.3 µm and 200 µm respectively) or as drops from a catheter tube at concentrations of 0 or 0.5%

(viscosity had no affect on the size of drops). Sprays tended to be deposited in the anterior of the nasal cavity and there were no apparent differences in the initial pattern of deposition of the 0% and 0.25% solutions but the initial deposition of the 0.5% solution was shifted anteriorly probably due to the considerable increase in particle size at this concentration. Administration of sprays resulted in 50% clearance in 65, 75 and 43 minutes at concentrations of 0%, 0.25% and 0.50%, respectively. Drops were deposited more evenly over the nasal cavity and pharynx although the initial deposition was more localised for the 0.5% solution and solution viscosity did not affect clearance with 50% of the administered dose being cleared after about 20 minutes. To explain the results, it was suggested that viscosity had a maximal effect on the retention time which was related to the droplet size. The effect of solution viscosity in relation to the intranasal absorption of desmopressin was later assessed in human volunteers (Harris et al.,1989). Desmopressin solutions containing 0% or 0.25% methylcellulose were administered intranasally from a metered dose pump. The authors reported that the addition of methylcellulose resulted in slower and sustained absorption although this claim is not convincing from the published results. The previous study by this group (Harris et al., 1988b) had shown that there was only a small difference in the rate of clearance of solutions containing either 0% or 0.25% methylcellulose. It would be interesting to evaluate nasal absorption and biological effect when the differences in clearance rates are much more pronounced.

Pennington et al. (1988) also investigated the influence of solution viscosity, using hydroxypropyl methylcellulose (HPMC) as the viscosity enhancer, on the deposition and clearance of nasal sprays containing radiolabelled DTPA, in humans. Three solutions containing either 0.6%, 0.9% or 1.25% HPMC (kinematic viscosities of 36, 120 and 430 $mm^2.S^{-1}$, respectively),were compared and each formulation was found to be deposited in the anterior of the nasal cavity, predominantly in the atrium, with no significant differences in the pattern and area of deposition. The half lives of clearance of the solutions (0.6%, 0.9% and 1.25%) were reported as 1.0, 1.7 and 2.2 hours, respectively and clearance was mainly along the inferior meatus to the pharynx. Clearance rates were not significantly different due to large inter-subject variation, although the results indicate that increasing formulation viscosity increases the residence time of the dose in the nasal cavity and thus may be a means by which the therapeutic efficacy of nasal spray preparations are prolonged. The clearance pattern following the administration of the 0.6% solution was biphasic which was suggested to be due to a relatively fast clearance of dose which is deposited in the ciliated posterior regions and the slower clearance of dose which is deposited in the less sparsely ciliated or non-ciliated anterior regions. Particles deposited in non-ciliated regions will be cleared by a very slow drag from contiguous mucus which will usually transport particles in an anterio-posterior direction although movement anteriorly may also occur where they may be removed by blowing, wiping or sneezing (Proctor et al., 1973). The differences observed in the clearance of the more viscous solutions may be due to the formation of a continuous film on the nasal mucosa which will tend to be slowly dragged from the site of deposition in contrast to less viscous solutions which may be film forming but these are likely to break up due to

mucus drag and be transported at faster rates (Pennington et al., 1988).

Illum et al. (1987) investigated the deposition and subsequent clearance of three radiolabelled microsphere powder systems (albumin, starch and DEAE-dextran microspheres) and two control systems (commercially available sodium cromoglycate powder or solution formulation) in human volunteers. Initial deposition of the powders, administered from a nasal insufflator, was mainly in the anterior part of the nasal cavity with little of the dose being deposited in the turbinate regions. In contrast, the nasal solution, administered from a spray pump, was deposited throughout the nasal cavity from the atrium to the nasopharynx. Both the control systems were rapidly cleared from the nasal cavity with half times of clearance of about 15 minutes. The microsphere formulations were cleared much slower from the nasal cavity with clearance half times for albumin and starch microspheres of about 3 hours. At this time, approximately 60% of the DEAE-dextran formulation remained in the nasal cavity. The slow clearance of the microsphere from the nasal cavity was proposed to be due to a combination of their mucoadhesive properties and also due to deposition in the non-ciliated anterior regions of the nasal cavity. The control powder, despite being deposited in the anterior of the cavity was rapidly cleared probably because it did not exhibit mucoadhesive properties.

Wolff et al. (1993) investigated the effect of varying the site of deposition on nasal clearance in anaesthetised (ketamine anaesthetic) rhesus monkeys. Radiolabelled sulphur colloid solution administered to the turbinate region of the nasal cavity, using a catheter tube, was shown to be cleared more rapidly than that administered to the anterior regions with 27 ± 4% and 87 ± 7%, respectively, of the dose being retained in the nasal cavity after 30 minutes which may be due to initial deposition in predominantly ciliated and non-ciliated regions, respectively.

The clearance of drug formulations from the nasal cavity may be reduced in patients with pathological conditions such as chronic sinusitis, nasal polyposis and infection such as the common cold which tend to impair mucociliary function (Lee et al., 1984, Chien and Chang; 1987). Lee et al. (1984) investigated the effects of nasal polyposis, causing obstruction of the nasal airway, on the deposition and clearance of radiolabelled HSA solution administered intranasally using a spray applicator (mean droplet diameter 51 μm). The pattern of deposition and clearance in patients with nasal polyposis was compared with that of normal healthy volunteers. The pattern of deposition of HSA solution did not differ between normal subjects and those will nasal polyposis with deposition in the anterior region of the nasal cavity. However, clearance was considerably slower in patients with nasal polyposis and clearance did not improve following polypectomy which suggested that the physical obstruction of the polyps to mucus flow was not responsible for the slow clearance which was presumed to be due to defects in ciliary action, mucus secretion or a combination of the two. Thus, pathological conditions are an important consideration if the nasal route is to be utilised for systemic drug delivery since inappropriate drug absorption (too great or too little) may be achieved depending on the distribution of the drug at the absorption site. It may not be an

appropriate alternative to parenteral administration if controlled blood concentrations are required (Lee et al., 1984).

1.6.1.2 Penetration of the mucus layer and epithelial membrane

The mucus layer protects the underlying epithelial membrane and is vital for the mucociliary clearance mechanism as outlined above. The mucus layer presents the first physical barrier to drugs deposited in the nasal cavity. Drugs must penetrate the mucus layer, particularly the viscous gel layer, to reach the epithelial cell membrane before the mucociliary clearance mechanism removes the deposited drug from the nasal cavity. The role of nasal mucus as a diffusional barrier for peptide and polypeptide drug absorption has not been established (O'Hagan and Illum, 1990, Lee et al., 1991). It has been suggested that mucus is a selective barrier to macromolecules (Edwards; 1978). Kearney and Marriott (1987) showed that the rate of transport of tetracycline across rat small intestinal epithelium was significantly increased if the layer of endogenous mucus was removed which was attributed to mucus as a diffusional barrier to drug transport rather than due to binding of the drug with mucus glycoproteins. However, since the thickness of the mucus layer in the nasal cavity is about one hundredth of that of the intestines, then the mucus layer may not present a serious diffusional barrier for drugs. There are possibilities that drugs may interact with mucus particularly the mucus glycoproteins which tend to be negatively charged due to the sialic acid and sulphate groups of the acid glycoproteins (anionic polyelectrolytes).

The mucolytic agent N-acetyl-L-cysteine, has been shown to improve the intranasal absorption of human growth hormone in rats (O'Hagan et al.,1990) with bioavailability, relative to a subcutaneous dose, improved from about 7% to 12%. N-acetyl-L-cysteine finds clinical application in the treatment of bronchopulmonary diseases by reducing the viscosity and tenacity of mucus thus facilitating its removal. Thus, improved absorption may be due to a reduction in the intermolecular interactions of growth hormone with mucus glycoprotein chains (Illum, 1992). However it is not known if N-acetyl-L-cysteine has a direct effect on the permeability of the nasal epithelial membrane (Lee, 1991). Illum (1992) suggests that relative to many of the other factors which limit nasal absorption, mucus does not constitute a very significant barrier.

Following its passage through the mucus layer, for absorption to take place the drug molecule must be able to traverse the epithelial membrane. The nasal epithelium is a highly efficient barrier to the absorption of peptide and protein drugs. Absorption across epithelial membranes, irrespective of the mucosal site in which they are located is proposed to occur by either transcellular or paracellular routes. In the transcellular route, molecules pass directly through cells by passive diffusion or by active processes which may involve carrier mechanisms. Alternatively transcytosis may occur whereby molecules are taken up in vesicles by endocytosis at the cell surface and cross the cell discharging their contents into the intercellular space. In the paracellular route, which may also be referred to as transport via aqueous channels or pores, molecules pass through the tight junctions and spaces between adjacent cells. Aqueous pores in the cell

membrane have also been described (Harvey, 1975, Gardner, 1984). However, there is no anatomical evidence to support the existence of aqueous pores in the cell membrane and the likely candidate are the intercellular tight junctions (Fisher, 1994). The properties of the drug molecule and the characteristics of the cellular barrier will determine whether or not molecules are able to cross the barrier by transcellular or paracellular routes (Raub et al., 1993). McMartin et al. (1987) proposed that in nasal mucosa, there are two mechanisms for the transport of compounds. One, a fast mechanism which is dependent on the lipophilicity of the compound and the second, a slower mechanism which is dependent on the molecular weight of the compound. Lipophilic compounds tend to readily diffuse across biological membranes since they are able to partition into the lipid environment (bilayer) of the cell membrane and diffuse into and traverse the cell in the cell cytoplasm. Drugs may also diffuse in the cell membrane, crossing the cell from the apical to the basolateral surface (Raub et al., 1993).

A number of lipophilic drugs, some of which are mentioned below, have been shown to be completely or almost completely absorbed after intranasal administration, relative to the intravenous route. Absorption of the β-adrenoceptor antagonist propranolol has been shown to be rapid and complete following intranasal administration in rats, dogs and humans with blood concentrations similar to those following intravenous administration (Hussain et al., 1979, 1980a, 1980b). *In vitro* studies, using sheep nasal mucosal tissue, showed that propranolol was transported at a rate approximately five times that of mannitol which has a similar molecular weight but is less lipophilic (Wheatley et al., 1988). It was suggested that propranolol traverses the nasal membrane by the trancellular route and mannitol by the paracellular route both by passive processes. The absorption of naloxone, a narcotic antagonist, buprenorphine, an opiate analgesic and testosterone, a natural steroid hormone, were 101%, 95% and 99% (25 μg dose) respectively in rats (Hussain et al., 1984). The bioavailability of 17α-ethinyloestradiol, a synthetic steroid, was shown to be 80%, 85% and 84% at dose concentrations of 1.75, 3.5 and 7 μg/kg, respectively (Bawarshi-Nassar et al., 1989). The difference in bioavailability of the nasal route compared to the intravenous route was attributed to metabolism in the nasal mucosa.

The correlation between lipophilicity and nasal absorption has been demonstrated using several compounds. The lipophilicity of a compound may be indicated by the partition coefficient which measures the partitioning or distribution of molecules between organic and aqueous solvents. The greater the value of the partition coefficient then the higher is the lipid solubility of the compound. Hussain et al. (1985) and Huang et al. (1985) demonstrated the effect of lipid solubility in rats using a series of barbiturates at pH 6.0. The intranasal absorption of the barbiturates was greater as the chloroform/water partition coefficient increased. However, a fifty-fold difference in the partition coefficient resulted in only a four-fold difference in the extent of absorption. The pH is an important consideration since at pH 6.0 the barbiturates are almost entirely in their non-ionised form (pKa 7.6). Generally unionised molecules will tend to partition from aqueous into organic phases whereas ionised molecules tend to remain in aqueous phases where they

are hydrated and highly soluble (Florence and Attwood, 1988). Thus, unionised molecules will tend to be more readily absorbed across mucosal membranes than ionised species. Most drugs are weak electrolytes, containing ionisable groups, which will tend to diffuse across lipid membranes mainly in their unionised, lipid soluble, form. The ionised, hydrophilic form will remain in aqueous phases. The pH will influence the ratio of ionised and unionised species as given by the Henderson-Hasselbach equation below:

$$pH = pKa + (\log \text{ionised} / \log \text{unionised})$$

Where pKa is the negative logarithm of the dissociation constant of the drug

Acidic drugs will tend to be predominantly unionised and basic drugs predominantly ionised at pH values below their pKa. The pH dependency of the absorption of weak electrolytes is the basis of the pH-partition hypothesis. The intranasal absorption of aminopyrine, a weakly electrolytic lipophilic compound, has been shown to follow the pH-partition theory (Hirai, 1981a). However, the same authors showed that although salicylic acid was absorbed to a greater extent in the unionised form, there was considerable absorption of the ionised species and clearly does not follow the pH-partition theory. Similarly, benzoic acid was absorbed to a greater extent in its ionised form but the compound was also absorbed when it was almost fully ionised (Hussain et al., 1985). Kaneo (1983) investigated the intranasal absorption of hydralazine, an antihypertensive basic drug, in rats (in-situ model) showing that the absorption of hydralazine increased linearly as values of pH and the fraction of the unionised species increased. Minimum absorption was demonstrated when the compound was fully ionised ($pH << pKa$) and it was suggested that the presence of aqueous channels in the nasal membrane allowed the absorption of the ionised species. Fisher (1990) considered that these results may be explained if absorption occurs via both lipoidal (transcellular) and aqueous (paracellular) routes. The unionised species will cross the membrane via the lipoidal route and the ionised species will cross the membrane via the aqueous route.

Gibson and Olanoff (1987) showed that the intranasal rate of uptake of steroids in rats increased as the octanol/water partition coefficient increased and was found to be pH independent. The same authors investigated the nasal absorption of alkanoic acids (decanoic (C10), octanoic (C8) and hexanoic (C6) acids), each having a pKa value of 4.85, over the pH range 2.5 to 10. The nasal absorption rates of each compound were similar and did not correlate to the partition coefficient (partition coefficient increased in the order $C6<C8<C10$) although absorption rates were pH dependent with maximum values between pH 4.5-5.0. Maximum absorption rates would be expected to occur at pH values less than the pKa value when the compound is largely unionised. The apparent right shift in the peak absorption rates as a function of pH together with the lack of correlation to partition coefficient was suggested to be due to the presence of an aqueous boundary layer over the lipid membrane. Thus, it is the aqueous layer rather than the lipid membrane which, under certain circumstances, influences the nasal absorption of charged compounds. Fisher (1990) points out that these compounds do

not follow the classical pH-partition theory and the reasons may be due to absorption via lipoidal and aqueous routes. Corbo et al. (1989b) in a study in rabbits, demonstrated that the rate and extent of absorption of progesterone and its monohydroxy, dihydroxy and trihydroxy derivatives decreased as the lipophilicity decreased. These findings were inconsistent with previous absorption studies in rabbits using progesterone and its hydroxy derivatives (Chien et al., 1988, Corbo et al., 1989a) where the extent of absorption was shown to be a hyperbolic function of the nasal mucosa partition coefficient (the degree of partitioning of the compound into excised samples of rabbit nasal tissue) rather than the octanol/water partition coefficient. The bioavailability of progesterone was lower than that of its less lipophilic monohydroxy derivative. Corbo et al. (1990) studied the barrier properties of nasal, rectal and vaginal membranes and found that the nasal membrane had the highest *in vitro* transport of mannitol (model hydrophilic compound) and progesterone (model lipophilic compound). The *in vitro* transport of progesterone and its hydroxy derivatives was shown to decrease as lipophilicity decreased and although the nasal membrane was found to have 'hydrophilic character' it was found to be primarily lipophilic in nature. Gibson and Olanoff (1987) concluded that for non-ionised molecules the nasal membrane is essentially a modified lipophilic transport barrier.

Although lipophilicity has been demonstrated to be of some importance in the intranasal absorption of several compounds, McMartin et al. (1987) suggested that the correlation between absorption and lipophilicity (indicated by charge and hydrophobic balance) was weak compared to the correlation between absorption and molecular weight. However, it was pointed out that the relatively weak correlation of charge and polarity may be consequential of the relationship with molecular weight since lipophilic compounds at the lower end of the molecular weight range were studied.

Hydrophilic, polar compounds are thought to be absorbed by the paracellular route via the intercellular tight junctions and the spaces between cells (aqueous pores). The presence of aqueous pores has been described by several authors (Hirai et al., 1981, Kaneo, 1983, Hayashi et al., 1985, McMartin et al., 1987, Tengamnuay and Mitra, 1988, Fisher et al., 1992). Gibson and Olanoff (1987) dismissed the presence of aqueous pores in the nasal membrane since the in-situ absorption of mannitol, over a 60 minute period was shown to be negligible. Mannitol has been shown to slowly permeate the ovine nasal membrane *in vitro* over a 60 minute period (Wheatley et al., 1988) and Corbo et al. (1989) showed that mannitol was readily absorbed *in vitro* over a 24 hour period.

A correlation between molecular weight and intranasal absorption has been demonstrated using of a variety of compounds (McMartin et al., 1987). The nasal membrane was found to be readily permeable to hydrophilic compounds up to a molecular weight of about 1000. This molecular weight dependency suggests that transcytosis is not responsible for the absorption of these compounds since endocytotic vesicles would be unlikely to restrict compounds of molecular weight less than than several hundred

thousand. Absorption via membrane pores and carrier systems was also thought to be unlikely since permeation of compounds of molecular weight of ≥1000 would disrupt the ion concentration gradients and therefore the internal environment of the cell. Also, this method would require that compounds are absorbed across the cell membrane into the cytoplasm containing a variety of peptidases which would probably destroy peptide drugs. Thus, it is likely that small hydrophilic compounds are absorbed via the aqueous pores.

The nasal membrane has been reported to be richer in water channels of smaller pore size compared to the jejunum or rectum and water influx across the nasal membrane was shown to be three times greater than that in the jejunum and six times greater than in the rectum (Hayashi et al., 1985). Transport of water across the numerous glands in the nasal mucosa may effect drug absorption through solvent drag (Kotani et al., 1983). In the study by Hayashi et al. (1983), inulin (molecular weight 5200) was used as a non-absorbable marker although Fisher et al. (1987) showed that inulin was significantly absorbed and dextran (molecular weight 70000) was absorbed to some extent following intranasal administration. Kotani et al. (1983) also found that inulin and fluorescein isothiocyanate-labelled dextran (molecular weight 20000) were not absorbed nasally and concluded that they were suitable for use as volume indicators in nasal perfusion studies. Volume indicators allow changes in perfusate volume, which may occur due to water transport, to be assessed since this, as well as drug absorption, will alter perfusate drug concentration. In the studies mentioned above, the conflicting results for inulin and dextran absorption may be explained by the different times allowed for contact between the drug and the nasal mucosa in the respective studies. Kotani et al. (1983) and Hayashi et al. (1985), using *in vivo* and/or *in situ* rat models, evaluated absorption over a period of 1 hour compared to 6 hours in the *in vivo* study by Fisher et al. (1987) suggesting that a contact time of more than 1 hour is required to achieve significant absorption of inulin. Nasal absorption may also be influenced by molecular shape allowing linear and flexible compounds such as dextran and to a lesser extent inulin to pass 'end-on' through pores which are narrower than values predicted from their molecular radii (Fisher, 1990).

Fisher et al. (1987) investigated the effect of molecular size on the intranasal absorption of a range of water soluble compounds in the rat finding that there was a direct correlation between the log of the molecular weight and the log of the proportion absorbed. Maitani et al. (1989) studied the effects of molecular weight and charge on intranasal absorption in rabbits using two dextran derivatives of various molecular weights. However, the dextrans were not absorbed without coadministration of the bile salt sodium glycocholate as an absorption enhancer. The absorption of a neutral dextran decreased as dextran molecular weight increased supporting the inverse relationship between molecular weight and intranasal absorption. In contrast, the absorption of polycationic dextrans increased with molecular weight for dextrans of molecular weight less than 10000 which was suggested to be due to the increased binding of the polycation to the negatively charged mucus. However, O'Hagan and Illum (1990) argue that there was no significant difference between the absorption profiles of the charged and neutral dextrans at

molecular weights below 10000. In addition, absorption of the dextrans was not achieved without the use of sodium glycholate which has been proposed to function by producing aqueous pores or reverse micelles in the nasal membrane (Gordon et al., 1985). Fisher et al. (1992) investigated the intranasal absorption of a homologous series of water soluble fractionated di-iodo-L-tyrosine-labelled dextrans in rats. The dextrans had similar physicochemical properties, being inert and having a uniform charge, and the size range of each of the 13 dextrans was small. This was in contrast to the 5 water soluble compounds used in the previous study (Fisher et al., 1987) which were differently charged, some being pharmacologically active, some metabolised and the larger compounds were heterogeneous. An inverse relationship between dextran molecular weight and intranasal absorption was demonstrated. Hosoya et al. (1993) investigated the *in vitro* permeability of rabbit nasal, buccal and various gastrointestinal mucosa, to fluorescein isothiocyanate-labelled dextrans of molecular weight 4400-71200. It was shown that the nasal mucosa had the lowest membrane resistance and the highest permeability to the dextrans which suggested that the nasal mucosa was a 'leaky' structural barrier. The higher permeability of the nasal mucosa was attributed partly to the shorter diffusion path since the thickness of the nasal mucosa (337 ± 35 μm) was approximately half that of the jejunal (740 ± 26 μm) and upper colonic (710 ± 25 μm) mucosae. The permeability of each mucosa was shown to decrease with increasing molecular weight.

Peptides are generally poorly absorbed following intranasal administration which may be attributed to their polar nature and hence the production of more lipophilic peptide prodrugs (drug precursors) could be useful in improving peptide bioavailability. The effects of polarity have been investigated using a model amino acid; L-tyrosine having three polar functional groups, each of which may influence nasal absorption (Hussain et al., 1985, Huang et al., 1985b). *In situ* perfusion studies in rats were used to investigate the effect of structural modifications of L-tyrosine on nasal absorption. Preliminary studies showed that the intranasal absorption of L-tyrosine, existing in its zwitterionic form over the pH range studied, was concentration dependent but pH independent and occurred by a carrier-mediated process. Derivatives of L-tyrosine were used to study the effect of the different polar functional groups on nasal absorption. Increasing the lipophilicity of L-tyrosine by O-acyl esterification did not improve absorption which was attributed to the polarity of the carboxylic and/or amino groups since the O-acyl derivatives also existed in zwitterionic form. N-acetyl-L-tyrosine, used to mask the amino group, had a similar partition coefficient to L-tyrosine and was absorbed at a similar rate. Carboxylic acid esters of L-tyrosine, used to mask the carboxylic group, were found to be more lipophilic than L-tyrosine and were absorbed at a faster rate. The rate of absorption of a carboxylic acid ester was shown to be four times faster than an O-acyl ester having a partition coefficients of the same order of magnitude suggesting that the enhanced nasal absorption of the carboxylic acid esters of L-tyrosine was due to masking the negative charge on the carboxylate moiety. Carboxylic acid esters were shown to be hydrolysed in the perfusion medium although the rate of disappearance of the compound from the perfusate was an order of magnitude less than that accountable

for by absorption. Hussain et al. (1985), suggested that the intranasal absorption of the polar quaternary ammonium compound clofilium tosylate, an antiarrhythmic drug, does not support the concept of low peptide absorption due to their polar nature. Clofilium tosylate was shown to be nasally absorbed in the *in vivo* rat model which was suggested to occur by simple diffusion (Su et al., 1984, Su and Campanale, 1985). However, the drug caused mild tissue necrosis, even with lowest dose tested, which may account for the nasal absorption observed for this compound.

The intranasal absorption of peptides is generally low without co-administration with absorption enhancing compounds with bioavailabilities obtained in the region of 1-2% when administered as simple solutions (O'Hagan and Illum, 1990). Several peptides have been shown to be absorbed without the use of enhancers. The intranasal absorption of thyrotropin-releasing hormone (TRH), a tripeptide neurotransmitter, has been shown to be approximately 20% relative to the intravenous route in rats (Sandow and Petri, 1985). The synthesis of lipophilic analogues of TRH and other peptides has been proposed as a means by which their transmembrane permeability may be enhanced (Muranishi et al., 1991). However, it was also pointed out that the biological activity of these derivatives may be reduced compared to the parent compound. Su et al. (1985b) demonstrated the intranasal absorption of pentapeptide drugs in rats using two enkephalin analogues (metkephamid and DADLE). Metkephamid (molecular weight 661) was completely absorbed (bioavailability relative to the intravenous route 102%) but DADLE (molecular weight 570) was absorbed to a lesser extent (bioavailability relative to subcutaneous route 59%), the difference explained as being due to metkephamid having greater stability and less susceptibility to enzymatic degradation. The effect of peptide dose on the intranasal absorption of metkephamid was investigated. The amount of metkephamid absorbed was directly proportional to the dose administered and the results suggested that the compound was rapidly absorbed by diffusion. Faraj et al. (1990), based on published reports and their own studies, point out that although there is variation between peptides in the fraction of the dose absorbed, peptide absorption is rapid. The initial rate of peptide absorption was suggested to be as rapid as the absorption of lipophilic compounds such as propranolol, although the total amount of peptide absorbed was said to be usually small and self limited. Assuming that peptides are absorbed via aqueous pores, it was speculated that the self limiting step was due to blockage of the pores by peptides or their hydrolysis products.

1.6.1.3 Enzymatic degradation

The low bioavailability of peptides administered via the nasal route can be attributed in part to poor transport across the nasal mucosa but enzymatic degradation in the nasal cavity may also constitute a significant barrier to peptide absorption. As previously discussed, intranasal drug absorption avoids the 'first pass' metabolism associated with poor bioavailability via the oral route but proteolysis before, during or following intranasal absorption also appears to limit intranasal peptide drug absorption. The nasal route was initially regarded as being devoid of the enzyme systems which limit oral absorption but Zhou (1994) considered that proteolytic activities are the most important

barriers preventing the intranasal absorption of peptide drugs. The various enzyme systems associated with the nasal cavity have previously been reviewed. Peptide drugs are most likely to be susceptible to the exopeptidases, cleaving peptides at their N and C termini, or endopeptidases, cleaving the internal peptide bonds, which are present in the nasal cavity. The importance of *in vivo* peptide degradation by nasal enzyme systems has not been fully elucidated (O'Hagan and Illum; 1990, Illum; 1992). Various studies have investigated peptide degradation during *in situ* or *in vitro* animal studies the results of which generally indicate that for many peptides, particularly smaller peptides, enzymatic degradation is likely to be a significant barrier to their absorption. Many studies have investigated the stability of peptides *in vitro* following incubation of peptides with nasal homogenates. However, with these studies there is an over exposure of peptides to the various enzymes present in the nasal tissue, since the homogenates will contain enzymes which are liberated from all mucosal cells and also those from subcellular fractions including the cell cytosol and lysosomal enzymes. Homogenate studies may be useful for comparing overall peptide degradation at various mucosal sites and to assess the likelihood of degradation of specific peptides at these sites. However, they do not take into account the susceptibility of peptides during absorption relative to the normal structural organisation of enzymes (Kashi and Lee; 1986, Illum; 1992).

Hirai et al. (1981) showed that insulin was rapidly degraded in rat nasal homogenates with approximately 50% and 9% of the insulin remaining after incubation for 10 and 60 minutes respectively. Hayakawa et al. (1889) investigated the hydrolysis of various concentrations of insulin in rabbit nasal homogenates. As insulin concentration was increased from 5 to 100 μl the susceptibility to proteolysis was shown to decrease (indicated from the increasing half life of degradation) and this apparent self-stabilisation was attributed partly due to self-association of insulin as concentration increased but also due to saturation of the proteases by insulin since the rate of proteolysis increased as the protein content of the tissue homogenate increased. At a tissue protein content of 10 mg/ml, the half lives of hydrolysis of 5 and 100 μM insulin were 9 minutes and 429 minutes, respectively. The vasopressin analogue desmopressin (DDAVP), a nonapeptide, was shown to be degraded by enzymes in rabbit nasal homogenates (Critchley, 1989). Hussain et al. (1985) investigated the *in situ* absorption of the dipeptide L-tyrosyl-L-tyrosine in rats finding that it underwent extensive hydrolysis in the nasal perfusate which was attributed to the presence of peptidase enzymes in the nasal cavity since the compound was stable in the perfusate medium. The dipeptides L-glycyl-L-tyrosine and L-glycyl-L-tyrosinamide and the more lipophilic dipeptide ester, L-tyrosyl-L-tyrosine methyl ester, were also shown to be rapidly hydrolysed demonstrating that the enzymatic hydrolysis was not specific for L-tyrosyl-L-tyrosine. The poor bioavailability of intranasally administered peptides was attributed to enzymatic hydrolysis in the nasal cavity during the absorptive process.

Kashi and Lee (1986), investigated the hydrolysis of pentapeptides in rabbit nasal homogenates using various enkephalins. Methionine enkephalin and leucine enkephalin were shown to be rapidly hydrolysed (half lives of degradation were approximately 16

minutes and 20 minutes respectively) which was attributed mainly to aminopeptidases with contribution from dipeptidyl peptidase and dipeptidyl carboxypeptidase. [D-Ala] Met-enkephalinamide, being resistant to aminopeptidase mediated cleavage, was much less susceptible to hydrolysis (half life of degradation of about 162 minutes). Chun and Chien (1995) investigated the effect of pH on the degradation of methionine enkephalin in rabbit nasal mucosal extracts reporting that the fastest degradation of methionine enkephalin occurred at about pH 7 indicating optimal activity of enkephalin-degrading enzymes at this pH.

The enzymatic degradation of leucine enkephalin has also been investigated using an *in situ* rat perfusion model (Hussain et al., 1985, Hussain; 1989, Faraj et al., 1990). The disappearance of leucine enkephalin from the nasal perfusate (half life of about 30 minutes) was shown to be almost entirely due to its hydrolysis in the perfusate rather than to absorption from the nasal cavity. Leucine enkephalin was also shown to be rapidly hydrolysed in buffer solution which had been pre-circulated through the rat nasal cavity indicating that active nasal enzymes were extracted into the buffer. The hydrolysis of leucine enkephalin was also demonstrated in rats using an *in vivo-in situ* technique, in which small volumes of drug solution were administered to the nasal cavity which was washed-out at appropriate time intervals and the nasal washings collected and analysed for drug and metabolite content, which was suggested to simulate the use of nose drops in humans. The rate and extent of formation of the metabolite depended on the concentration of leucine enkephalin with greater conversion of the parent compound to metabolite at lower concentrations suggesting that the enzymes which hydrolyse leucine enkephalin become saturated at higher drug concentrations. Using the same *in vivo-in situ* technique, it was found that the hydrolysis of leucine enkephalin was reduced by the inclusion of di- or tripeptides which were suggested to compete for peptidase enzymes in the nasal cavity. The same research group observed the hydrolysis of leucine enkephalin (half life of disappearance was about 40 minutes) in human nasal washings demonstrating the presence of loosely bound peptidase enzymes in the human nasal cavity (Hussain et al., 1990). Irwin et al. (1994) investigated the degradation of leucine enkephalin and its metabolite des-tyrosine leucine enkephalin in sheep nasal washings and mucosal homogenate preparations finding that the half life of degradation was 40 and 12 minutes for leucine enkephalin and 13 and 7 minutes for des-tyrosine leucine enkephalin respectively. With reference to leucine enkephalin, this study outlines the greater exposure of peptides to enzymes in studies involving nasal homogenates. Irwin et al. (1995) demonstrated the hydrolysis of di-, tri- and tetrapeptides in sheep nasal homogenates. Each of the peptides investigated was shown to be degraded following incubation with the nasal homogenates but there was considerable variation in their susceptibility to degradation.

1.6.2 Overcoming the barriers to peptide or protein drug delivery

The various absorption barriers must be overcome in order to achieve an effective degree of absorption of peptide and protein drugs via the nasal route. Much attention has been focused on the use of compounds, in the form of absorption enhancing agents, which

will promote the absorption of peptide and protein drugs administered nasally and these are discussed below. An alternative approach is to modify the structure of peptides and protein drugs in order to improve their absorption characteristics although investigation of such systems is limited. Hashimoto et al. (1989) synthesised palmitoyl derivatives of insulin which were more lipophilic than native insulin. Following intravenous administration to rats, a monopalmitoyl derivative resulted in a hypoglycaemic response which was similar in magnitude but more prolonged than that following the intravenous administration of native insulin. In contrast, a dipalmitoyl derivative was less effective in lowering plasma glucose concentrations following intravenous administration. However, both the mono- and the dipalmitoyl derivatives were much less effective than native insulin in lowering plasma glucose concentrations following intramuscular administration. Asada et al. (1992 and 1995) showed that the transport of insulin across intestinal membranes was improved by chemical modification with fatty acids which enhanced lipophilicity of the insulin. However, as lipophilicity was increased the pharmacological availability was decreased although several of the derivatives exhibited pharmacological activity similar to that of native insulin. Insulin derivatives were shown to be more stable in small intestine mucosal homogenates. This suggests that the stability of peptide and proteins, administered by non-parenteral routes, may be improved by the use of chemically modified derivatives.

Many compounds have been investigated as absorption enhancers for peptide and protein drugs. These compounds are usually classified into a number of enhancer systems based on the way in which they principally act on the mucosa and promote drug absorption. However, compounds may enhance drug absorption via a combination of several mechanisms. The classification system is generally used for all compounds which are used as enhancers via non-parenteral routes. The main types of enhancer system are:
(i) bile salts and derivatives such as sodium deoxycholate, sodium glycocholate and sodium taurodihydrofusidate; (ii) surfactants such as sodium lauryl sulphate and polyoxyethylene-9-lauryl ether; (iii) chelating agents such as ethylenediaminetetraacetic acid (EDTA), citric acid and salicylates; (iv) fatty acids such as sodium caprylate and sodium laurate; (v) enzyme inhibitors such as bestatin and amastatin (vi) novel enhancer systems such as bioadhesives, cyclodextrins and chitosan. (Lee and Yamamoto; 1990, O'Hagan and Illum; 1990, Illum; 1991).

1.6.2.1 Bile salts

The common naturally occurring bile salts are steroids which are synthesised in the liver from cholesterol. Bile salts are amphipathic molecules possessing distinct nonpolar and polar regions. In aqueous solution at low concentration, bile salts exist as monomers but above the critical micellar concentration, bile salts will self associate to form colloidal aggregates referred to as micelles. In bile salt micelles, the hydrophilic groups are exposed to the aqueous medium and surround a core formed by the nonpolar hydrophobic groups and as a consequence the whole structure is water soluble. Micelles consisting of only bile salts are referred to as simple micelles. However, mixed micelles are formed if other lipids are solubilised into the core structure of simple micelles. Bile

salts have surface active (surfactant) properties enabling them to solubilise lipids. This is of biological importance in the digestion and absorption of dietary lipids (Gordon et al., 1971, Carey and Small; 1972, Shiau; 1987).

Hirai et al. (1978) showed that a solution formulation containing 1% sodium glycocholate enhanced the intranasal absorption of insulin in dogs. In a later study several bile sodium salts (taurocholate, cholate, deoxycholate, glycocholate, chenodeoxycholate) were shown to be equally effective in enhancing the nasal absorption of insulin in rats (Hirai et al., 1981b). The absorption promoting effect of bile salts was suggested to be due not only to the increase in membrane permeability, i.e. a direct effect on the nasal mucous membrane, but also due to their inhibitory effect on proteolytic enzymes since sodium glycocholate significantly inhibited the enzymatic hydrolysis of insulin in rat nasal tissue homogenates (Hirai et al., 1981c). Moses et al. (1983) showed that coadministration of 1% sodium deoxycholate enhanced the intranasal absorption of insulin in human volunteers. However, following nasal administration, a solution containing sodium deoxycholate was reported to cause mild nasal irritation and slight nasal congestion which lasted about 15 minutes. The same study group investigated the effect of dihydroxy bile sodium salts (deoxycholate, glycodeoxycholate, taurodeoxycholate, ursodeoxycholate, chenodeoxycholate) and trihydroxy bile sodium salts (cholate, glycocholate and taurocholate) on the nasal absorption of insulin in human volunteers (Gordon et al., 1985, Flier et al., 1985). In contrast to the work reported by Hirai et al. (1981b), the bile salts differed in their absorption promoting efficacy and the bile salts glycocholate, taurodeoxycholate, deoxycholate and chenodeoxycholate appeared to be the most effective. Sodium deoxycholate was shown to be only slightly effective at a concentration of 2.5 mM, 50% effective at 6 mM and maximally effective at 12 and 24 mM. The optimal effect of deoxycholate was exhibited at a concentration well above the critical micellar concentration of approximately 3 mM. Each of the bile salts investigated in the study resulted in local nasal irritation (burning sensation in the nose lasting for up to 5 minutes) although the degree of irritancy did not correlate with enhancing activity since ursodeoxycholate was inactive but resulted in the most irritation. The taurine and glycine conjugates tended to be less irritative.

Longenecker et al. (1987) showed that the bile salt 'derivative' sodium taurodihydrofusidate (STDHF), at a concentration of 1%, enhanced the nasal absorption of insulin in sheep (bioavailability approximately 16%). Absorption enhancement appeared to occur only in the presence of micelle formation and it was suggested that solubilization of insulin in STDHF micelles plays a major role in its activity as an absorption promoter. An erythrocyte haemolysis assay showed that STDHF was less haemolytic indicating that it caused less membrane damaging effects, than sodium deoxycholate and much less haemolytic than the surfactant polyoxyethylene-9-lauryl ether (Laureth-9). STDHF (1%) was shown to improve intranasal insulin bioavailability from 0.9% to 5.2% in rabbits and from 0.3% to 18.0% in rats (Deurloo et al., 1989, Verhoef et al., 1989). The above studies illustrate the difference in intranasal insulin absorption with the animal model employed since the bioavailability obtained by coadministration of

the formulation containing 1% STDHF was greater in the anaesthetised rat model than in the conscious rabbit model (sedated during dose administration) although there were no significant differences between the animal models in the absence of the enhancer. STDHF was shown to induce ciliostasis at concentrations of 0.3% and above although it was less ciliostatic than sodium deoxycholate or Laureth-9 (Hermens et al., 1990). A formulation of insulin with 1% STDHF decreasd cilia beat frequency from 100% to about 20% after 10 minutes. The authors suggested that long-term application of irreversibly ciliostatic formulations should be avoided in nasal drug delivery.

Maitani et al. (1986) showed that bile salts at 3% w/v enhanced the intranasal absorption of β-interferon in rabbits and that sodium glycocholate and sodium cholate were more effective than sodium deoxycholate in promoting absorption. Sodium glycocholate at 0.5% was shown to increase the bioavailability of intranasally administered methionyl-human growth hormone from less than 1% to between 7-8% (Daugherty et al., 1988). Aungst et al. (1988) investigated the effect of sodium glycocholate on insulin absorption in the rat following nasal, rectal, buccal and sublingual administration finding that insulin absorption was improved in the order nasal > rectal > buccal > sublingual. Tengamnuay and Mitra (1990) showed that a bile salt-fatty acid mixed micellar solution (15 mM sodium glycocholate with 5 mM linoleic acid) was marginally more effective than the bile salt alone in promoting intranasal insulin absorption in the rat. Bile salt-lipid mixed micelle formulations have been shown to improve the intestinal absorption of normally poorly absorbed non peptide drugs such as streptomycin and gentamicin in animal models (Muranishi et al., 1979). The absorption of gentamicin was also shown to be improved via the nasal route in human volunteers following coadministration with 1% sodium glycocholate (Rubinstein, 1983) and in rabbits by coadministration with several different bile salts (Duchateau et al., 1986). In a later study in rabbits, Duchateau et al. (1987) showed that bile salts effectively improved the nasal bioavailability of phenol red. Duchateau et al. (1986) showed that dihydroxy bile salts, particularly deoxycholate, were more ciliotoxic than trihydroxy bile salts. Ciliotoxicity is an important consideration in intranasal drug delivery since absorption enhancers should not affect the normal mucociliary clearance mechanisms. However, it is not clear how impairment of cilia function may manifest *in vivo* although increased susceptibility and recurrence of respiratory infections are known to result in patients with 'immotile' cilia syndrome (Hermens et al., 1990).

The mechanism by which bile salts enhance drug absorption has not been fully elucidated although it appears that there may be a combination of effects (O'Hagan and Illum; 1990). Several mechanisms by which bile salts enhance drug absorption have been proposed. Gordon et al. (1985), in nasal absorption studies with insulin, proposed that bile salts act by forming transmembrane channels or as mobile carriers for insulin due to the formation of reverse micelles. In reverse micelles, the hydrophilic surface of the bile salt faces inwards and the hydrophobic surface faces outwards towards the lipid environment of the membrane. The different degrees of potency of the bile salts was suggested to be due to their ability to penetrate the membrane, and self-associate to form

reverse micelles. The formation of insulin / bile salt mixed micelles, resulting in the complete solubilization of insulin, was speculated to move down an aqueous concentration gradient through the nasal epithelial cells, into the intercellular space and into the blood circulation. The authors favoured the channel type mechanism rather than the mobile carrier since absorption enhancement became saturated at bile salt concentrations of approximately 12 mM probably indicating local membrane saturation. The reverse micelles were suggested to stack end-to-end and span the lipid bilayer with ionised polar groups projecting into the aqueous environment on either side of the membrane. Thus, the stacked reverse micelles form a temporal aqueous pore through which solubilised insulin can be transported. It has also been suggested that ion-pairing between anionic bile salts and the positive residues on peptides and proteins would render them more lipophilic and hence increase membrane permeability (Lee; 1990). Passive diffusion of bile salt micelles into hamster intestinal mucosa has been demonstrated *in vitro* (Gordon et al., 1971). Maitani et al. (1991) demonstrated the permeability of rabbit nasal mucosa to bile salts by investigating their effect on membrane potential. The nasal membrane, carrying a net negative charge, became slightly more negatively charged by pretreatment with bile salt solution due to the penetration of the bile salt into the nasal mucosa. Uchida et al. (1991a and 1991b) showed that bile salts increased the membrane permeability to water soluble compounds and suggested that this is due to the formation of a temporal pore in the membrane. Gordon et al. (1985), proposed that pores may form in the lipoidal membrane, thereby enhancing transcellular transport. Alternatively, formation of an intercellular 'pore' would increase permeability via the paracellular route. Lee (1990) suggests that the rapid nature of bile salt induced absorption enhancement of water soluble compounds would tend to favour a mechanism involving the paracellular route.

The mechanism of absorption enhancement of bile salts may in part be due to their ability to chelate calcium ions similar to the mode effect of EDTA which is thought to chelate calcium and magnesium ions in the regions of the tight junctions, thus opening the tight junctions and promoting absorption via the paracellular route (Murakami et al., 1984). In the study investigating the rectal absorption of ampicillin in rats, formulations containing calcium chloride were shown to decrease the absorption enhancing efficacy of sodium deoxycholate although the presence of magnesium ions had no affect suggesting that the bile salt binds membrane calcium ions but not magnesium ions. Shiga et al. (1987), in a study investigating colonic absorption, also suggested that the absorption enhancing mechanism of sodium taurocholate is by chelated depletion of metal ions in regions of the tight junctions similar to that of EDTA. Bile salts (deoxycholate, taurodeoxycholate, glycocholate) have also been shown to be potent mucolytic agents resulting in a decrease in the viscosity and elasticity of human bronchial mucus (Martin et al., 1978). The effect on mucus was more prominent as bile salt concentration increased and at the highest concentration investigated (20 mM), liquefaction of gelled mucus occurred. For intranasally administered compounds to reach the nasal epithelium, they must first penetrate the mucus layer which will be facilitated if the consistency of mucus is adversely affected.

Hirai et al. (1981c) demonstrated that bile salts inhibit the enzymatic degradation of insulin in the nasal mucosa suggesting this as a possible mechanism by which they promote absorption. Lee and Kashi (1987) showed that bile salts were more potent as inhibitors of aminopeptidase activity at concentrations above their critical micellar concentration. However, no correlation existed between aminopeptidase inhibition and the rank order of absorption enhancing potency suggesting that the inhibition of proteolytic enzymes is not a principal mechanism.

Several studies have shown that bile salts have a membrane damaging effect which may be another reason for their effectiveness as absorption promoters. Chadwick et al. (1979) showed that dihydroxy bile acids (deoxycholic acid and chenodeoxycholic acid) induced fluid secretion, increased mucosal permeability and resulted in mucosal damage during *in vivo* perfusion of the rabbit colon. Hirai et al. (1981c), using scanning electron microscopy, showed that nasal villi were slightly denuded at the tip 2 hours after the administration of 1% sodium glycholate although complete recovery had occurred by 24 hours. Bile salts (glycholate, cholate, taurocholate) were also shown to cause membrane damage (assessed by measuring protein release from the nasal mucosa and erythrocyte haemolysis) although the extent of the damage was reported to be mild compared to many of the other compounds investigated. However, it must be pointed out that the concentrations of the compounds used in the study were much lower than the concentrations which had been demonstrated to promote insulin absorption. Shao and Mitra (1992) showed that 15 mM sodium deoxycholate caused the greatest membrane damage (protein release), while STDHF, cholate, glycocholate and taurocholate, in descending order, exhibited less membrane damaging effects. Hersey and Jackson (1987) using *in vitro* techniques, showed that 0.5% sodium deoxycholate caused a rapid 4 to 5 fold increase in nasal permeability which was accompanied by extensive histological damage to the epithelium suggesting that bile salts work by removing the epithelial layer which constitutes a major barrier to drug absorption. Damage to the nasal epithelium would obviously cause concern particularly during chronic therapy. Merkus et al. (1993) showed that the dihydroxy bile salts deoxycholate and taurodeoxycholate caused much greater morphological damage to rat nasal mucosa following 5 minutes exposure than did glycocholate after 10-15 minutes exposure (all assessed at a concentration of 1%). The study also showed that there was a good correlation between morphological damage and arrest of cilia beat frequency which was suggested to be a valuable tool in the search for safe absorption enhancing compounds. Clearly, ciliotoxicity would tend to prolong the residence time of the formulation in the nasal cavity and may be another factor for the absorption enhancing efficacy of bile salts.

1.6.2.2 Surfactants

The term surfactant or surface-active agent applies to compounds which tend to accumulate at the boundary between two phases resulting in a modification of the surface energy of the interface (Florence and Attwood, 1988, Hills, 1988). In solution, surfactants will lower the surface and interfacial tension of water and promote the association into micelles and foam formation (Schott, 1995). Many compounds display

surfactant properties including the bile salts previously mentioned and fatty acids such as phospholipids. The efficacy of saponin, a sapogenin glycoside, in promoting the nasal absorption of insulin was demonstrated as early as 1932 by Collens and Goldzieher. The potency of surfactants in enhancing the intranasal absorption of insulin has been demonstrated in dogs (Hirai et al., 1978) and in rats (Hirai et al., 1981b). In dogs, the non-ionic surfactant Laureth-9 and saponin, at concentrations of 1%, increased the intranasal absorption of insulin. In rats, various compounds with surfactant activity including bile salts were investigated. The absorption enhancing efficacy of a range of non-ionic polyoxyethylene -ether and -ester surfactants were evaluated and it was shown that the esters did not perform as well as the ethers. Of the ether type surfactants, those with an hydrophile-lipophile balance (HLB) of between 8-14 were shown to be more effective and this group included Laureth-9 (HLB 11.5) which caused a decrease in plasma glucose concentrations of about 60%. An increasing value of HLB corresponds to increasing hydrophilic character (Helenius and Simons; 1975). Hirai et al. (1981b) also showed that anionic surfactants, such as sodium laurylsulphate and potassium laurylsulphate, saponin and surfactin (a peptidelipid) effectively promoted the nasal absorption of insulin but the efficacy was lower than that of Laureth-9. Laureth-9 was shown to be a more potent enhancer with a maximal absorptive effect observed at a concentration of about 0.3% compared to about 0.5% for both saponin and the bile salt sodium glycocholate.

Salzman et al. (1985) investigated the efficacy of a nasal insulin formulation containing Laureth-9 in normal volunteers and in Type I diabetic patients. In fasted subjects, insulin was rapidly absorbed from nasal formulations containing 1% Laureth-9 and in normal volunteers and diabetic patients, plasma glucose concentrations were lowered to 50% of basal values in about 45 and 120 minutes, respectively. Following a meal, an insulin formulation containing 1% Laureth-9 was more effective in controlling postprandial hyperglycaemia than formulations containing 0.1% or 0.25% Laureth-9. The absorption profile of insulin and subsequent control of postprandial hyperglycaemia following intranasal administration of the insulin / Laureth-9 formulation simulated more closely endogenous insulin secretion compared to a subcutaneous dose of insulin. However, the degree of nasal irritation, although highly variable between subjects, increased with increasing concentration of Laureth-9.

Several other studies have assessed surfactants, mainly Laureth-9, as an absorption enhancer for insulin. Pontiroli et al. (1986) reported that Laureth-9 enhanced the absorption of insulin and glucagon in normal subjects following intranasal administration. Aungst and Rogers (1988) showed that 5% Laureth-9 promoted the nasal, buccal and rectal absorption of insulin in rats. Formulations of insulin with Laureth-9 were also shown to markedly improve intranasal insulin absorption in rats (Chandler et al., 1991b, Jabbal Gill et al., 1994b). Chandler et al. (1991b) administered an insulin dose of 8 IU/kg in a formulation containing 1% Laureth-9 and reported an 87% decrease in blood glucose concentrations 60 minutes after dosing. Insulin was not absorbed nasally without the presence of absorption enhancers. Jabbal Gill et al. (1994b)

administered 4 IU/kg insulin with 0.9% Laureth-9 and blood glucose concentrations were shown to decrease to about 55% of the basal value compared to 86% for the insulin control solution. Duchateau et al. (1987) reported that 1% Laureth-9 improved the absolute bioavailability of phenol red in rabbits from about 22% to 87%. Laureth-9 at a concentration of 1% (17 mM) has been shown to enhance the absorption of recombinant methionyl human growth hormone (Met-hGH) across the rat nasal mucosa (Daugherty et al., 1988). The bioavailability of Met-hGH, compared to an intravenous dose varied between about 60-80% depending on the dose administered. This was a marked improvement on the bioavailability of 7-8% achieved using the bile salt sodium glycocholate.

Surfactants are able to modify the properties of biological membranes and alter their permeability or transport characteristics (Gibaldi and Feldman; 1970). However, the mechanism of absorption enhancement of surfactants such as Laureth-9 appears to correlate with the damage that it causes to the integrity of the nasal mucosa. Surfactants, including bile salts, have been shown to have membrane solubilising properties and have been used to extract membrane components for biochemical study (Helenius and Simons; 1975, Florence; 1981). The solubilization and subsequent disruption of membrane integrity by surfactants appears to be at the centre of many of the observed biological effects caused by these compounds (Florence; 1981).

Hirai et al. (1981c) investigated the possible mechanisms for the enhanced nasal absorption of insulin by surfactants. Surfactants, such as polyoxyethylene ethers, including Laureth-9, sodium lauryl sulphate and saponin, were shown to have a potent haemolytic or protein releasing effect on biomembranes indicating the disruption of the integrity of the membranes which correlated well with their absorption enhancing potency. For polyoxyethylene ethers it was found that the degree of membrane damage decreased as the value of HLB either increased or decreased from a value of 11.5 (the HLB value of Laureth-9). In rat nasal homogenates, the activity of leucine aminopeptidase decreasd as the concentration of Laureth-9 increased although it had only a slight inhibitory effect on the degradation of insulin. At concentrations of 0.27%, non-ionic surfactants resulted in less than about 23% inhibition of leucine aminopeptidase compared to about 98% for the ionic surfactant sodium laurylsulphate and 85-87% for bile salts. The recovery of the nasal membrane from the hyperabsorptive state induced by 1% Laureth-9 was also assessed by intranasally administering Laureth-9 solution to conscious rats followed by the intranasal administration of 10 IU/kg insulin solution to anaesthetised rats at various time intervals. Insulin absorption was shown to decrease as the time interval between surfactant and insulin administrations increased. The recovery from the hyperabsorptive state following the administration of the bile salt sodium glycocholate (1%) was faster than that following the administration of Laureth-9 indicating the greater membrane disturbing effects of the latter. Scanning electron microscopy confirmed these findings by showing that the membrane damage caused by Laureth-9 was significant at 2 hours and had not recovered by 24 hours whereas sodium glycocholate caused only slight membrane damage at 2 hours and there was complete

recovery by 24 hours. Following the intranasal administration of Laureth-9 or sodium glycocholate solutions 3 times daily for one month, Laureth-9 was shown to be more irritative, causing a morphological change in the nasal microvilli, compared to sodium glycocholate, which resulted in no morphological change. The studies of Hirai et al. (1981c) appear to indicate that absorption enhancement by surfactants such as Laureth-9, sodium lauryl sulphate and saponin is due mainly to disruption of the nasal membrane.

Longenecker (1986) and Longenecker et al. (1987) showed that Laureth-9 was a highly potent haemolytic agent finding that it was approximately 20 and 100 fold more haemolytic than sodium deoxycholate and STDHF, respectively. Laureth-9 at a concentration of 0.3%, was shown to cause rapid and irreversible ciliostasis of human nasal cilia (Hermens et al., 1990). Daugherty et al. (1988), using histological techniques, showed that nasal solutions containing 1% Laureth-9 severely disrupted the cellular permeability barrier. In the rat nasal cavity, the entire epithelial monolayer was shown to be removed at certain locations, as early as 30 minutes after dose administration, and was accompanied by multifocal necrosis, inflammation and exudation. In comparison, sodium glycocholate was reported to be less destructive on the integrity of the epithelial lining. Laureth-9 was found to cause severe disruption of the rat nasal mucosa after only 5 minute exposure (Ennis et al., 1990). This was confirmed by Chandler et al. (1991a) who demonstrated that 1% Laureth-9 caused epithelium disruption, cell loss and reduction in epithelium height after 5 minutes exposure and became worse as contact time was increased. Jabbal Gill et al. (1994b) have also demonstrated damage to the rat nasal membrane in rats using histological techniques. Sodium lauryl sulphate and Laureth-9, at a 1% concentration, were shown to severely disrupt the integrity of the rat nasal epithelium which was generally accompanied by an increase in the permeability of the mucosa to a range of polyethylene glycols (PEG) of molecular weight about 600 to over 2000 daltons (Donovan et al., 1990). In contrast, 0.1% Laureth-9 caused only slight damage to the nasal mucosa. It was suggested that the surfactant may alter or extract membrane proteins and lipids resulting in severe disruption of the integrity of the membrane at higher concentrations which correlates with their ability to improve absorption.

In view of the severity of the epithelial damage caused by surfactants such as Laureth-9 and sodium lauryl sulphate, at concentrations which are required to significantly improve drug absorption, it is unlikely that these compounds will be successfully exploited for nasal delivery systems for peptide and protein drugs. However, surfactants remain a useful research tool against which alternative enhancers can be assessed (O'Hagan and Illum; 1990).

1.6.2.3 Chelating agents

Compounds such as EDTA, salicylic acid and citric acid have metal ion chelating properties. Critchley (1989) showed that the absorption of desmopressin was significantly improved in rats by coadministration of a formulation containing 1% EDTA. Values of bioavailability calculated after administering either control solution or solution

containing EDTA were approximately 16% and 59% respectively. In contrast, Aungst and Rogers (1988) showed that the degree of enhancement of insulin absorption by coadministration of 5% EDTA or sodium salicylate was low via the nasal and buccal routes compared to the rectal route. EDTA was shown to increase the permeability of rabbit nasal mucosa *in vitro* (Uchida et al., 1991a and 1991b). Adjei et al. (1992) showed that EDTA improved the nasal absorption of leuprolide acetate, a luteinizing hormone releasing hormone agonist, in rats. Following coadministration of EDTA with cyclodextin, the bioavailability of leuprolide acetate was 47% compared to 37% following coadministration of cyclodextin alone and only 13% for a control solution of leuprolide acetate. Yamamoto et al. (1993) investigated the effects of sodium salicylate and EDTA on the nasal absorption of phenol red and a series of fluorescein isothiocyante dextrans (FITC-dextrans, having mean molecular weights of 4400, 9400 and 71200) in rats. Sodium salicylate was shown to be ineffective in promoting the nasal absorption of phenol red and the FITC-dextrans and EDTA only effectively enhanced the nasal absorption of the 4400 molecular weight FITC-dextran. Citric acid has also been proposed as a calcium ion chelating agent for transepithelial transport following *in vitro* studies (Cho et al., 1989). Citrate (0.1, 1.0 and 10 mM) was shown to significantly decrease the transepithelial electrical resistance across a cell monolayer. This was assumed to be an indication of the citrate opening tight junctions in the epithelium thereby enhancing paracellular solute transport.

The absorption enhancing activity of chelating agents is related to their ability to open intercellular tight junctions by chelating divalent cations thereby increasing transport by the paracellular route (Murakami et al., 1984). Divalent cations, particularly Ca^{2+} and Mg^{2+}, are important for maintaining both the structure and rigidity of cell membranes and ciliary activity (Cassidy and Tidball, 1967). The integrity of epithelial tight junctions is dependent on extracellular Ca^{2+} and following exposure to absorption enhancers, the ability of Ca^{2+} to restore the mucosal barrier function has been used to indicate a paracellular mechanism (Lee et al., 1991c). Although the chelation of divalent cations is associated with the opening of tight junctions, the site of action of the chelating agents is not known. Lee et al. (1991c) suggested that chelating agents act on other elements of the junctional complex rather than directly on the tight junction itself. One such junctional element is the Ca^{2+} dependent cell adhesion molecule uvomorulin. In addition to increasing the size of intercellular spaces, chelating agents may also increase water influx via the intercellular space. Increasing water influx may increase the absorption of hydrophilic solutes by a solvent-drag mechanism, increasing blood flow at the site of absorption or increasing the concentration gradient.

If the chelating agent decreases ciliary activity thereby mucociliary clearance, then the residence time of the dose at the site of delivery will be increased. Batts et al. (1989) demonstrated that 0.1% EDTA irreversibly halted mucociliary transport over the frog palate following one or two 10 minute applications. However, in the presence of an excess of calcium ions, sufficient to saturate the chelation sites of EDTA, the compound

was well tolerated. In a later study, 0.1% EDTA was shown to be non-ciliotoxic to explants of chick embryo (Batts et al., 1990). Furthermore, EDTA did not affect mucus rheology which is another factor, in addition to a direct action on cilia, which could influence mucociliary clearance. The differences observed between the frog palate and the chick embryo trachea were explained as being due to the differences between the two models. In the frog palate, the cilia beat in response to mechanical stimulation and are otherwise inactive. In contrast, the cilia of higher vertebrates beat in the absence of stimulation and appear to maintain a basal activity. In addition, the activity of the cilia of the frog palate are apparently more dependent on an extracellular source of calcium than chick embryo trachea. Batts et al. (1991) also evaluated the effect of EDTA on nasal clearance in human volunteers using a modified saccharin test with concomitant gamma scintigraphy (Batts et al., 1991). A solution of 0.1% EDTA (0.3 ml) was administered to the volunteers, as drops, one hour prior to the administration of a radiolabelled saccharin solution by spray (0.1-0.2 ml ejection volume). The deposition and subsequent clearance of the radiolabel was monitored by gamma scintigraphy. The time of perception of a sweet taste, indicating the presence of saccarin in the nasopharynx, was also recorded. EDTA was shown to be well tolerated by the nasal mucosa and did not significantly alter the rate of nasal spray clearance. However, the authors suggested possible explanations for the lack of toxicity of EDTA in the human study. The calcium concentrations in nasal secretions may be sufficiently high to complex with EDTA and reduce ciliotoxicity. In addition, EDTA may bind to mucus glycoproteins thereby preventing toxic amounts from reaching the cilia over the experimental period.

1.6.2.4 Fatty acids and fatty acid derivatives

Fatty acids are organic compounds which are the principal component of most lipids and consist mainly of straight chains of an even number of carbon atoms. Mishima et al. (1987) investigated the effect of medium chain fatty acid salts; sodium caprylate (C8), sodium caprate (C10) and sodium laurate (C12) on the intranasal absorption of insulin in rats. Sodium caprate at 1% was shown to be the most effective and resulted in a 10-fold increase in insulin absorption compared to an insulin solution without enhancer. Optimal absorption enhancement was shown at a sodium caprate concentration of 1%. Bioavailabilities, relative to a subcutaneous dose, for 1% sodium caprylate, caprate and laurate salts were in the region of 15%, 50% and 30% respectively.

Acylcarnitines have also been used to enhance drug absorption. These are a class of fatty acid derivatives consisting of a fatty acid connected to a quaternary amine which carries a free carboxyl group (O'Hagan and Illum; 1990). Endogenous acylcarnitines, formed from carnitine and the fatty acyl group of acyl coenzyme A, are important in the transport of long chain fatty acids across the mitochondrial membrane and are thus important in the regulation of fatty acid metabolism (Fix et al., 1986). Fix et al. (1986) showed that 2% palmitoylcarnitine was highly effective in promoting the rectal absorption of somatostatin analogue, gentamicin, cefoxitin, theophylline and methyldopa in rats. LeCluyse et al. (1991) showed that the efficacy of acylcarnitines as absorption promoters for cefoxitin administered rectally was dependent on their fatty acid chain length. Acylcarnitines with

chain lengths between 2-8 carbon units were ineffective compared to those containing 12-18 carbon units and palmitoylcarnitine (C16) resulted in maximal absorption enhancement. The presence of 1% palmitoylcarnitine in solution formulations has been shown to increase the bioavailabilitiy of human growth hormone, relative to a subcutaneous dose, from about 7% to 22% following intranasal administration in rats (O'Hagan et al., 1990).

Lysophospholipids are another class of fatty acid derivatives which have been shown to be potent absorption enhancers for a variety of drugs. Lysophospholipids are formed naturally in biological membranes by the action of phospholipase enzymes on membrane phospholipids (Stafford and Dennis; 1988). They are usually found at low concentration in most biomembranes although cellular damage and inflammatory processes can cause activation of phospholipase enzymes and increased production of lysophospholipids which have been shown to be able to stimulate macrophage production (Yamamoto and Ngwenya; 1987, Stafford and Dennis; 1988). Lysophospholipids are ampiphilic compounds consisting of a large polar head group and one long chain acyl group joined to a glycerol backbone (Stafford and Dennis; 1988). The polar head group is commonly a choline or ethanolamine moiety attached to the glycerol backbone via a phosphate ester although other moieties such as glycerol, serine and inositol are possible. The non-polar long chain acyl group consists of a saturated or unsaturated hydrocarbon chain which is attached to the glycerol backbone via an ester linkage. The length of the acyl chain may vary but most naturally occurring lysophospholipids are 16 or 18 carbon units in length. Lysophosphatidylcholine (LPC, lysolecithin) generally describes a group of compounds which each contain a choline moiety but which differ in the length of the acyl side chain. Naturally occurring LPC which is commercially derived from egg yolk phosphatidylcholine will usually contain a mixture of LPCs of different side chain length such as stearoyl (C18) and palmitoyl (C16). Relatively pure LPC homologues having specific side chain lengths and degrees of saturation can be derived synthetically.

Several studies have demonstrated the absorption promoting efficacy of LPC for drugs delivered via the nasal route. Hansen et al. (1988) showed that 0.5% lauroyl-L-α-LPC enhanced to intranasal absorption of insulin in rabbits but was found to irritate the nasal mucosa in human volunteers. Didecanoyl-L-α-LPC, administered as a microemulsion, was non-irritant to the volunteer's nasal mucosa and enhanced the intranasal absorption of insulin in rabbits. Illum et al. (1988) showed that LPC enhanced the intranasal absorption of gentamicin from a liquid formulation in the anaesthetised rat model but was less effective in the conscious sheep model. These differences were explained as the difference between the *in vivo* rat model, where mucociliary clearance is impaired, and the conscious sheep model which is physiologically more relevant since mucociliary clearance mechanisms are not impaired. However, the incorporation of LPC in a lyophilised powder formulation of gentamicin and starch microspheres improved the bioavailability of gentamicin from about 10% to 57% following nasal administration to sheep. Similar increases in nasal drug absorption by coadministering LPC with starch microspheres were demonstrated by Farraj et al. (1989) and Critchley et al. (1994). A

powder formulation of LPC with starch microspheres increase the absolute bioavailability of insulin from about 5% to 13% in sheep (Farraj et al., 1989). LPC in a solution formulation resulted in only a slight improvement in insulin bioavailability from about 1% to 2%. Critchley et al. (1994) showed that 0.2% LPC increased the nasal absorption of desmopressin in the rat model with absolute bioavailability improved from approximately 16% to 26%. In sheep, LPC had only a slight effect on desmopressin absorption improving the nasal bioavailability from about 1% to 3% compared to an improvement from about 5% to 10% in a powdered starch microsphere formulation.

In a later study, 0.5% LPC was shown to improve the nasal absorption of insulin (16.7 IU/kg) in rats (Illum et al., 1989). In the study, an LPC composed mainly of a mixture of palmitoyl (72%) and stearoyl (24%) components was shown to be as effective as LPC palmitoyl or LPC stearoyl. Chandler et al. (1994) investigated the efficacy of a range of synthetic LPC homologues in promoting intranasal insulin (8 IU/kg) absorption in rats. It was found that coadministration of LPCs which possessed 10 or more carbon groups in their fatty acid chain were effective enhancers although LPC caproyl (C6) did not appreciably promote insulin absorption. At equimolar concentrations (11.9 mM), it was shown that LPC decanoyl (C10) and LPC stearoyl (C18) were equally effective in promoting insulin absorption but that LPC caproyl (C6) was not significantly different to a control insulin formulation (without enhancer). The absorption enhancing efficacy of three concentrations of LPC decanoyl (15.2, 11.9 and 5 mM), in terms of lowering blood glucose concentrations, increased as LPC concentration decreased although the rate of fall of glucose concentrations decreased with decreasing LPC concentration. However, blood samples were only collected up to 40 minutes and so this may not fully reflect the efficacy of the various concentrations of LPC particularly since glucose concentrations after coadministration of insulin with 5 mM LPC reached a plateau or were levelling off at 40 minutes in contrast to the glucose concentrations which were still falling at the 40 minute time point when 11.9 mM LPC was coadministered. Lysophosphatidylglycerol (LPG) at 0.0625% and 0.5% was shown to be equally effective in promoting the nasal absorption of insulin despite an approximately 10-fold difference in the LPG concentration. The extent of insulin absorption was shown to be similar to that obtained with LPCs containing 10 or more carbon groups on the fatty acid chain.

LPC at 0.2% was shown to be more effective in enhancing the absorption of human growth hormone (hGH), via the nasal route, than 1% palmitoylcarnitine (O'Hagan et al., 1990). In studies in rats, the presence of LPC increased the bioavailability of 1 mg/kg hGH, relative to a subcutaneous dose, from about 7% to 29%. Fisher et al. (1991) further investigated the effect of LPC on the intranasal absorption of hGH in three animal species finding that the co-administration of LPC with hGH consistently increased hGH absorption. Bioavailability of hGH was improved from about 2% to about 18% in rats with 0.5% LPC (1 mg/kg hGH) and in sheep from 0.2% to about 16% with 0.585% LPC (0.308 mg/kg hGH) and the amount of hGH absorbed was shown to be proportional to the concentration of LPC in the nasal solution. In rabbits, a concentration of 0.2% LPC (0.308 mg/kg hGH) increase bioavailability from about 1% to 73%.

The mechanisms by which fatty acids and fatty acid derivatives act as absorption enhancers are not fully understood. Mishima et al. (1987) demonstrated the membrane damaging activity of medium chain fatty acid salts by investigating their haemolytic activity. Sodium laurate was shown to have the highest haemolytic potency resulting in approximately 75% haemolysis at 0.1% compared to only about 4% haemolysis for 0.1% sodium caprate although at 0.5% both compounds resulted in a similar degree of haemolysis (approximately 68-74%). In contrast, sodium caprylate resulted in only about 2% haemolysis at concentrations up to 1%. Mishima et al. (1987) also investigated the ability of the fatty acid salts to sequest calcium ions finding that sodium caprylate, sodium caprate and sodium laurate (concentration of 1M) were able to bind 0.02, 0.35 and 0.42 M of calcium ions respectively, compared to about 1.08 M for the same concentration of EDTA. The sequestration of ions was shown to increase with increasing alkyl chain length of the fatty acid salts. The fatty acid salts were also shown to inhibit leucine aminopeptidase activity in rat and rabbit nasal homogenates in the order; sodium laurate > sodium caprate > sodium caprylate. The results are not conclusive, suggesting that absorption enhancement may involve a combination of factors including damage to the nasal membrane, sequestration of ions and hence the opening of tight junctions and inhibition of proteolytic enzymes in the nasal mucosa.

The damaging effects of acyl carnitines on erythrocyte membranes have been demonstrated by Cho and Proulx (1969 and 1971). The acyl carnitines were generally shown to be potent haemolytic agents on rat and human erythrocytes (Cho and Proulx; 1969). Haemolytic potency was shown to decrease as the fatty acid chain length of the acyl carnitine decreased with little or no haemolytic activity for chain lengths of C10 and below. The haemolytic action of the acyl carnitines coupled with their ampiphilic nature suggests that absorption enhancement is due to a surfactant-like effect on mucosal membranes (O'Hagan and Illum, 1990). Fix et al. (1986) demonstrated that enhancement of the rectal absorption of sodium cefoxitin by palmitoyl carnitine was reversible within 60-120 minutes indicating no gross mucosal damage. Furthermore, histological examination of intestinal or rectal tissue showed that even after prolonged exposure to 1% palmitoyl carnitine there was only slight alteration in the mucosal structure. Significant rectal absorption enhancement was observed at concentrations of palmitoyl carnitine which did not alter mucosal structure. LeCluyse et al. (1991) investigated the relationship between *in vivo* enhancement of drug absorption and *in vitro* perturbation of brush border membranes from rat intestine by acyl carnitines of varying chain length (C2-C18). Acyl carnitines of chain length less than 12 carbon units were ineffective in enhancing cefoxitin absorption and did not result in a decrease in the lipid order of brush border membranes used as an indication of the membrane perturbing effects. Acyl carnitines of chain length 12-18 carbon units effectively promoted cefoxitin absorption and decreased lipid order. However, the membrane perturbing effects differed among the C12-C18 acyl carnitines and did not correlate with their absorption enhancing efficacy. The authors concluded that the acyl carnitines must possess a critical chain length, above 10 carbon units, in order to partition into the membrane and must perturb the membrane lipid order beyond a threshold value (15-20%) to effectively

enhance absorption. Richardson et al. (1989) showed that the intravaginal administration of 1% palmitoyl carnitine to ovariectomised rats resulted in moderate epithelial damage which was suggested as a partial mechanism for enhanced gentamicin absorption. Although the thickness of vaginal epithelia was reduced compared to control animals by treatment with palmitoyl carnitine the damage was not as severe as that following administration of Laureth-9 and LPC which both resulted in extensive cell loss. The literature suggests that acyl carnitines, like other fatty acid 'derivatives', act as absorption enhancers due to their surfactant-like effect on mucosal membranes which may also result in epithelial disruption. However, other mechanisms such as enzyme inhibition and calcium ion chelation may also be involved to some extent.

Lysophospholipids, being ampiphilic surface-active agents probably act as absorption enhancers by a mechanism similar to that of the surfactants (O'Hagan and Illum; 1990). LPC has been demonstrated to be a potent haemolytic agent (Cho and Proulx; 1971, Weltzien et al., 1977, Weltzien; 1979, Martin et al., 1992, Jabbal Gill et al., 1994a, Chandler et al., 1995). Martin et al. (1992) showed that LPC caused 50% haemolysis at a concentration of 0.07 mM (pH 6 and 8) and 0.09 mM (pH 7). Jabbal Gill et al. (1994a) demonstrated that LPC was a more potent haemolytic agent than other surfactants such as Laureth-9 and GDC causing 100% haemolysis at a concentration as low as 0.04 mM (0.002%). Chandler et al. (1995) compared the membrane activity of three LPC homologues (stearoyl C18, decanoyl C10 and caproyl C6). LPC caproyl, investigated up to concentrations of 20 mM did not result in erythrocyte haemolysis (results corrected for background haemolysis). LPC decanoyl was haemolytic at concentrations greater than about 5 mM (0.21%) and resulted in approximately 50% haemolysis at a concentration of 20 mM. In contrast, LPC stearoyl was a potent haemolytic agent resulting in 50% haemolysis at 0.01 mM and approximately 100% haemolysis at concentrations above 0.02 mM (0.0011 %). Thus decreasing the length of the LPC fatty acid side chain decreased the haemolytic activity and supported the findings of Weltzien (1977).

Previously, Chandler et al. (1994) showed that at concentrations of 11.9 mM LPC stearoyl and LPC decanoyl were equi-effective in promoting intranasal insulin absorption in the rat but that LPC caproyl was ineffective which together with the results from the haemolysis studies suggests that disruption of the nasal membrane is a prominent mechanism for the absorption enhancing efficacy. LPC caproyl did not promote insulin absorption, even at a concentration of 17.6 mM (0.625%) and lacked haemolytic activity. In contrast, LPC stearoyl was shown to be a potent haemolytic agent at a concentration well below that which enhanced insulin absorption. However, other mechanisms may be involved since LPC decanoyl was also shown to be an effective enhancer at a concentration (5.0 mM) at which it was not haemolytic. The enhancing efficacy along with haemolytic activity increased with increasing concentration of LPC decanoyl. As pointed out by Chandler et al. (1995), care must be taken when extrapolating the results from studies using a model membrane system, such as erythrocyte haemolysis studies, to the effects on the nasal membrane.

Chandler et al. (1991b), using histological techniques, showed that 0.625% LPC (stearoyl/palmitoyl mixture) caused severe damage to rat nasal mucosa. The thickness of the nasal epithelium was reduced in parts to a thin layer of squamous cells and cells were lost from the epithelium and were observed in the nasal lumen mixed with large quantities of mucus. In further histological studies in the rat, Chandler et al. (1994) evaluated the nasal membrane damaging effects of formulations containing insulin and LPC caproyl or LPC decanoyl at equimolar concentrations (11.9 mM). LPC caproyl, which did not promote insulin absorption, showed signs of slight membrane interaction but this was regarded as comparable to that which resulted from administration of a control solution of insulin. Membrane disruption following the administration of LPC decanoyl was only marginally increased compared to LPC caproyl. However, LPC decanoyl promoted insulin absorption to a similar extent as the LPC stearoyl/palmitoyl mixture which was shown to severely disrupt the nasal epithelial membrane. In addition, LPG at 0.5% was shown to cause membrane damage which was comparable to that caused by LPC stearoyl/palmitoyl mixture but that 0.0625% LPG resulted in only slight membrane damage, comparable in that caused by the control solution of insulin, despite enhancing the absorption of insulin to a similar extent as 0.5% LPG. Thus, it appears that drug absorption is possible without severe membrane disrupting effects by careful selection of the lysophospholipid and the concentration used.

Martin et al. (1978) showed that LPC had mucolytic activity and that the viscosity of mucus decreased with increasing LPC concentration. In contrast, phosphatidylcholine showed no mucolytic activity. At concentrations of 6 and 20 mM, mucus viscosity was decreased in the region of 50% and 80% respectively. Thus, mucolytic activity may be an important contribution to the absorption enhancing mechanism of lysophospholipids.

The mechanisms by which fatty acids and fatty acid derivatives acts as absorption enhancers has not been fully elucidated although membrane perturbation appears to be an unfortunate feature of these compounds. This would severely limit the use of these compounds for commercial application.

1.6.2.5 Enzyme inhibitors

The exact nature of the nasal enzymatic barrier *in vivo* and the extent to which it affects intranasal peptide delivery has not been fully elucidated. However, *in vitro* and *in situ* studies indicate that enzymatic degradation may constitute a significant barrier to peptide absorption in the nasal cavity particularly for smaller peptides (Illum, 1992). Enzyme inhibitors have been extensively shown to inhibit the degradation of peptides in mucosal homogenates but few studies have reported the use of enzyme inhibitors to improve the bioavailability of peptides.

Several of the classes of compounds previously mentioned, such as bile salts, surfactants and fatty acids, have been shown to possess enzyme inhibitory action which may contribute to their overall efficacy as absorption enhancers. However, enzyme inhibition does not appear to be the main mode of action of these compounds. Aprotinin is a

protease inhibitor which has been used to prevent the proteolytic degradation of proteins and polypeptides during purification from biological fluids (Hewlett; 1990). Aprotinin will inhibit a variety of enzymes belonging to the serine protease group, such as trypsin, chymotrypsin, plasmin and kallikrein, by binding to the active site of the enzyme. The oral absorption of vasopressin in rats was shown to be increased by coadministration with aprotinin which was attributed to the inhibition of proteolytic enzymes, such as trypsin, in the gastrointestinal tract thereby preventing or limiting the degradation of vasopressin (Saffran et al., 1988). Morimoto et al. (1992) showed that aprotinin enhanced the nasal absorption of salmon calcitonin in rats. However, in the same study, the same concentrations of aprotinin did not enhance the intranasal absorption of vasopressin and desmopressin. A later study reported that aprotinin improved the bioavailability of salmon calcitonin after nasal administration from 5.4% to 7.5% at pH 4 and from 3.2 to 6.9% at pH 7 respectively (Morimoto et al., 1995).

Bestatin is an enzyme inhibitor which has been investigated as an absorption enhancer. Bestatin has been reported to specifically inhibit aminopeptidase B and leucine aminopeptidase *in vitro* and furthermore it has been shown to have low toxicity following intraperitoneal injection (300 mg/kg) in mice (Umezawa et al., 1976). Critchley (1989) reported that bestatin (10 μg/ml) increased the bioavailability of desmopressin, compared to a control solution, by 8%. However, following nasal administration to rats, bestatin at a concentration of 0.015% did not enhance the absorption of hGH (O'Hagan et al., 1990). Hanson et al. (1986) showed that bestatin did not improve the nasal absorption of salmon calitonin in rats. This was recently confirmed in studies by Morimoto et al. (1995). O'Hagan et al. (1990) also studied the effects of amastatin, a potent inhibitor of leucine aminopeptidase and a less potent inhibitor of aminopeptidase A, at a concentration of 0.015%. Amastatin was shown to improve intranasal hGH absorption in rats, relative to a subcutaneous dose, from about 7% to 29%. Critchley (1989) also demonstrated that puromycin, which had been shown to inhibit the degradation of enkephalin *in vitro* by Barclay and Phillipps (1978), improved the bioavailability of desmopressin, compared to a control solution, by 4%. *In situ* studies have shown that boroleucine, an α-aminoboronic acid derivative, is 100 and 1000 times more effective than bestatin and puromycin, respectively in preventing the degradation of leucine-enkephalin in nasal perfusate (Hussain et al., 1989). Furthermore, in contrast to bestatin and puromycin, boroleucine was shown to be a potent but reversible inhibitor of nasal aminopeptidase enzymes (Hussain et al., 1990). However, effective absorption promotion using α-aminoboronic acid derivatives has not been demonstrated. Bacitracin, a less potent inhibitor of aminopeptidase activity than bestatin or puromycin which has been used as an antimicrobial agent, has been shown to enhance the nasal absorption of two analogues of growth hormone releasing hormone (GH-RH) in rats (Raehs et al., 1988). This may indicate that there are additional mechanisms involved in the absorption enhancing efficacy of this compound. *In vitro* studies showed that bacitracin irreversibly arrested cilia motility within one hour after exposure for 20 minutes. Differences in the absorption promoting efficacy of enzyme inhibitors appears to be also dependent on the characteristics of the drug molecule. In the study by Morimoto et al. (1992), the

inhibition of trypsin activity in the nasal mucosa was reported to be useful for enhancing the absorption of salmon calcitonin but not for the absorption of vasopressin or desmopressin. Likewise, in studies in rats, bestatin was reported by Critchley (1989) to increase the nasal absorption of desmopressin but Morimoto et al. (1995) showed that it was ineffective for the nasal delivery of salmon calcitonin.

Despite the limited number of studies performed, the degree of absorption promotion of peptide drugs by coadministration with enzyme inhibitors does not appear to have the same magnitude as that demonstrated by utilising other enhancing systems. Although enzyme inhibition may be an important factor contributing to the mechanism of action of some absorption promoting compounds, enzyme inhibition alone does not appear be a sufficient factor to increase the bioavailability of intranasally administered drugs.

1.6.2.6 Other absorption enhancers

Cyclodextrins

Cyclodextrins are cyclic oligosaccharides which have been used to improve the physical and chemical properties of drug molecules in solution through the formation of inclusion complexes (Irie et al., 1992). During the formation of peptides / cyclodextrin inclusion complexes, the hydrophobic residue of the peptide guest molecule penetrates into the 'cavity' of the cyclodextrin host molecule (Irwin et al., 1994). This will reduce the concentration of free drug in solution and may have a significant effect on aqueous solubility and chemical stability. The poor water solubility of lipophilic drugs can be overcome by the formation of guest-host complexes (Rao et al., 1990). The common types of cyclodextrin are α- (α-CD), β- (β-CD) and γ-cyclodextrin (γ-CD) containing 6, 7 or 8 glucopyranose units, respectively, which are linked forming a torus-shaped, apolar, electron-rich hydrophobic cavity (Jabbal Gill et al., 1994a). Chemically modified cyclodextrins are also available, such as hydroxy-propyl-β-cyclodextrin (HP-β-CD) and dimethyl-β-cyclodextrin (DM-β-CD), which have improved solubility characteristics compared to the parent compound (Yoshida et al., 1988).

DM-β-CD was shown to significantly enhance the nasal absorption of 17β-estradiol, compared to a suspension of 17β-estradiol, in both rats and rabbits (Hermens et al., 1990). Compared to a suspension of 17β-estradiol, coadministration with DM-β-CD improved the absolute bioavailability of estradiol from about 23% to 67% in rats and 25% to 95% in rabbits. The effect of DM-β-CD as a solubilising agent for the poorly water soluble estradiol was attributed as an important mechanism for its absorption enhancing efficacy. DM-β-CD has surfactant activities, in-contrast to the underivatized parent β-CD, which may also be important mechanism for its absorption enhancing properties. DM-β-CD was shown to have only a minor effect on the cilia beat frequency on human adenoid tissue and the authors suggested that the formulation containing DM-β-CD is biocompatible and suitable for chronic nasal administration. The same study group investigated the effect of various cyclodextrins on the intranasal absorption of insulin in rats (Merkus et al., 1991). All cyclodextrins were investigated at concentrations of 5%

with the exception of β-CD which owing to its lower solubility was investigated at 1.8%. DM-β-CD was the most effective giving rise to an insulin bioavailability of about 110% compared to an intravenous dose. Coadministration with α-CD was less effective (absolute bioavailability approximately 28%) and β-CD, HP-β-CD and γ-CD were ineffective (absolute bioavailabilities of about 2.9, 1.2 and 0% respectively). The influence of the cyclodextrins on ciliary beat frequency was investigated using chick embryo tracheal tissue and both DM-β-CD and α-CD were shown to induce ciliostasis 30-40 minutes after exposure. The ciliostatic effect of DM-β-CD appeared to be reversible (reversibility of ciliostasis induced by α-CD was not evaluated). The cilia beat frequency, 60 minutes after exposure to β-CD, HP-β-CD and γ-CD was reduced to 63, 78 and 77% respectively. In a later study in rabbits, DM-β-CD was shown to be more effective as a nasal absorption enhancer for insulin when coadministered in a powder formulation rather than in a liquid formulation (Schipper et al., 1993). The insulin / DM-β-CD formulation which had previously been shown to result in complete insulin absorption in the rat was ineffective when tested in the rabbit giving rise to an insulin bioavailability of approximately 1%. This was attributed to substantial inter-species differences in the absorption enhancing effect of DM-β-CD. However, the administration of a powder formulation of insulin and DM-β-CD improved insulin bioavailability to about 13%.

Jabbal Gill et al. (1994a) showed that cyclodextrins may be useful as protective agents against the potential damage induced by absorption enhancers used in nasal delivery systems. Using rat erythrocytes as a model biomembrane, enhancers were shown to be potent haemolytic agents in the order LPC > Laureth-9 > GDC. The two cyclodextrins investigated, HP-β-CD and γ-CD, showed low degrees of haemolysis. By combining the cyclodextrins with the enhancers it was possible to reduce or prevent haemolysis. As the haemolytic potency of the enhancer increased, greater concentrations of cyclodextrins were required to prevent haemolysis. The coadministration of cyclodextrins without absorption enhancers did not dramatically improve insulin absorption compared to a control solution. The enhancers, LPC, Laureth-9 and GDC, were shown to increase insulin absorption but in the presence of cyclodextrins, with the exception of Laureth-9 in which the enhancing effect was maintained, that of LPC and GDC was considerably reduced. However, sustained absorption of insulin was observed following coadministration of GDC with γ-CD. Histological studies, evaluating Laureth-9, GDC and the cyclodextrins, did not consistently correlate with insulin absorption results. However, the histological results complemented the findings of the haemolysis studies and the enhancer / cyclodextrin combinations were observed to cause the least damage to the rat nasal epithelium. Enhancer / cyclodextrin combinations may provide the necessary activity and safety required for nasal drug delivery systems.

Shao et al. (1992) investigated the possible mechanisms of action of cyclodextrins in promoting the nasal absorption of insulin by using a rat *in situ* perfusion method. Concentrations of 5% were used with the exception of β-CD which was used at 1.8%. The relative effectiveness of the cyclodextrins in promoting insulin absorption was

reported to be in the order γ-CD < HP-β-CD < β-CD < α-CD < DM-β-CD. The absorption enhancing efficacy of the cyclodextrins correlated with the release of both protein and phospholipid from the nasal membrane, indicating the degree of membrane damage, which was measured in the perfusate. The magnitude of cyclodextrin induced damage, in terms of solubilising membrane components, was further evaluated by measuring enzymic activity of membrane-bound 5'-nucleotidase (5'-ND) and intracellular lactate dehydrogenase (LDH) following perfusion of DM-β-CD or HP-β-CD. It was shown that the activity of 5'-ND and LDH was considerably increased by perfusion with DM-β-CD confirming the findings that DM-β-CD induces relatively more membrane damage. In contrast, HP-β-CD resulted in only minimal disruption of the epithelial membrane. The membrane disrupting effect of DM-β-CD was shown to depend on the cyclodextrin concentration with less membrane disruption observed with 1.8% DM-β-CD compared to 5% DM-β-CD. Cyclodextrins were also shown to be able to dissociate insulin hexamers into smaller aggregates which by increasing the diffusion of insulin across the nasal membrane may provide an additional mechanism for the absorption enhancing efficacy of cyclodextrins. In addition to membrane disruption, other factors may contribute to the absorption enhancing properties of the cyclodextrins. Complex formation with drug molecules may improve the aqueous solubility of some drugs and may also protect the drugs to some extent from proteolytic degradation. Cyclodextrins may also have a direct inhibitory effect on proteolytic enzyme activity. Irwin et al. (1994) demonstrated that the stability of leucine enkephalin in nasal enzymic systems (mucosal homogenates or nasal wash fluid) was improved by β-CD. However, γ-CD did not stabilise leucine enkephalin which was attributed to the larger cavity diameter of the γ-CD resulting in less complexed peptide.

Bioadhesive systems

Rapid mucociliary clearance of drug formulations which are deposited in the nasal cavity is thought to be an important factor for the low bioavailability of drugs administered intranasally (Illum et al., 1987). Increasing the residence time of the drug formulation in the nasal cavity will increase the time of contact between the drug and the nasal mucosa which may increase drug absorption. The clearance of solutions administered in the form of a spray from the nasal cavity has been shown to decrease as solution viscosity is increased by the inclusion of hydroxypropyl methylcellulose and has been suggested as a possible means by which the therapeutic effect of nasal spray preparations may be increased (Pennington et al., 1988).

Several approaches have been used to increase the residence time of drug formulations in the nasal cavity. Compounds which increase the viscosity of solution formulations may be used to prevent drainage of the formulation and slow-down mucociliary clearance from the nasal cavity. Compounds having bio- or mucoadhesive properties, enabling them to interact with the mucus layer, have also been used in solution or powder delivery systems to increase the residence time of drug formulations in the nasal cavity. Bioadhesion involves intimate contact between- and interpenetration of the bioadhesive and the mucus layer with the formation of chemical bonds (Duchêne et al., 1988, Gu et

al., 1988). The bioadhesive properties of a compound will depend on the nature of the compound and on the nature of the surrounding media. Viscosity enhancers and bioadhesives used in drug delivery systems are usually polymeric materials. Many of the compounds used have both bioadhesive and viscosity enhancing properties when in solution.

Morimoto et al. (1985) used carbopol, a derivative of polyacrylic acid which forms aqueous gels at low concentrations, to enhance the intranasal absorption of insulin and calcitonin in rats. Carbopol has been used to increase the viscosity of solutions and has also been reported to have excellent mucoadhesive properties (Smart et al., 1985, Duchêne et al., 1988, Junginger; 1990). Park and Robinson (1987) suggest that the bioadhesive properties of carbopol are due to the formation of hydrogen bonds between carbopol carboxy groups and suitable groups in the mucus. In the study by Morimoto et al. (1985) both 0.1% and 1.0% carbopol promoted insulin absorption. However, absorption with 1.0% carbopol was slower than that observed using 0.1% carbopol which was attributed to an optimum concentration / viscosity of the gel base. Thus, at concentrations above the optimum, the increased viscosity slows down the release of insulin from the gel base. Carbopol at a concentration of 0.1% also increased calcitonin absorption. Morimoto et al. (1987) showed that carbopol gel at concentrations of 0.05, 0.1 and 0.5%, were ineffective in promoting the intranasal absorption of nifedipine in rats. However, sustained absorption of nifedipine was observed by coadministration in a gel composed of 0.05% carbopol in 50% PEG.

Critchley (1989) showed that coadministration of 2% carbopol improved the nasal bioavailability of desmopressin from about 16% to 78% in rats but it had no effect on gentamicin absorption which was suggested to be due to an interaction between positively charged gentamicin and negatively charged carbopol. In addition, DEAE dextran, a cationic polymer exhibiting viscosity enhancing and mucoadhesive properties, was shown to improve desmopressin absorption (absolute bioavailability of 60%). The difference in the performance of carbopol and DEAE dextran was suggested to be due to DEAE dextran (a polycation) becoming tightly bound to the mucus layer (containing negatively charged binding sites) due to electrostatic interactions which create a barrier to drug release. Binding may also occur between the polycation and anionic sites on the mucosal surface which are a common feature of membranes (Quinton and Philpott; 1979). Chu et al. (1991) described a carbopol sustained release gel system for intranasal drug delivery. The formulation was of low viscosity for ease of spray administration to the nasal cavity but formed a highly viscous gel on contact with the moist nasal mucosa due to the change in solvent composition. The authors reported a correlation between viscosity and bioavailability following the evaluation of nasal formulations of propranolol in dogs which was suggested to be due to the increased residence time of the viscous formulations in the nasal cavity.

Carboxymethylcellulose is another compound which has been reported to have excellent bioadhesive properties (Duchêne et al., 1988, Junginger; 1990). However, a liquid

formulation containing 1% carboxymethylcellulose did not promote nasal insulin absorption in the rat despite having a similar viscosity to the carbopol formulation which had been shown to promote insulin absorption (Morimoto et al., 1985). This suggested that viscosity and bioadhesive properties were not the only factor which affected insulin absorption. The authors suggested that water absorption from the gel base (water influx) would allow the movement of hydrophilic and macromolecular compounds through intracellular channels. Thus, polyacrylic acid was suggested to promote drug absorption via the paracellular route rather than through the transcellular route. Junginger (1990) has suggested that carbopol may bind calcium ions thus opening intracellular tight junctions in the nasal mucosa. Cellulose ethers such as methylcellulose and hydroxypropylmethylcellulose (HPMC) have been shown to improve the nasal absorption of desmopressin in the rats (Critchley 1989). The study was intended to investigate the effect of viscosity enhancing agents rather than bioadhesives although the bioadhesive properties of methylcellulose and HPMC have been described as excellent and satisfactory respectively (Junginger; 1990) and hence it is difficult to distinguish between viscosity and bioadhesive effects. Critchley (1989) showed that the absolute bioavailabilities of desmopressin from formulations containing 2% methylcellulose (viscosity at 37°C 185.2 mPas) or 2% HPMC (viscosity at 37°C 20.6 mPas) were approximately 28% and 51% respectively. The lower absorption for the methylcellulose formulation was attributed to its higher viscosity which would limit the spreading of the solution in the nasal cavity resulting in a longer path length for diffusion of the drug. Harris et al. (1988b) in a study in humans, showed that the addition of 0.25% methycellulose to a solution of desmopressin resulted in an increase of the residence time of the formulation in the nasal cavity. However, this was not accompanied by an increase in the bioavailability of desmopressin compared to a control solution although slower and more prolonged absorption was reported from the methylcellulose formulation (Harris et al., 1989).

Nagai et al. (1984) investigated powder dosage forms of insulin for nasal administration in dogs. Various excipients were evaluated in this study including the viscosity enhancing agents hydroxypropylcellulose (HPC) and neutralised carbopol prepared as blends of excipient and freeze-dried insulin. The use of neutralised carbopol resulted in the sustained absorption of insulin which was explained as being due to the excipient forming a viscous gel incorporating insulin on contact with the nasal mucus which adhered to the mucosa and increased the residence time of the formulation in the nasal cavity. Insulin was absorbed to about the same extent as in the neutralised carbopol formulation by using HPC although the effect was not sustained which may be due the HPC forming a less viscous gel in the nasal cavity and also being a poorer bioadhesive material. Freeze-dried formulations containing insulin and carbopol resulted a dramatic lowering of plasma glucose concentrations, used as an indication of insulin absorption, following nasal administration in the dogs and it was found that greater sustainment of insulin absorption was observed by increasing carbopol concentration. The excipients used in the study were reported to be non-irritant to nasal mucosa following administration to human volunteers.

Bioadhesive microsphere powder delivery systems for intranasal drug delivery have been extensively investigated. The retention of powdered microsphere systems (albumin, starch and DEAE dextran) in the nasal cavity following administration to human volunteers has been investigated by Illum (1986) and Illum et al. (1987). The retention of the microsphere systems in the nasal cavity was attributed to the microspheres imbibing water, swelling and forming a gel like layer on contact with the nasal mucosa. The three microspheres systems were described as having good bioadhesive properties with binding suggested as being via hydrogen bonds for albumin and starch microspheres and ionic bonding for DEAE dextran. A study in rats evaluated degradable starch microspheres (mean diameter of 45 µm) for the nasal delivery of insulin finding that the bioavailability of insulin, compared to an intravenous dose was about 30% (Björk and Edman; 1988). Illum et al. (1988) showed that the bioavailability of intranasally administered gentamicin administered as a powder with degradable starch microspheres (mean diameter 48 µm) was approximately 10% compared to about 1% for a simple solution of gentamicin. The nasal dose of starch microspheres was 2.5 mg/kg. The absolute bioavailability of gentamicin was further improved to approximately 57% by a combination of starch microspheres and LPC. Although the bioavailability of gentamicin from a nasal solution with LPC was not given the absorption profile was very much similar to that of the simple gentamicin solution. The same study group showed that the starch microsphere delivery system improved the absorption of insulin in sheep (Farraj et al., 1990). The absolute bioavailability of insulin administered with starch microspheres (2.5 mg/kg) was 4.5% compared to about 1% for a solution of insulin without absorption enhancers. The absorption of insulin was further improved by the coadministration of LPC with starch microspheres with an absolute bioavailability of about 13% and bioavailability relative to the subcutaneous route of almost 32%. The coadministration of LPC with insulin in a solution formulation resulted in an absolute bioavailability of about 2%. However, the dose of LPC administered to sheep from the solution formulation was considerably less (0.02 mg/kg) than from the powder formulation (0.2 mg/kg). A combination of LPC and starch microspheres (2.5 mg/kg) has also been shown to improve the nasal absorption of hGH (Illum et al., 1990) and desmopressin (Critchley et al., 1994) in sheep. For hGH, bioavailabilities, relative to the subcutaneous route, by coadministration with LPC as a solution, or in the form of powders with starch microspheres or LPC / starch microspheres were 0.1%, 2.7% and 14.4%, respectively. For desmopressin, bioavailabilities, relative to intravenous route, were 4.7% with starch microspheres and 9.6% with LPC / starch microspheres.

It has been reported that degradable starch microspheres are biocompatible and do not induce serious histopathological changes to nasal mucosa (Björk et al., 1991). Starch microspheres were administered twice daily to the left nostril of rabbits (10 or 20 mg doses) for 2, 4 or 8 weeks. The right, undosed, nostril of each animal was used for control purposes. There were no macroscopic alterations in the nasal mucosa (such as changes in the mucosal architecture, inflammatory reaction or ulcerations) due to the administration of starch microspheres and scanning electron microscopy showed that nasal epithelial cells and cilia remain intact. The authors concluded that starch

microspheres have negligible effects on nasal mucosa. In the study by Björk et al. (1991) the dose of starch microspheres administered to rabbits was 5 or 10 mg/kg for 10 or 20 mg doses, respectively, which is much higher than the doses of about 2.5 mg/kg which were shown to effectively promote drug absorption in the studies by Illum et al. (1988, 1990), Farraj et al. (1990) and Critchley et al. (1994).

Björk et al. (1992) showed that that the administration of 10 mg degradable starch microspheres into each nostril of 15 healthy volunteers once daily for eight days was well tolerated and there was no significant changes in mucociliary transport within this period. *In vitro* studies using a cell model, originating from a human intestinal epithelial (Caco-2) cell line, showed that degradable starch microspheres pre-applied to the mucosal side of the cell monolayer increased the transport of both mannitol and insulin (applied at later stages) across the cell layer (Artursson; 1990). The effect of the starch microspheres on the permeation of mannitol and insulin was shown to be reversible. Transmission electron microscopy showed that the cells and cilia of the cell monolayer following exposure to starch microspheres were intact but that the intracellular tight junctions were opened to a higher degree. The tight junctions were shown to be closed again after 180 minutes. Thus, the opening and closing of the tight junctions correlated with the reversible effect of starch microspheres on the permeation of mannitol and insulin indicating absorption via the paracellular route. In a recent paper by this study group, starch microspheres coated with insulin were shown to induce a pulsed delivery of insulin across Caco-2 cells *in vitro* which lasted for 1-2 hours (Björk et al., 1995). The pulsed delivery correlated with a reversible separation of the intracellular tight junctions. The effect on tight junctions was thought to involve hydration of the starch microspheres since prehydrated microspheres had no effect on transport across the cell monolayer. The hydration capacity of the starch microspheres is 8-10 times their own dry volume and thus on hydration the mean diameter increases from 20 μm to 45 μm. Hydration of the microspheres was suggested to cause a hydrostatic pressure within the intracellular space that results in the structural separation of the tight junctions.

Other types of microsphere have also been investigated. In studies in rats, powder formulations incorporating insulin and dextran microspheres (Sephadex) or DEAE-dextran microspheres (DEAE-Sephadex) have been investigated as systems for nasal delivery (Rydén and Edman; 1991 and 1992). Sephadex was shown to promote insulin absorption, assessed from the lowering of plasma glucose concentrations, although DEAE-Sephadex had no affect on glucose concentrations and these results were shown to correlate with the *in vitro* release characteristics of insulin from the microsphere systems. However, Sephadex appeared to be less effective in promoting insulin absorption in rats than the starch microspheres used previously (Björk and Edman, 1988). Dextran microspheres which were coated with insulin were show to be more effective in terms of promoting insulin absorption in the rat than when insulin was loaded into the microspheres (Pereswetoff-Morath and Edman; 1995). Incomplete or slower release of insulin contained within the microspheres is expected since moisture must penetrate into the microspheres for complete swelling of the dextran matrix prior to dissolution and

diffusion of the insulin into the nasal cavity. In contrast, insulin coated onto the surface of the microspheres can begin to dissolve on contact with the moist nasal mucosa. Bioadhesive microspheres prepared from human serum albumin have been reported to sustain the absorption of propranolol administered intranasally to dogs (Vyas et al., 1991). Illum et al. (1994) have investigated hyaluronic acid ester (Hyaff 11) microspheres for the nasal delivery of insulin in sheep. These are derived from hyaluronic acid, a natural water-retaining and lubricating polysaccharide found in high concentrations in soft body tissues (Benedetti; 1994). The Hyaff 11 microspheres (mean diameter 10 μm) were shown to effectively promote insulin absorption to a degree similar to that obtained by Farraj et al. (1990) in sheep using a starch microsphere powder system. The bioavailability of intranasally administered insulin increased as the dose of Hyaff 11 microspheres was increased (For doses of 0.25, 0.50, 1.0 and 2.0 mg/kg Hyaff 11 microspheres values of bioavailability, relative to a subcutaneous dose, were 7.7, 10.0, 11.5 and 14.5% respectively). The mechanism of action of these microspheres is unclear. However, some degree of bioadhesiveness, an appropriate size range and the ability to release the drug from the microspheres *in vivo* were considered as factors. The effect of Hyaff 11 microspheres on intracellular tight junctions was not known.

1.6.3 General problems in the development of nasal delivery systems

The main barriers preventing the use of the nasal route for the delivery of the majority of peptide and protein drugs are rapid clearance or degradation of the administered drug formulation from the nasal cavity and/or poor absorption of the drug across the nasal epithelium. The very low bioavailabilities obtained severely restricts the commercial exploitation of the nasal route for the delivery of the majority of peptide and protein drugs. Exceptions are compounds, such as calcitonin nasal spray developed by Sandoz Pharma, which, although poorly absorbed via the nasal route (bioavailability of approximately 1%) are marketable owing to their high potency and relatively low molecular weight. Thus, therapeutic plasma concentrations of these drugs can be achieved at relatively low doses. By using absorption enhancers, used here as a 'loose-term' to describe any compound which can modulate nasal absorption, it may be possible to commercially exploit the nasal route for many other peptides and proteins. Ideally, the absorption enhancer should be rapid-acting, result in transient and reversible modulation of the absorptive properties or physiology of the nasal mucosa and not be absorbed systemically. The degree of absorption enhancement should be predictable and reproducible. Ultimately the compound must be safe for nasal administration particularly as many nasal preparations of peptide and protein drugs would be used in chronic therapeutic regimens which could require one or more daily doses. A full evaluation of the safety of potential enhancers must be made before it is possible to exploit such compounds on a commercial basis. However, many of the enhancers investigated have been shown to cause damage to the nasal mucosa at the concentrations required to promote nasal absorption. Thus, research groups are continually investigating novel materials or better use of existing materials to fit the criteria of the ideal absorption enhancer. The novel use of chitosan, a versatile biomaterial that finds many commercial

applications (Sandford 1989), as an absorption enhancer in nasal drug delivery systems appears promising based on various published reports and the findings of this project. A review of chitosan is presented below.

1.7 Chitosan

1.7.1 Sources of chitosan

Certain fungi contain chitosan as part of their cell walls although it is seldom found in nature (Arcidiacono et al., 1989). Commercially it is obtained by the N-deacetylation of chitin, a biopolymer widespread in nature. The abundance of chitin is second only to cellulose to which it is structurally similar. Chitin is a major component of the cuticular or exoskeletal structures of most invertebrates. It is found in the cell walls of most fungi and yeasts and is synthesised by some unicellular organisms (Jeuniaux et al., 1988). It is estimated on a global basis that krill, marine crustacea of the order Euphausiacea, can potentially provide the highest yield of annually accessible chitin (Brine, 1984). However, commercially, the highest yields of chitin, per total dry weight of material, are obtained from the calcified cuticles (shells) of decapod crustaceans mainly crabs and shrimp (also prawn, lobster and crayfish). These provide a readily available source of chitin as part of the waste material from shellfish processing plants. Reliability of the source for chitin production is ensured because of the seasonal harvesting of shellfish (Sandford, 1989). In several countries, including the USA, Japan, Norway, Italy and India, shellfish waste is commercially exploited for the production of chitin or chitosan (Nicol, 1991). The processing of shellfish waste currently satisfies the demand for chitin and chitosan. However, with increasing commercial applications for chitin and chitosan, alternative sources may be required in the future. With advances in biotechnology, microbially produced chitin and chitosan would appear an attractive alternative to that derived from shellfish. The cell walls of certain fungi such as the Mucorales contain both chitin and chitosan (Shimahara, 1989). Microbial derived chitosan can be prepared more readily than chitosan derived from shellfish waste. Also, with genetic manipulation of microorganisms there is the potential for the production of a more consistent and well defined material (Arcidiacono et al., 1989).

In nature, chitin usually exists as a complex with other materials. In fungi, chitin forms a complex with other polysaccharides such as glucans or mannans and may constitute up to about 45 % of the total dry weight of organic matter. In the calcified cuticle of crustacea, it is tightly associated with proteins, inorganic salts mainly calcium carbonate, lipids, pigments and trace metals (Jeuniaux 1971, Austin 1988). Cuticular chitin may contribute between about 58-85 % of the total dry weight of organic matter and the protein constituent may range from about 10-32 %. Although chitin is responsible for much of the structural integrity of the cuticle it is the overall chitin complex to which many of the mechanical and physical properties can be attributed. Chitin is prepared by removing other materials from the chitin complex. The method of preparation may considerably alter the chemical and physical properties of chitin. In addition there is a species variation in the properties of chitin. Thus a range of chitins exist which vary depending on the

species, method of preparation and application requirements and may therefore be better described as chitin isolates (Brine 1981 & 1981b, Austin, 1988).

1.7.2 Structures of chitin and chitosan

Chitin is a polymer which consists mainly of unbranched chains of β-1,4 linked-2-acetamido-2-deoxy-D-glucopyranose which can also be referred to as poly N-acetyl-D-glucosamine. Chitin is structurally similar to cellulose (β-1,4 linked-D-glucopyranose). The difference is that the cellulose hydroxyl group at carbon position 2 (C2) is replaced by an acetamido group in chitin. The disaccharide repeating unit of chitin is $C_{16}H_{26}O_{10}N_2$.

Chitosan is the deacetylated derivative of chitin and consists mainly of poly [β-1,4 linked-2-amino-2-deoxy-D-glucopyranose] also referred to as poly-D-[glucosamine]. In chitosan an amine group replaces the chitin acetamido group at C2. The disaccharide repeating unit of chitiosan is $C_{12}H_{22}O_8N_2$. These structures represent idealised structures, but the N-acetylation of chitin is usually incomplete and chitosan will be partly N-acetylated. Thus chitin and chitosan exist as copolymers of N-acetyl-D-glucosamine and glucosamine. It is not fully established if naturally occurring chitin, existing as part of a complex, is partially deacetylated or whether deacetylation results from hydrolysis during chitin extraction and purification (Roberts 1992). The pure homopolymer chitin is often denoted as chitin [1.0] and pure chitosan as chitosan [0.0] where the figure in brackets gives the mole fraction of N-acetyl-D-glucosamine residues. Thus, chitosan [0.15] would denote chitosan having 85% decetylation. The structures of chitin and chitosan are shown in Figure 1.3.

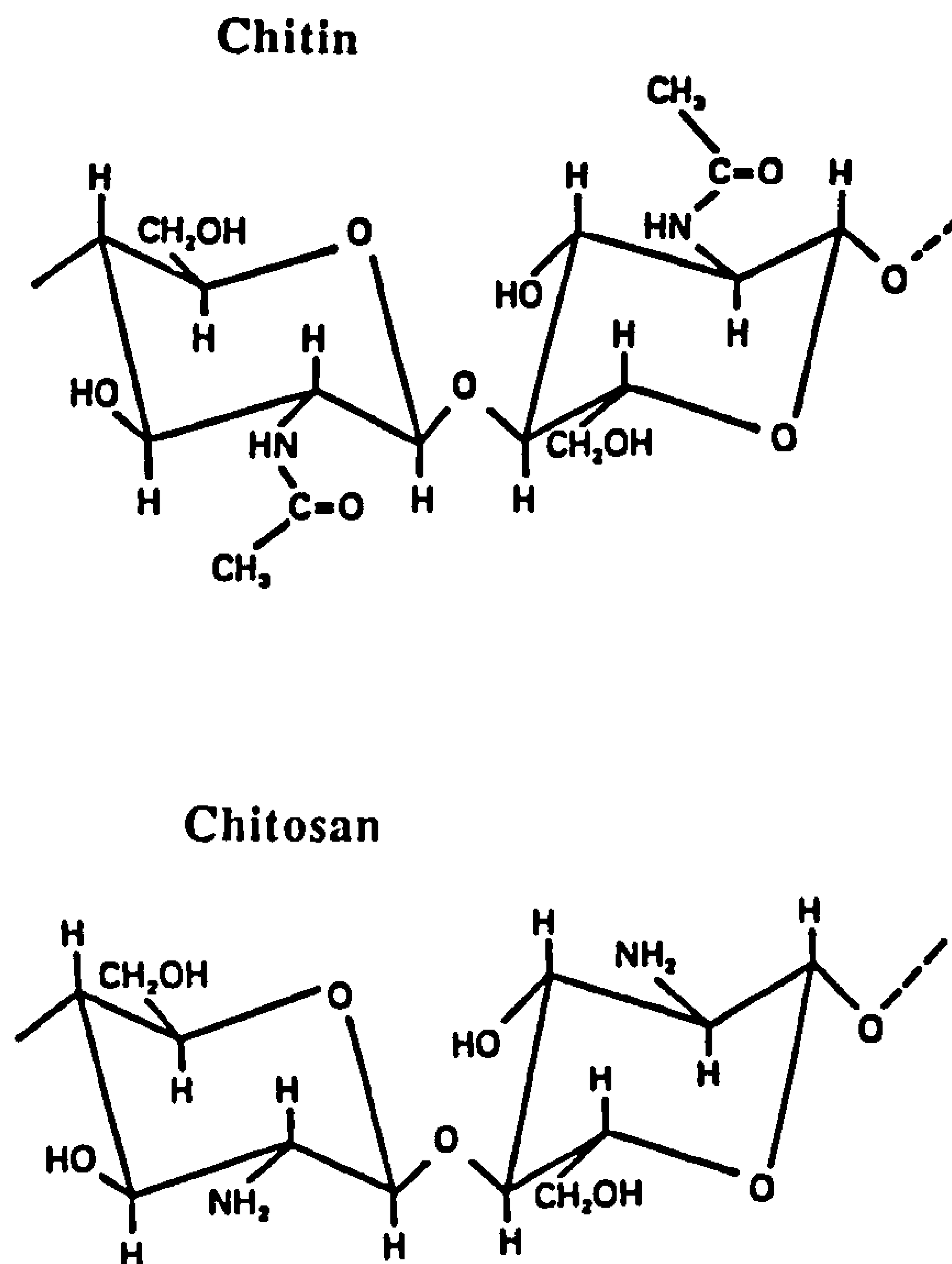
1.7.3 Preparation of chitosan

Chitosan is produced by the alkaline deacetylation of chitin which is itself produced commercially, from the shells of crustaceans. Chitin must first be isolated from the other materials, mainly proteins, with which it forms a complex. Several methods are used to extract and purify chitin but these will usually involve deproteination, demineralisation and removal of lipids and pigments. The methods employed in the production of chitin will often depend on the commercial application of the chitin. Muzzarelli (1977) outlines a number of methods for the isolation of chitin. The raw chitinous starting material i.e. crab, prawn or lobster shells will usually first be washed, crushed and dried prior to deproteinisation commonly using aqueous sodium hydroxide which may partially deacetylate the chitin if strong alkaline treatment is used (Roberts 1992). Residual amino acids in the chitin may subsequently affect the properties of the chitosan. Demineralisation will often involve treatment with hydrochloric acid but this will often be accompanied by some deacetylation and depolymerisation and less harsh agents such as EDTA have been shown to result in less denatured chitin although there is incomplete removal of inorganic salts (Shimahara and Takiguchi, 1988). Pigments, mainly carotenoids, may also be extracted with ethanol or acetone or destroyed with bleaching agents (Roberts 1992).

Deacetylation of the processed chitin to produce chitosan occurs by hydrolysis of the C2 acetamido group. Most commercially produced chitosans will not be 100 % deacetylated since extreme conditions are usually required if the degree of deacetylation is to exceed about 85 %. Typically chitosans are produced which are 75-85 % deacetylated. The acetamido group can be hydrolysed by alkaline or acidic treatment. However acidic hydrolysis will be accompanied by considerable hydrolysis of glycosidic links leading to depolymerisation (Muzzarelli 1977, Roberts 1992). The characteristics of the chitosan product will depend largely on the acidic treatments used during the initial isolation of the chitin since this will considerably determine the degree of depolymerisation. Resistance of the chitin acetamido group to hydrolysis is increased by the trans arrangement of the C2-C3 substituents (Horton & Lineback 1965). Recent publications describe the kinetics of depolymerisation and the prediction and control of depolymerisation of chitosan by the use of nitrous acid for the reliable manipulation of the chitosan molecular weight (Allan and Peyron, 1995a &b). The degree of deacetylation of chitin can be controlled to some extent by regulating the conditions under which the chitin is treated, mainly the concentration of alkali used (commonly aqueous NaOH) and the temperature and duration of the treatment. Increasing the temperature and / or the duration of the treatment will increase the degree of deacetylation but result in considerable chain degradation (Muzzarelli 1977). Increasing the concentration of aqueous alkali allows lower temperatures and / or treatments of shorter duration to be used. For example an acid soluble chitosan (approximately 60 % deacetylated or above) may be obtained with either 5 % NaOH at 150 °C for 24 hours, 40 % NaOH at 100 °C for 18 hours or 50 % NaOH at 100 °C for 1 hour. The latter treatment gave a product which was 82 % deacetylated. Increasing the duration of the treatment resulted in considerable chain degradation. If the degree of acetylation of the final chitosan end product is to be carefully controlled for its commercial application, then often the best way to achieve this is to re-acetylate either highly deacetylated chitosans (typically 5% acetylated) or moderately deacetylated chitosans (typically 15-40 % acetylated) to the required acetyl content.

The chitosan end product obtained will depend on subsequent treatment following deacetylation. Acid soluble chitosan flakes are produced if the deacetylated chitin is dried. These may then be milled into a chitosan powder which can be dry-blended with organic acids (e.g. acetic, formic, lactic and adipic acids) to produce self dissolving chitosan/acid blends. The purity of the chitosan end product may be improved by dissolution of the deacetylated chitin in acid followed by filtration to remove extraneous substances then precipitation, washing and drying to obtain the purified acid soluble chitosan in its free amine form. Alternatively, drying (e.g. spray-drying) after the filtration step will yield chitosan salts (chitosonium acid salts), the nature of which will depend on the acid used for dissolution (Sandford, 1989). Water soluble chitosan salts are produced if acids such as acetic, lactic, hydrochloric, formic and glutamic acid are used in the dissolution step (Muzzarreli, 1977, Lang and Clausen, 1989).

Figure 1.6. Structure of chitin and chitosan



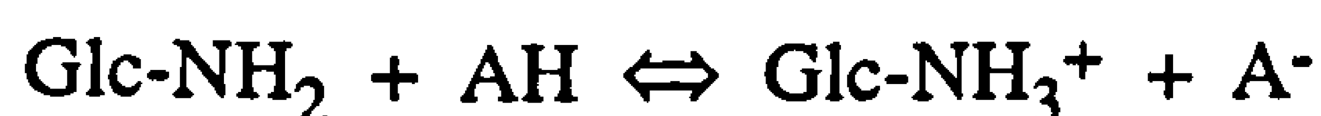
1.7.4 Solubility and solution properties of chitosan

Chitosan, in its free amine form ($-NH_2$), is insoluble in water at neutral or alkaline pH but will dissolve under suitable acidic conditions (Sandford 1989). During dissolution, the free amine groups become protonated forming cationic amine groups ($-NH_3^+$). Since chitosan is a weak base salts (polyelectrolytes) are formed with acids (Roberts, 1992). The solubility of the chitosan salt is dependent on the nature of the anion involved. In addition, particularly with slightly soluble salts, solubility will be influenced by molecular weight, degree of N-acetylation and the temperature. The amount of acid present will also influence solubility since a minimum amount of acid is required to protonate the amine groups. Thus, more acid is required to solubilise 5 % chitosan compared to 1 % chitosan (Pronova Biopolymers, Chitosan Product literature). Chitosan is soluble in dilute solutions of mineral acids although solubility is limited by the concentration ratio of chitosan : acid. Increasing the concentration of acid (e.g. with HCl) may cause precipitation of the chitosan. Hydrochloride salts of chitosan are soluble whereas the nitrate and phosphate salts are slightly soluble and the sulphate salt is insoluble at room temperature (Muzzarelli, 1977). Chitosan is soluble in solutions of most organic acids and forms soluble salts such as acetate, formate, glutamate, lactate, malate and pyruvate.

The water solubility of chitosan in salt form will be influenced by the degree of acetylation and distribution of acetyl groups of the chitosan (Rinaudo & Domard, 1989, Aiba, 1991). Water soluble chitosans tend to have degrees of acetylation between 0 and 30%. Chitosans having degrees of acetylation between 30 and 70% will usually be soluble in dilute acids. However, water solubility of these chitosans will mainly depend

on both the degree and distribution of acetyl groups since this will affect the intermolecular associations that occur between acetyl regions. In addition, glucosamine residues being hydrophillic in nature will become charged at acid pH which will increase the interaction with the solvent. An irregular arrangement of acetyl groups along the length of the chitosan chain (referred to as block type copolymers) tends to decrease chitosan solubility since associations between acetyl regions of chitosan chains will be increased resulting in aggregation . In contrast, solubility will tend to be increased in chitosans having a regular arrangement of acetyl groups (referred to as random type coploymers) since associations between chains, and hence aggregation will tend to be reduced. Chitosans which are more than 70% acetylated tend to be refered to as partially deacetylated chitins. These chitosans will not usually be soluble in water but are usually soluble under suitable acid conditions.

In an acid medium a chemical equilibrium reaction will be established as given below (Rinaudo & Domard, 1989).



$$\text{Glc-NH}_2 + \text{AH} \Leftrightarrow \text{Glc-NH}_3^+ + \text{A}^-$$

The reaction directly depends on the pKa. Chitosans will usually be soluble below pH 5.5-6. For polyamines, the ease of dissociation of conjugate acid groups ($-NH_3^+$) is increased by the presence of adjacent NH_3^+ groups thereby increasing the dissociation constant of the conjugate acid (Ka) and decreasing pKa (Roberts, 1992). Thus, the pKa depends on the charge density of the polymer which is proportional to the degree of deacetylation and on the degree of neutralisation of the acid. For samples having the same fraction of NH_3^+ groups neutralised, values of pKa will decrease with an increase in the degree of deacetylation of the chitosan. Values of pKa in the literature range from about 6.3 to 7 (Muzzarelli, 1985, Roberts, 1992). However, these measurements do not take into account the effect of charge density of the chitosan. The intrinsic pKa of chitosan was reported to be 6.5 using Katchalsky's equation and extrapolating to zero charge (Rinaudo & Domard, 1989). Measurment of intrinsic pKa takes into account the degree of acetylation and the degree of neutralisation of the chitosan.

The solution properties of chitosan will depend on the degree of N-acetylation and molecular weight of the chitosan and on the ionic strength and pH of the solvent (Rinaudo & Domard, 1989, Roberts, 1992). The rheological behaviour of chitosan can be used to predict the conformation of chitosan in solution (Lang and Kienzle-Sterzer, 1982). The flow properties of polyelectrolytic polysaccharides depends on the overall molecular conformation and on the degree of hydrogen bonding or electrostatic repulsion between neighbouring chain segments.

Ionic strength (salt concentration) strongly influences the molecular configuration of chitosan in solution. In solutions of low ionic strength, chitosan tends to adopt an extended, rodlike, configuration due to the electrostatic repulsion between charged glucosamine groups resulting in a stiffening of the chitosan chain. Thus, decreasing the degree of deacetylation would tend to reduce the degree of chain stiffening (Anthonsen et

al., 1993). At low ionic concentration, solution viscosity is increased due to the increase in intramolecular repulsion between charged groups resulting in an elongated conformation and enlargement of the hydrodynamic volume (Muzzarelli, 1977). As ionic strength increases the chitosan chain will tend to become more flexible and adopt a random coil conformation due to the shielding of charged glucosamine groups by the salt counter-ions resulting in a reduction in the electrostatic repulsion between the charged groups. This will decrease the hydrodynamic volume of the chitosan molecule, thereby reducing solution viscosity. The stiffness of the chitosan molecular backbone increases with an increase in the degree of ionisation of the chitosan amino groups and with a decrease in the ionic strength of the media (Kienzle-Sterzer et al., 1984). Chain flexibility will also increase with temperature, thus solution viscosity will tend to decrease (Roberts, 1992). The degree of ionisation of chitosan will depend on the concentration and degree of deacetylation of the chitosan and on the pH and ionic strength of the solvent. At high ionic strength, a decrease in the degree of deacetylation will tend to favour an extended chain conformation since the bulky N-acetyl groups have a steric effect and will also interfere with intramolecular hydrogen bonding (Anthonsen et al., 1993). Chitosan can be precipitated out of solution by an increase in the concentration of electrolytes such as NaCl and KCl or by the addition of concentrated HCl . Precipitation with electrolytes is due largely the increase in ionic concentration rather than the accompanying decrease in pH. Phosphate salts and polyphosphoric acid or its salts will also precipitate chitosan.

At low ionic concentration, a reduction in pH will drive the equilibrium reaction (given above) to the right favouring the presence of Glc-NH_3^+ (Filar and Wirick, 1977) Thus, chitosan molecules will tend to stiffen and adopt an extended configuration resulting in an increase in solution viscosity. Once the chitosan molecule has adopted the extended conformation further decrease in pH will tend to have very little effect on solution viscosity although high concentrations of acids such as HCl can precipitate chitosan from solution resulting in a dramatic decrease in viscosity. Conversely, an increase in pH favours the presence of free amine groups and the chitosan molecule will tend to adopt a random coil conformation resulting in a decrease in solution viscosity. Above a certain pH chitosan will precipitate out of solution due to the reduction in the solubility of chitosan as free amine groups predominate. The susceptibility of chitosan to changes in solution pH will be highly dependent on the degree of deacetylation and concentration of the chitosan. A low degree of deacetylation will tend to improve the stability of chitosans at higher pH. In contrast, high concentration will tend to decrease chitosan stability at higher pH. The magnitude of the viscosity change can be reduced by using a more highly ionised acid or by increasing ionic concentration. At higher ionic concentration, the effects of pH tend to be masked due to the shielding of charged moieties by salt counter-ions.

Chitosan solutions behave as non-Newtonian fluids since the viscosity of chitosan will vary depending on the rate of shear applied (Lang and Kienzle-Sterzer, 1982). Chitosan solutions display shear thinning properties with viscosity decreasing as shear rate

increases although at low shear rates chitosan solutions will show Newtonian behaviour. Solution viscosity will increase with increases in chitosan molecular weight and degree of ionisation of amine groups of chitosan and a decrease in the ionic concentration of the solution which will each increase the hydrodynamic volume of chitosan. Viscosity will also decrease with heating chitosan solutions (Rinaudo and Domard, 1989).

A reduction in the viscosity of chitosan solutions tends to occur with aging due to degradation. Degradation may be due to a number of factors such as hydrolysis, free radical oxidation / reduction reactions and microbial attack. The stability of chitosan in solution may be influenced by a number of factors such as the presence of other excipients, particularly preservatives which would prevent microbial growth, pH and storage temperature. The degradation of chitosan in solution during storage is accelerated by increasing the storage temperature. Chitosan is degraded by lysozyme which is an important property in the utilisation of chitosan for pharmaceutical and medical applications (Nordtveit et al., 1994). Biodegradation of chitosan by lysozyme is influenced by the degree of deacetylation of chitosan with initial degradation rates tending to increase with the degree of acetylation. Degradation is dramatically reduced when the degree of deacetylation is high since lysozyme enzymes act on acetyl groups. Thus, the pure homopolymer chitin will be the most susceptible to lysozyme.

Chitosan displays a number of useful solution and chemical properties which have been exploited commercially and these have been comprehensively reviewed by Muzzarelli (1977) and Roberts (1992). The commercial applications of chitosan are detailed in Section 1.7.7. Chitosan, being a polyelectrolyte, will react with negatively charged polyelectrolytes usually forming insoluble complexes (Muzzarelli, 1977, Mireles et al., 1991, Roberts, 1992). Mireles et al. (1991) showed that complex formation with polyanions such as polyacrylic acid was influenced by solution pH although for polymers such as pectin, alginate and carrageenan complex formation was independent of pH. For each of these polyanions, complex formation was found to be independent of the ionic concentration of the solution. Electrostatic interaction between polyanionic COO^- or SO_3^- groups and polycationic NH_3^+ groups were attributed as the main cause of complex formation. Chitosan-polyanion complex formation was proposed as a means of obtaining large floculating molecules possessing both positive and negative charge characteristics. These could find potential application in the food industry. Rha et al. (1985) reported the biotechnological application of chitosan-polanion capsules for cell entrapment or immobilisation.

Chitosan has the ability to form complexes with transition metal and post-transition metal ions (but not with alkali and alkaline earth metal ions) and has been extensively reviewed by Muzzarelli (1973 and 1977) and Roberts (1992). Comparison of published data proves difficult due the different methods of investigation and/or conditions used by different research groups (e.g. differences in the mode of agitation or in the physical nature of the chitosan) and inadequate characterisation of the chitosans used. The capacity of chitosan to adsorp metal ions depends largely on the concentration of

accessible amine groups of the chitosan. This will depend on the degree of deacetylation of the chitosan and to some extent on the crystal structure of the chitosan.

1.7.5 Physicochemical characterisation of chitosan

The term chitosan can be applied to a family of polymers which usually contain both N-acetyl-D-glucosamine and D-glucosamine subunits. Thus, as no definite structure exists for chitosan, it is necessary to characterise chitosans usually in terms of their degree of N-acetylation and molecular weight since these will influence the properties and potential uses of the individual chitosans ((Rinaudo & Domard, 1989, Roberts, 1992). Various analytical methods used to characterise the degree of acetylation and molecular weight of the chitosans are described below. In addition to the characteristics of the polymer, for medical and pharmaceutical application, chemical and microbiological purity levels should be standardised (Knapczyk et al., 1989b). Measurement of the content of residual protein, colour, moisture, ash, nitrogen, heavy metals and percentage of insoluble materials are reviewed by Knapczyk et al. (1989b) and Roberts (1992).

1.7.5.1 Measurement of the degree of N-acetylation of chitosan

The degree of N-acetylation and N-deacetylation of chitosan are inversely related and may be determined by measurement of the proportion of the amide or amine functional group in the chitosan. Alternatively other analytical methods may be employed.

Determination of the N-acetyl content

Infra-red spectroscopy (IR)

Infra-red spectroscopy (IR) is frequently used to analyse chitosan since it is a relatively rapid technique for determining N-acetyl content and the instrumentation is widely used in analytical laboratories (Roberts, 1992). Also, if an internal reference peak is used to correct for variation in the amount of material in the beam, then the purity of the material does not have to be determined. This method can be used to analyse insoluble samples of chitosan. Several methods of IR have been reported in the literature which vary in the absorbance band ratio selected for measurement and also on the choice of standardisation method allowing a correlation between the degree of acetylation and absorbance to be determined (Rinaudo & Domard, 1989). Each method has limitations over the range of values of N-acetylation which can be measured. Sannan et al. (1979) used a band ratio (in cm^{-1}) of 1554/2878, Miya et al (1980) used 1655/2867 and Miya et al (1985) used 1554/897 (references reported by Aiba, 1986). However, Aiba (1986) showed that different results were obtained for the same chitosan depending on which of the above band ratios were used. Another disadvantage of this technique is that hydroxyl bands of polysaccharides and /or linked water molecules appear near 1640 cm^{-1} thus, these may be confused with the bands attributed to the N-acetyl group (Domard, 1987).

Ultra-violet spectroscopy (UV)

Muzzarelli and Rocchetti (1986) reported a method using first derivative ultra-violet spectroscopy (UV) at 199 nm to measure N-acetyl content of chitosan although

quantitative results were not given. N-acetyl-D-glucosamine solutions were used for calibration. Aiba (1986) measured N-acetyl content of chitosan using conventional UV and found that there was a better correlation of the results obtained to those of a colloid titration method (measures free amino group) than those obtained by IR. The uv absorbance at 220 nm of chitosan samples and acetylglucosamine oligomers was found to be linear. Thus, an average of the molar absorption coefficients of N-acetyl-D-glucosamine and di-N-acetyl-D-chitobiose (177 and 173, respectively) were used to determine the N-acetyl content of the chitosan samples. The molar absorption coefficient of glucosamine at 220 nm was found to be negligible. This technique is only suitable for chitosan samples which are soluble in dilute acidic solutions.

Circular dichroism (CD)

Domard (1987) describes circular dichroism (CD) as an easy and precise method for determining the N-acetyl content of chitosan samples. CD spectra of N-acetyl-D-glucosamine and its oligomers show two CD bands one close to 190 nm and the second, arising from the n-π* transition, close to 211 nm whereas in a fully deacetylated chitosan, glucosamine residues show only one CD band centred near 185 nm. In acidic solution, the CD band attributed to glucoseamine residues disappears due to protonation of the amine group. Thus, in dilute acid media, the CD spectrum will show only the bands attributed to the N-acetyl group. The band arising from the n-π* transition in the range 200-250 was used to deduce the N-acetyl content of chitosan samples using chiotobiose, which is readily available commercially, for reference. The transition state is independent of ionic strength and pH of the chitosan solution and only small amounts of sample are required to prepare solutions for analysis. Domard (1987) found that the results following measurement of N-acetyl content by CD gave good agreement with those reported by Miya et al. (1980) using IR.

High-field nuclear magnetic resonance spectroscopy (NMR)

High-field nuclear magnetic resonance spectroscopy (NMR) has been described as a simple and rapid technique which can be applied routinely to measure the proportion and distribution of N-acetyl groups in chitosan samples (Varum et al., 1991a and b). In the method using ^{1}H-NMR described by Varum et al. (1991a), limited depolymerisation of the chitosan samples with nitrous acid (attacks D-glucosamine units but not N-acetyl-D-glucosamine units) was performed to reduce solution viscosity, improve solubility and narrow the line widths of the signals. Solutions of chitosan were prepared in D_2O and ^{1}H-NMR spectra recorded at either 99.6 MHz (to confirm that the relative areas of the peaks observed represented the relative occurrence of chitosan) or 500 MHz (to identify the resonances of the spectrum and thus, determine the degree of N-acetylation). By calculating the proportions of the four possible diads that occur in chitosans (GlcNAc-GlcNAc, GlcNAc-GlcN, GlcN-GlcNAc, GlcN-GlcN where GlcNAc and GlcN represent the N-acetyl-D-glucosamine and D-glucosamine units, respectively) the distribution of the acetylated residues can be determined. A method using ^{13}C-NMR to measure the acetylation sequences of chitosan has also been reported ((Varum et al., 1991b). Advantages of this technique are that small sample weights are required for analysis and

measurement is not influenced by the water content of the chitosan. However, NMR is only suitable for chitosans having a degree of N-acetylation above 5% and like IR relies on the production of good quality films of chitosan (Rinaudo & Domard, 1989).

Gel permeation chromatography (GPC)

Aiba (1986) used gel permeation chromatography (GPC) with UV detection at 220 nm to analyse chitosan samples prepared in dilute acetic acid. The peak area were found to be dependent on the degree of N-acetylation, the technique was reproducible and a linear relationship existed between the peak areas and the concentration of chitosan or acetylglucosamine oligomers. Calibration with acetylglucosamine oligomers was found to be unsuccessful and thus, chitosan samples of known degree of N-acetylation (fairly consistent values obtained following analysis by IR, UV and colloid titrimetry) were used. The results obtained by GPC were shown to be fairly consistent with those determined by colloid titrimetry and IR using the methods of Miya et al. (1980 & 1985). Aiba (1986) concluded that GPC was a reliable technique for the determination of the degree of acetylation of chitosan and furthermore, molecular weights could also be measured concurrently. Roberts (1992) points out an unusual feature of the study by Aiba (1986) in that the calibration samples had the same retention time regardless of molecular weight.

Hydrolytic techniques

In this technique the amount of acetic acid liberated by acidic or alkaline hydrolysis of the N-acetyl groups of chitosan is quantified and used to calculate the degree of N-acetylation. Rutherford and Austin (1978) used 50% NaOH high temperature to hydrolyse the chitosan followed by acidification with phosphoric acid and fractional distillation. The volume of acetic acid in the distillate was determined by titrimetry and this was related to the % N-acetyl content of the chitosan sample. Niola et al (1993) reported a method using acid hydrolysis (sulphuric and oxalic acid mixture) at high temperature. The acetic acid produced was separated from the reaction mixture and quantified by HPLC. This technique can be applied to chitosan samples regardless of degree of N-acetylation and solubility.

Determination of the amine group content

Acid-base titrimetry

Roberts (1992) described an acid-base titrimetry method (first reportedby Broussignac in 1968) in which the chitosan was dissolved in a known excess of acid and titrated potentiometrically with NaOH giving a titration curve with two inflection points. The difference between the two inflection points on the abscissa corresponded to the amount of acid required to protonate the amine groups from which the proportion of amine groups hence the degree of N-acetylation was determined. Since there is a tendency for chitosans to precipitate in the neutral pH range titration methods are imprecise, sometimes erroneous and often uneasy to use (Domard and Rinaudo, 1989). Hayes and Davies (1978) titrated solutions of chitosan hydrochloride with sodium hydroxide. The titration

was followed by three methods; either potentiometrically; or by using phenolphthalein as an indicator; or with silver nitrate using 2,7-dichlorofluorescein as an indicator. Values of N-acetyl content were shown to be in close agreement using each of the three titrimetric methods.

Colloid titration

Colloid titration is based on the reaction between cationic and anionic polyelectrolytes in aqueous solution where neutralisation of electrical charge proceeds stoichiometrically (Roberts, 1992). With knowledge of the concentration of ionic groups in the anionic polyelectrolytes, the concentration of ionic groups in chitosan (cationic polyelectrolyte) can be calculated from the relative volumes at the end point. The end point is detected using a dye such methylene blue (3.1) or toluidine blue (3.2) which undergo a metameric colour change in the presence of free anionic polyelectrolyte. Aiba (1986 & 1991) used colloid titrimetry to determine the degree of N-acetylation of samples of chitosan finding that the results obtained were generally in agreement with those obtained using UV.

Metachromatic titration

In metachromatic titrimetry, the absorbance (at λ_{max}) of a dye showing polyelectrolyte induced metachromasy will decrease with increase in concentration of added polyelectrolyte until a minimum value is obtained where further additions of polyelectrolyte will not affect absorbance (Roberts, 1992). The intersection of a plot of Absorbance versus volume of polyelectrolyte solution added indicates the point at which an equivalent number of dye ions and charged groups on the polyelectrolyte are present. Gummow and Roberts (1985) reported a quantitative method for the determination of either the concentration of a chitosan of known structure or the amine group content of chitosan of unknown structure to be elucidated using an anionic dye (sodium 2'-hydroxy-1,1'-azo-naphthalene-4-sulfonate).

Other methods

Domszy and Roberts (1985) reported a method in which the free amine groups of chitosan react with an excess of salicylaldehyde (in a 1: 3 mole ratio), in methanol/ acetic acid, to give a yellow Schiff's base (N-salicylidenechitosan). The salicylaldehyde remaining at the end of the reaction period was determined by spectroscopy. A method using dye adsorption was reported by Maghami and Roberts (1988). In this technique the amine groups of chitosan which have been protonated with acid act as sites for the adsorption of anionic dyes. At equilibrium there is a 1:1 stoichiometry for the interaction between the protonated amine groups of chitosan and the sulphonic acid groups on the dye thus the quantity of dye adsorbed will depend on the ionic charge of the dye. The quantity of dye adsorbed at equilibrium can be determined from the initial and final concentrations in the reaction mixture and therfore the proportion of amine groups in the chitosan sample determined. The results have been shown to compare reasonably well with those obtained by IR. This technique can be used with chitosans which are not fully soluble. A drawback to this method is that aggregation of the dye can lead to erroneous calculation of the degree of N-acetylation. A colorimetric assay was described by Hayes

and Davies (1988) which was an adaptation of a technique reported by Ride and Drysdale (1972) for estimation of fungal chitin in the presence of plant tissues. Deamination of glucosamine units with nitrous acid yields 2,5-anhydromannose which can be measured colorimetrically. Techniques using picric acid adsorption and reaction with 2,4-dinitrofluorobenzene have also been described (refer to Roberts, 1992).

Nanjo et al. (1991) described an enzymatic method for determining the degree of N-acetylation of chitosan. Chitosan was completely hydrolysed to D-glucosamine and N-acetyl-D-glucosamine using chitosanolytic enzymes and chitosanase and the subsequent monosaccharides determined by colorimetric assays or HPLC. HPLC was found to be the best method for quantitative analysis in terms of its simplicity and rapidity although its application is limited due to interference by impurities in the chitosan samples or in the enzyme preparations. The results obtained for this technique agreed reasonably well with those obtained by colloid titration.

Determination of degree of acetylation based on the overall composition

Elemental analysis has been used to calculate the degree of N-acetylation of chitosan (Aiba, 1986 & 1991). Aiba showed that for some of the chitosan samples studied, the degree of N-acetylation calculated by elemental analysis compared well with other methods although there were inconsistencies with some of the chitosan samples tested. The nitrogen content of pure chitin and pure chitosan is 6.89% and 8.69%, respectively (Roberts, 1992). Thus, with knowledge of the nitrogen content of the sample the degree of N-acetylation can be determined. The use of the nitrogen / carbon ratio may be preferable due to the presence of moisture and residual inorganic materials in chitosan samples (the ratio is 0.194 and 0.146 for pure chitin and chitosan respectively). Since the nitrogen and carbon content of proteins will be very different to that of chitosan, residual protein must not be present in the chitosan sample. Other methods such as gas chromatography and pyrolysis have been reported (Hayes and Davies, 1988, Roberts, 1992).

1.7.5.2 Determination of the molecular weight of chitosan

The physicochemical properties of chitosan in solution will be influenced by the average molecular weight and the molecular weight distribution of the chitosan (Beri et al., 1993). A reliable method for the measurement of molecular weight is essential for quality assurance of the product on a commercial basis (Muzzarelli et al., 1987). However, precise determination of chitosan molecular weight remains difficult due to polymer-polymer or polymer-solvent interactions and difficulty in determining the concentrations used (Rinaudo & Domard, 1989).

Measurement of intrinsic viscosity

Measurement of intrinsic viscosity ($[\eta]$) is a rapid and relatively simple method for the determination of the molecular weight of polymers (Roberts & Domszy, 1982). Intrinsic viscosity and molecular weight (M) are related by the Mark-Houwink relationship given below.

$$[\eta] = K M^a$$

Where K and a are empirical constants which are independent of molecular weight but depend on the degree of N-acetylation of the chitosan, the solvent system used and the temperature (Roberts & Domszy, 1982, Maghami & Roberts, 1988, Wang et al., 1991, Roberts, 1992). Values of K and a are usually determined from a plot of log [η] versus molecular weight for a number of homologous chitosan fractions having a narrow molecular weight distribution. Conveniently values of K and a can be obtained for chitosan from the literature (Rinaudo & Domard, 1989). However, careful selection of values of K and a is required to match the particular chitosan-solvent systems used in the viscometry (Wang et al., 1991). A disadvantage of viscometry is that it is not an absolute technique for determining chitosan molecular weight since it relies on pre-determination of the constants K and a and there are wide discrepancies in the literature for these values. However, viscometry remains a popular and precise method of analysis of chitosan not only for the determination of chitosan molecular weight but also to investigate the behaviour of chitosan in solution.

Laser light scattering spectophotometry (LLS)

Static laser light scattering spectrophotometry (LLS) has been used to study the molecular weights of chitosan (Muzzarelli et al., 1987). An advantage of using LLS is that it does not require the use of reference materials and thus, provides an absolute measurement of molecular weight. However, this technique has been reported to generally overestimate chitosan molecular weights due to aggregation of chitosan chains which occurs on standing (Rinaudo & Domard, 1989, Roberts, 1992) although problems of aggregation were not apparent in the study reported by Muzzarelli et al. (1987). LLS also requires that a precise determination of the concentration dependence of the refractive index (dn/dc) for chitosan obtained (Rinaudo & Domard, 1989, Roberts, 1992). Values of dn/dc reported in the literature appear to depend on the chitosan and/or the solvent used. This technique also requires that several Zimm plots are obtained from which molecular weights are determined (Beri et al., 1993). LLS cannot determine molecular weight distribution of chitosan unless time consuming fractionation of the sample is performed. LLS has been coupled to GPC to improve the accuracy of molecular weights determinations (Domard and Rinaudo, 1984). However, coupling GPC to multiple-angle laser light scattering (MALLS) allows molecular weights and molecular weight distributions to be determined (Beri et al., 1993).

Gel permeation chromatography (GPC)

Gel permeation chromatography (GPC) or size exclusion chromatography (SEC) is frequently used to measure the molecular weight and molecular weight distribution of polymers and has been applied to chitosan (Roberts, 1992). In GPC, separation of molecules is based on molecular size with larger molecules eluting first since smaller molecules are able to move in and out of the pores in the column matrix (Freifelder, 1982). To determine the molecular weight of chitosan by GPC requires that there is no interaction (either attraction or repulsion) between the gel and the chitosan (Rinaudo & Domard, 1989, Roberts, 1992, Terbojevich et al., 1993). Since many conventional gel

matrices tend to be negatively charged then adbsorption of cationic chitosan to the gels due to electrostatic attraction may cause problems in measuring molecular weights by this method. Wu (1988) described a method to measure the molecular weight distribution of chitosan by high-performance liquid chromatography (HPLC) using a conventional gel (Glycophase-G/CPG) columns which were preconditioned by injection of chitosan to deactivate any residual active sites on the column. Rinaudo and Domard (1989) reported that a cationic silica gel (porous silica beads grafted with quaternary ammonium groups) and an eluent containing a low molecular weight electrolyte (0.05ammonium acetate) to suppress repulsion between chitosan and the gel gave a good determination of molecular weight using light scattering detection.

Beri et al. (1993) reported that SEC coupled to multiple-angle laser light scattering detection (MALLS) was an accurate, reliable and reproducible technique for the determination of molecular weight and molecular weight distribution in the molecular weight range 10,000-500,000. For chitosans having molecular weights above 100,000 the technique could also be used to determine the conformation of chitosan in solution. Terbojevich et al. (1993) reported a high-performance GPC method for determining the molecular weight distribution of chitosan. A difficulty encountered by this method was that calibration with sodium polystyrene sulphonate standards was not possible under the conditions required for the elution of chitosan. Since chitosan standards of narrow molecular weight distribution are not commercially available, attempt was made to produce calibration standards by fractionation of chitosan. However, this was not successful and hence calibration was performed with chitosan fractions having a broad molecular weight distribution. Hasegawa et al. (1994) reported a method for determining the molecular mass distribution of chitosan using pullulan standards. Dextrans have frequently been used as calibration standards in conventional GPC studies although this is likely to lead to erroneus molecular weight values for chitosan, particularly overestimation of values, since the difference in the conformation of the two polymers in solution leads to difference in their behaviour on the column during GPC (Beri et al., 1993). An additional source of error using conventional GPC is due to changes in elution volume if the column is overloaded which will affect the values of molecular weight obtained. By coupling GPC to laser light scattering the pitfalls associated with using secondary calibration techniques such as the use of dextrans and possible potential problems due to column overloading are avoided.

Sedimentation equilibrium

Sedimentation equilibrium is a technique which has been widely used to determine the molecular weight and molecular weight distribution of solutes and has been used to measure the molecular weight of chitosan (Errington, 1993, Errington et al., 1993, Aspden, 1996). This technique was also used in this project details of which are given in Chapter 10. However, this technique is intricate and time consuming and furthermore, great care in sample preparation is required since errors in polymer concentration measurement can affect the accuracy of molecular weight measurments (Beri et al., 1993).

1.7.6 Commercial applications of chitosan

Chitosan is a versatile material that has many potential commercial applications (Sandford, 1989). These include applications in industrial waste management, paper technology, agriculture, food, cosmetics and biotechnology as well as biomedical and pharmaceutcal uses.

1.7.6.1 Industrial waste management

Increased public awareness of environmental pollution by heavy metals and potentially other toxic materials such as pesticides which through accumulation via the food chain can have serious impact on ecological systems and ultimately on health has led to a demand for the purification of industrial effluent prior to their discharge or reuse (Knorr, 1991). However, conventional methods for removing heavy metals from industrial waste waters by using methods such as precipitation, oxidation, filtration, electrochemical treatment, ion exchange and evaporation tend to be either ineffective at removing all traces of metal ion contamination or expensive particularly if contaminating levels of metal ions are low (Volesky, 1987). Chitosan has excellent chelating properties for many potentially harmful metals and thus, the use of chitosan may provide a commercially viable alternative to the use of conventional purification methods (Muzzarelli, 1977, Sandford, 1989). Chitosan has been shown to chelate metals such as copper, nickel, chromium, cadmium, manganese, cobalt, lead, mercury, zinc, uranium and silver. Treatment of industrial waste effluent to remove metal ions does not require the use of highly processed and purified chitosans which would considerably reduce the cost of their application (Coughlin et al., 1990). The use of chitin and chitosan to remove contaminants such as dyestuffs from processing effluents has also been reported (Knorr, 1991). Chitosan has flocculant properties and thus may be used to clarify waste water in preference to synthetic polymer flocculants which may contain hazardous monomers (Sandford, 1989). In Japan, clarification of waste water is the largest single use of chitosan.

1.7.6.2 Paper and textile technology

Use in paper manufacture has been described as the greatest potential application of chitin and chitosan (Nicol, 1991). Muzzarelli (1977) reviewed chitosan as a paper and textile additive. Adding chitin and chitosan to pulp increases the strength of paper, speeds up the rate of water drainage from pulp and increases the quantity of fibres retained when manufacturing sheets of paper. This enables the use of cheaper, weaker fibres without reducing quality and considerably reduces the cost of beating the paper pulp. Paper containing chitin and chitosan is also easier to print on. Chitin and chitosan also increase the wet strength of paper which has implications in the manufacture of disposable nappies, shopping bags and paper towels. Chitosan has been used as a thickener and fixing agent in the preparation of printing pastes. Domszy and Roberts (1986) investigated ionic interactions between chitosan and oxidised cellulose which have obvious implications in the paper industry. In the textile industry the use of chitin to improve cellulose fibres has been investigated. Also, plastic fibres have been prepared

using chitosan. The use of chitosan as a sizing agent for fabrics is reported to have advantages over conventional agents such as starch and gums and one of the main features of this is the resistance of chitosan to laundering. Applications of chitosan in the dying and printing of textiles is also an important applcation.

1.7.6.3 Agriculture

There are a wide variety of potential applications of chitosan in agriculture including use as an animal feed, to coat seeds (e.g. wheat, rice and peas) and as a fertiliser (Sandford, 1989). The use of chitinous material as a feeding supplement for animals such as cattle (Patton & Chandler, 1975), rats (Landes & Bough, 1976) chickens (Austin et al., 1981), hens and rabbits (Hirano et al., 1990) and fish (Kono et al., 1990) has been investigated. Landes and Bough (1976) showed that the addition of up to 5% chitosan in the diet of rats had no adverse effects. However, growth reductions were observed in animals fed a diet containing 10-15% chitosan and this was accompanied by alteration of the tissue composition of liver. Hirano et al. (1990) reported that a hen fed a high dose of chitosan (20 g/day) suppressed appetite due to aggregation of chitosan in the upper small intestine although the animal made full recovery within one week of stopping the dietary intake of chitosan. Chitosan is used at low concentration to recover proteinaceous material from food processing waste waters which is subsequently used as animal feed although the chitosan content of the feed will usually be low (less than 0.1%).

Chitosan has been shown to be fungicidal against many pathogenic fungi except those in which chitosan is present as a component of the cell wall (Allan & Hadwiger, 1979). This has implications for the use of chitosan for pre- and post harvest protection of crops. Increased crop yields have been reported after coating wheat seeds with chitosan (Sandford, 1989). Cuero et al (1991) demonstred that chitosan inhibited the growth and toxin production of the mould *Aspergillus flavus* in preharvest maize which was suggested to be due to chitosan chelating essential metal ions which are required by fungi for normal growth. The use of chitosan as a postharvest protection against fungi which are a major cause of food spoilage during shipping and storage is encouraging in view of public concern about the use of chemical pesticides. Leuba and Stossel (1986) investigated the antifungal activity and interaction of chitosan with biological membranes. Chitosan was shown to reduce fungal growth, disturb the membrane function of a filmentous fungus at acid pH and cause agglutination of sheep erythrocytes *in vitro* which may have implications for the agglutination of yeasts. As well as a direct action on fungal growth, chitosan may also have an indirect effect by inducing the accumulation of phytoalexins (low molecular weight anitmicrobial agents) and proteinase inhibitors, increase in cell wall phenolic compounds, deposition of calloses and changes in membrane permeability in plant cells. Due to their film forming and gelling properties and their mechanical robustness, use of chitosans for the controlled release of agrochemicals has been suggested, thus, overcoming the potential problems of leaching, degradation and evaporation of conventional fertilisers and pesticides which often requires that they are applied in excess (Teixeira et al., 1990).

1.7.6.4 Cosmetic applications

Chitin, chitosan and their derivatives may have many commercial applications in cosmetics ranging from preparations for the skin and hair to preparations for oral hygiene and tooth care (Lang & Clausen, 1989). Muzzarelli (1989) reports a number of potential applications of water soluble modified chitosans (carboxymethyl- and carboxybutyl chitosan) in cosmetics. The moisturising, protective and smoothing properties of these chitosans due to their hydrophillic nature and film forming properties make them ideal for use in skincare products. Due to their antibacterial action, they can also be used in mouthwashes and liquid soaps for personal hygiene. The film forming properties of chitosan are well suited for its inclusion in haircare products such as setting lotions, blow-dry lotions and hair sprays and for use in nail varnishes (Lang & Clausen, 1989). The viscosity enhancing properties of chitosan enable it to be used in cosmetic gels and emulsions and being a substantive and conditioning polymer due to its polycationic nature they can be generally used in hair care (shampoos and hair conditioners) and skin care products. The use of chitosan in cosmetics is limited by its insolubility at neutral and alkaline pH and in the presence of anionic detergents. However, water soluble chitosan derivatives which are compatible with anionic detergents may enable the more widespread use chitosan in cosmetics.

1.7.6.5 Food applications

Chitosan is a non toxic biomaterial which is endogenously present at low concentration in foods such as prawns, soft-shelled crabs, cheeses, mushrooms and yeasts (Arai et al., 1968). In Japan it is already used as an ingredient in foods such as biscuits and noodles (Nicol, 1991). Chitosan has many potential uses in the food industry. The chelating/ flocculating properties of chitosan have been exploited for the clarification or fining of beverages such as apple or carrot juices and are reported to be as effective as, yet easier to use than, conventional silica sol/gelatin/bentonite treatment (Knorr, 1991). As well as reducing turbididty, chitosans have also been shown to reduce microbial counts in apple juice at ambient temperature which has important implications with consumer demant for less processed foodstuffs. As previously mentioned, chitosan is effective for the recovery of proteinaceous material in food (cheese, meat, poultry, fruitcake, shellfish) processing plants which may subsequently be used for animal feed. The emulsifying properties of chitosan are also being investigated in products such as mayonnaise and peanut butter (Nicol, 1991). Chitosan has also been shown to improve the stability of food emulsions susceptible to freeze-thaw breakdown (International Commission on Natural Health Products, 1995). The use of chitin and chitosan in the baking of bread were reported by Knorr (1982). Microcrystalline chitin was shown to increase specific loaf volume in breads. Chitosan films may be used to coat fruit and vegetables to preserve freshness and to prevent the growth of fungi which will may improve their shelf-life and also considerably reduce post-harvest losses during shipment and storage. Tough, non-toxic and biodegradable films for foods, such as sausages, and food packaging may also utilise chitosan and furthermore the chitosan component of these films may help to prevent the growth of bacteria which cause food-poisoning (Knorr, 1991, Nicol, 1991).

1.7.6.6 Biotechnology applications

In biotechnology, chitosan has important applications for the immobilisation of cells and enzymes and in the purification and recovery of biologicals (Muzzarelli, 1985, Sandford, 1989). The immobilisation of whole cells is a practical method of obtaining biocatalytic systems and is of particular use when multi-enzyme systems are required or enzyme isolation is too expensive (Protan Laboratories, Inc., 1987a). Chitosan ionotropic gels (gel formation requires the presence of a counterion) used for the entrapment of whole cells have advantages over conventional gels (e.g. calcium alginate, widely used for the entrapment of plant and microbial cells), since they are stable in phosphate buffers and in the presence of sodium and potassium ions. Unlike gels such as agarose and kappa-carrageenan they are gel forming at room temperature. Chitosan has been used to immobilise a wide variety of enzymes which have important applications in biotechnology (Muzzarelli, 1985, Protan Laboratories, Inc., 1987b). Enzymes may be immobilised using three general techniques; entrapment and ionic and absorptive binding; crosslinking chitosan solutions; and crosslinking insolubilised chitosan. Enzyme immobilisation using crosslinked chitosan systems are the preferred methods. Glutaraldehyde is commonly used to crosslink solutions containing chitosan and enzyme. Glutaraldehyde crosslinks the amino groups of the chitosan and enzyme via a reaction involving the formation of Schiff bases (aldimine bonds) which results in the formation of a gel which can be washed and collected as a granular moist product (90% water). A simpler technique is via crosslinking insolubilised chitosan. Glutaraldehyde is added to chitosan flakes in water resulting in crosslinked chitosan which following washing and air drying is insoluble in acid solutions. The crosslinked chitosan is suspended in buffer containing the enzyme and more glutaraldehyde is added resulting in the formation of complex between the chitosan and the enzyme which can be collected by filtration after washing and air drying. The crosslinking of the enzyme can be easily controlled and the ease of preparation, the high enzyme loading and the production of a dry product has advantages over other techniques.

Applications of chitin and chitosan in chromatography have also been reported including chromatographic techniques for the immobilisation of enzymes, separation of nucleic acids and amino acids, separation of wheat germ agglutinin and removal of coloured substances from foods such as tea, coffee and apple juice (Muzzarelli, 1977). Takeda (1978) reported that powdered chitin as an adsorbent in thin-layer chromatography was as good as, or superior to that of crystalline cellulose, silica gel and polyamide layers for the separation of phenols, amino acids, nucleic acid derivatives or inorganic ions.

1.7.6.7. Biomedical and pharmaceutical applications

Chitosan has many biomedical applications which make use of its non-toxic and biodegradable properties and also its physical, mechanical and bioactive properties (Olsen et al. , 1989, Sandford, 1989). Knapczyk et al. (1989b) described specifications of chitosan for biomedical and pharmaceutical application. Knapczyk et al. (1989a) investigated some biomedical properties of chitosan, with a view to determining 'is chitosan really a drug or an excipient ?', and concluded that chitosan was an excipient

with specific biomedical properties.

Cell binding

The cell binding properties of chitosan have led to its application as haemostatic, bacteriostatic and spermicidal agents (Olsen et al., 1989). As a haemostatic agent, chitosan is able to coagulate blood via agglutination of red blood cells which is independent of the normal blood clotting cascade. The agglutination is predominantly due to the interaction of the negatively charged neuraminic acid residues on the red cell membrane with positively charged chitosan. Malette et al. (1983) investigated the haemostatic activity of chitosan, *in vivo*, in dogs. The study looked at blood loss from porous vascular grafts (grafts sewn into the infrarenal aorta) pre-soaked in either saline solution, as a control, or chitosan solution. The control animals suffered immediate exsanguination (mean blood loss approximately 440 ml) and all animals died. In contrast, blood loss from grafts treated in chitosan solution was dramatically reduced (mean blood loss 44 ml) due to the plugging of the interstices of the graft by the chitosan coagulum and all animals survived. In the surviving animals killed up to a period of four months after the study, it was found that the graft material was encased in abundantly vasular smooth muscle which penetrated the graft and linked the outer layers to the inner subepithelial layers indicating healing. The potency of chitosan as a haemostatic agent is reported to be molecular weight dependent with the coagulum formed by lower molecular weight chitosan (35,000) being 'less firm' than that formed by chitosans of higher molecular weight (600,000 & 1,500,000) (Olsen et al., 1989). Chitosan derivatives have also been investigated as blood anticoagulants although compared to heparin the anticoagulant potency of these derivatives appears to be low (Muzzarelli, 1977, Nishimura and Tokura, 1987b). Dutkiewicz et al. (1990) investigated the coagulating properties of chitosan derivatives *in vitro*, finding that the clotting time of whole blood depended on the chemical modification of the polyaminosaccharide structure. The clotting time in the presence of the chitosans was significantly higher than that of the control.

Cell activation

Chitin and chitosan have been shown to possess immunological properties which is thought to be due to their ability to activate certain cell type (Muzzarelli, 1985). The role of chitin and chitosan in cell activation is thought to be as an adjuvant since there is no evidence to suggest that they have antigenic properties. Although chitosan has been reported to activate a number of cell types, it is the activation of macrophages which is believed to be most important for its immunomodulating properties (Nishimura et al., 1984 and 1985). Macrophage activation leads to the production of various superoxides and cytokines. The production of cytokines is known to influence the activities of other cell types such lymphocytes, fibroblasts and endothelial cells. The cytolytic and cytostatic activity of macrophages against leukaemia and carcinoma cells was reported. It was shown that a 70% deacetylated chitosan had the greatest immunoadjuvant and antitumour activities in mice and guinea pigs. This chitosan was reported to be digested by lysozyme more slowly than the chitosan derivatives tested. Macrophage activation

with chitosan (80% deacetylated) in the form of biodegradable multi-porous beads and multi-porous microspheres has been described (Nishimura et al., 1986 & 1987a). Stimulation of the production of interleukins and colony stimulating factor by macrophages was also demonstrated. This research group also discussed the possibilities of the use of these chitosan preparations as carriers for drugs such as antitumour drugs. Otterlei et al. (1994) investigated the effect of the neutral solubility and molecular weight of chitosan on its immunomodulating properties.

Wound healing

Chitin and chitosan have been investigated as wound healing accelerators (Muzzarelli, 1977). Knapczyk et al. (1989c) reported that chitosan in the form of a powder and solution exhibited no irritant and allergic effects on healthy or damaged skin. The mechanism of action of these materials as wound healing promoters has not been fully elucidated and there are several mechanisms by which chitosans could improve or modify wound healing (Olsen et al., 1989). The N-acetylglucosamine subunit of chitosan is a major component of dermal tissue and its presence in scar repair tissue is essential. Furthermore, glycoproteins, which are the predominant proteins in the initial phases of wound healing, contain abundant N-acetylglucosamine. Thus, chitinous material may provide a source of N-acetylglucosamine through their degradation by lysozymes which are present in wound fluid. The oral administration of N-acetylglucosamine has also been reported to accelerate wound healing and improve the tensile strength of scar tissue. In addition, since glycosaminoglycans, to which chitosans have similar structural characeteristsics, play an important role in the structural organisation of collagen in the wound giving structure and strength to newly formed collagen, it is possible that chitosans provide a recognised environment for wound healing to occur similar to that of glycosaminoglycans. The activation of cells such as macrophages by chitosan, thereby stimulating the inflammatory response through modulation of the activity of other cells or via the secretion of regulatory substances, may also be important for the wound healing process. Muzzarelli et al. (1988) report normal regeneration of the *dura mater* in cats by the application of chitosan (86.8% deacetylated). The role of chitosan was suggested to be as a primer on which normal tissue architecture is organised similar to the actions of glycosaminoglycans. Through its wound healing properties, chitosan could find application as an artificial skin which would be of particular benefit in the treatment of extensive skin loss occuring as a result of burns (Chandy and Sharma, 1990).

Several application of chitosan in bone healing, treatment of periodontal bone loss and dentistry have been reported (International Commission on Natural Health Products, 1995a). Malette et al. (1986) demonstrated the use of chitosan to enhance the regeneration of leg bone in dogs. Studies by Borah et al. (1992) showed that N-acetyl chitosan induced bone growth in large metacarpal / fibular defect areas in rabbits which alleviated the need for surgical bone grafting. Reconstruction of the periodontium using chitosan ascorbate, thus, avoiding more complicated and less tolerable treatments was reported by Muzzarelli (1989). In a patient exhibiting severe periodontitis, a chitosan gel was used to fill large pockets neighbouring the teeth and prevent dental mobility. Ito

(1991) also reported the application of chitosan as a bone-filling paste for dental treatment.

Biomedical membranes and ophthalmology

The use of chitosan in the preparation of artificial kidney membranes has been suggested (Muzzarelli, 1977). Chandy and Sharma (1990) reviewed the biomedical applications of chitosan membranes. A prerequisite for the use of materials as artificial kidney membranes is high tensile strength, selective permeability and blood compatibility. The use of albumin blended chitosan membranes for this role has been suggested owing to their superior permeability and blood compatibility properties compared to chitosan and standard cellulose membranes. The application of chitosan in ophthalmology (e.g. contact and intraocular lenses) has utilised the mechanical strength of chitosan, its wettable, non-abrasive and non-allergenic properties and its ability to allow oxygen and carbon dioxide to permeate to and from the cornea, respectively (International Commission on Natural Health Products, 1995b). Markey et al. (1989) described the use of chitosan and chitosan blends for the preparation of bandage contact lenses primarily for use in the protection of an acutely traumatised eye such as in intraocular surgery or a chronically compromised cornea. These materials were reported to exhibit properties desirable in a bandage lens.

Hypocholesterolaemic / hypolipidaemic agent

The potential use of chitosan as a hypocholesterolaemic agent was demonstrated in rats by Sugano et al. (1978) and later by Nagyvary et al. (1979) and Kobayashi et al. (1979). The hypolipidaemic activity of chitosan has also been demonstrated (Nagyvary et al., 1979). In rats fed a high cholesterol diet containing 2-5% chitosan for a period of 20 days plasma cholesterol concentrations were shown to be reduced by 25-30% without influencing food intake and growth of the animals (Sugano et al., 1980). The liver concentration of cholesterol and triglycerides was also shown to decrease by a dietary supplement of chitosan. The hypocholesterolaemic activity of chitosan was similar to that of cholestyramine, an aromatic polymer used as a bile salt sequestrant in the treatment of hypercholesterolaemia. Dietary chitosan was shown to increase the faecal excretion of exogenous and endogenous cholesterol. Jennings et al. (1988) demonstrated the hypocholesterolaemic activity of a diet containing 5% chitosan in rats and in contrast to feeding cholestyramine, chitosan did not have deleterious effects on the intestinal mucosa. LeHoux and Grondin (1993) showed that a diet containing 7.5% chitosan maintained adequate cholesterol homeostasis in rats fed a high cholesterol diet. In rats fed with cholestyramine there were changes in the weight or appearance of the liver, however, there were no such changes in chitosan fed rats. Hirano et al. (1990) confirmed the hypocholesterolaemic activity of a dietary intake of chitosan in hens and rabbits. The hypocholesterolaemic and hypolipidaemic activity of chitosan has mainly been attributed to the disruption and/or inhibition of micelles in which cholesterol, fatty acids or monoglycerides are normally incorporated in the upper intestine, thus preventing their absorption (Sugano et al., 1978 & 1980, Nagyvary et al.,1979, Furda, 1983). Dietary chitosan has been shown to impair iron absorption in rats, probably as a

consequence of chelation, resulting in depression of haemoglobin levels although there appeared to be little effect on the absorption of other metal ions (Gordon & Besch-Williford, 1984). However, liver iron levels were not significantly lower than those in control animals and the authors suggested that impaired iron utilisation may partially be attributed to residual EDTA used to prepare the chitosan sample. The hypolipidaemic application of chitosan is currently utilised in the USA in a dietary supplement (Fat Binder™, Genesis Research Corporation, IL, USA). Deuchi et al. (1995) investigated the effect of viscosity and degree of deacetylation of chitosan on faecal fat excretion in rats fed a high fat diet. It was shown that as the viscosity, hence molecular weight, or degree of deacetylation of the chitosan preparation increased, fat digestability decreased (i.e. faecal fat excretion increased). A supplement of ascorbic acid with chitosan in the diet significantly reduced fat absorption possibly due to the increase in the charge of the chitosan at reduced pH.

1.7.6.8 Applications in Pharmaceutical delivery systems

The pharmaceutical application of chitosan is receiving wide interest particularly for oral drug delivery. Studies on the use of chitin and/or chitosan in directly compressed tablets, in sustained release preparations of water soluble drugs and in ground mixtures containing poorly soluble drugs has been described (Nagai et al., 1984). The gel forming property of chitosan in tablet formulations at low pH was suggested as a promising vehicle for the sustained release of water soluble drugs in the gastrointestinal tract. In dogs, the oral bioavailability of the poorly soluble drug phenytoin (used in the treatment of epilepsy) was improved by co-grinding with chitosan which correlated with the *in vitro* dissolution profile of the phenytoin-chitosan ground mixture. Brine (1989) evaluated controlled release solid dose forms incorporating chitosan for drug delivery. Technical feasibility in the use of chitosan in a direct compression / sustained release matrix for the highly water soluble drug chlorpheniramine maleate (and in a controlled release wet granulation formulation for aspirin was demonstrated. Machida et al. (1989) reported the sustained absorption of prednisolone in dogs from buoyant oral dosage forms utilising chitosan. Buoyant formulations were suggested as a means of increasing the resisence time of orally administered dose forms in the stomach. The sustained release formulations utilised a chitosan granule, containing internal cavities, which was layered in a chitosan membrane. By changing the composition of the granules and/or the thickness of the chitosan membrane, the release properties of the drug were controlled. Knapczyk et al. (1989c) reported that the efficiency of treatment of vaginal fungal infections (vaginitis mycotica) with the antifungal agent clotrimazole was improved by the use of chitosan. A single application of a vaginal tablet composed of clotrimazole with chitosan was as effective as four applications (normally applied over a period of 12-14 days) of a commercial preparation of clotrimazole. Later, Knapczyk (1992) reported on the properties of tablets for buccal and vaginal delivery of antimycotic compounds. The proportion of chitosan in the tablet, the degree of acetylation of the chitosan and the addition of other excipients was shown to influence the properties of the tablet. Miyazaki et al. (1990) utilised the film-forming properties of chitosan to investigate oral drug delivery systems. Films which were prepared from chitosan and diazepam (0.5:1 ratio)

and loaded into hard gelatin capsules were shown to be as effective as a commercial tablet preparation.

Duoxian et al. (1991) examined the properties of chitosan-alginate microcapsules which were suggested for the sustained release of pharmaceuticals. The use of chitosan gel microspheres (chitosan gel core crosslinked with glutaraldehyde) in controlled release preparations for the antitumour drug 5-fluorouracil have also been reported (Ouchi et al. (1991). Imai et al. (1991) showed that the rate of absorption of the acidic drug indomethacin (anti-inflammatory) was improved following the oral administration of a 'kneaded mixture' containing a complex of indomethacin and low molecular weight chitosan which correlated with its increased *in vitro* dissolution rate. The use of chitosan or modified chitosan in pH-sensitive controlled-release cationic hydrogel systems have also been described (Amiji and Patel, 1994). These systems were suggested to be suitable for the selective delivery of drugs to different regions of the gastrointestinal tract. The use of chitosan (93% deacetylated) hydrogels as an ointment base for drugs such as lidocaine hydrochloride and clotrimazole has also been described (Knapczyk, 1993). Gel stability was reduced when less extensively deacetylated chitosans (66% deacetylation) were used which suggested to be due to the possible interactions with the drug. Polk et al. (1994) suggested the use of chitosan-alginate microcapsules for the delayed-release of protein drugs. Capsules prepared from high molecular weight chitosan or a combination of high and low molecular weight chitosan were shown *in vitro* to be the most effective in reducing the release of albumin during the first four hours. Increasing the alginate content of the capsules also decreased the release of albumin. The pH of the extracapsular fluid was shown to strongly influence the release profile with low release at low pH and high release towards neutral pH. The authors suggested the application of these capsules for the selective delivery of proteins to the neutral environment of the intestines. Alamelu and Rao (1994) investigated a potential delivery device for the lipophilic drug dapsone using liposomes sequestered in chitosan gel. *In vitro* studies demonstrated that the liposome-chitosan gel matrix provided a stable slow release system for dapsone (tested in phosphate buffer and mouse plasma). In contrast, the release of the hydrophillic marker bromothymol blue was more rapid. Akbuga and Durmaz (1994) investigated the release of furosemide from chitosan microspheres prepared using a water-in-oil emulsion technique with a view of evaluating candidate systems for drug delivery. Ritthidej et al. (1994) investigated the application of chitosan as a disintegrant in paracetamol tablets, finding that the tablets containing chitosan disintegrated faster than those containing the commonly used excipients corn starch and microcrystalline cellulose. Collagen-chitosan composite membranes for the controlled release of propranolol have been reported (Thacharodi and Rao, 1995. These authors showed that transdermal patches incorporating chitosan were successful for the delivery of propranolol. Crosslinked chitosan microspheres have also been investigated for the controlled release of drugs administered by parenteral injection (Jameela and Jayakrishnan, 1995). *In vitro* drug release was found to be controlled by altering the degree of crosslinking. *In vivo*, no significant biodegradation of placebo chitosan microspheres was observed following intramuscular injection in rats. However, the *in vivo* release profile of drug was not

reported.

1.7.6.9 Novel application of chitosan in nasal delivery systems

A universal characteristic of cell membranes is their predominantly net negative charge due to the presence of anionic radicals which are covalently linked to the molecular matrix of the membrane (Quinton and Philpott, 1973). These authors investigated the role of the fixed anionic sites in the cell membrane by looking at the effect of cationic polymers on membrane-membrane interactions, membrane structural rigidity and membrane permeability. The cationic polymers, poly-L-lysines, protamine and histone, were shown to produce similar changes in the structure of rabbit gall bladder. Induction of the fusion of cell membranes was reported which was suggested to be due to the neutralisation of the negative surface charge on the membrane or the formation of polymeric cation bridges which crosslink opposed membrane surfaces. The cationic polymers were reported to alter the morphology of membrane processes such as microvilli which was suggested to be due to the loss of membrane rigidity as a result of disruption of the ionic and hydrogen bonds which normally impair a degree of stiffness to the membrane. The cationic polymers were reported to increased membrane permeability. The authors suggested that the intermolecular interactions involving fixed anions which are critical to molecular rigidity are also critical to the molecular organisation and hence permeability properties of the membrane. Granger et al. (1986) also investigated the role of fixed anionic sites on the integrity of the rat intestinal capillary wall by looking at the effect of neutralising the negative charge of these sites by using polycations such as protamine sulphate, poly-L-lysine or polyethyleneimine. The polycations, administered by intravenous infusion, resulted in increased transmembrane efflux of proteins across the intestinal capillary wall. This was attributed to neutralisation of fixed anionic sites on the capillary wall although the authors also suggested that other mechanisms may be involved since polycations have been shown to distort the kidney glomerular basement membrane structure, thus, altering porosity. Increased nasal absorption of polycationic dextran derivatives compared to neutral dextran derivatives was reported following coadministration with sodium deoxycholate in rabbits (Maitani et al., 1989). It was suggested that the positive charges of the polycationic dextrans were available to interact and form complexes with the negatively charged nasal mucosa thereby increasing absorption through increased intimate contact with the nasal membrane.

The novel application of chitosan to improve the nasal absorption of polypeptides such as insulin and calcitonin was reported by Illum (1992). Later, Illum et al. (1994) published the results of nasal absorption studies in rat and sheep models. Some of these results were generated as part of this project. Chitosan glutamate solutions were shown to greatly enhance the nasal absorption of insulin in both species. Histological examination following exposure of the rat nasal mucosa to 0.5% chitosan for a period of 60 minutes showed that there were no significant changes in normal nasal morphology. Rentel et al. (1993) demonstrated that chitosan glutamate enhanced the *in vitro* absorption of a vasopressin analogue (molecular weight 1412) across the rat intestinal mucosa from a

suspension formulation.

The mechanism (s) by which chitosan enhances nasal absorption has not been elucidated. Illum et al. (1994) suggested that the mucoadhesive properties of chitosan may contribute to the mechanism of absorption enhancement although other factors such as the effect of chitosan on the gating properties of epithelial tight junctions may be involved. As detailed above, neutralisation of the fixed anionic sites on cell membranes has been shown to influence membrane permeability. The mucoadhesive properties of chitosan were reported by Lehr et al. (1992). It was suggested that mucoadhesion probably results from an ionic interaction between the positively charged amino groups in chitosan and the negatively charged sialic acid residues in mucus. Fiebrig et al. (1994) demonstrating the interaction between chitosan and pig gastric mucin, a major component of gastric mucus, using the technique of sedimentation in an analytical ultracentrifuge. Chitosan is also a viscosity enhancing agent and the viscosity of chitosan solutions is a function of its molecular weight and concentration as well as on the properties of the solvent used (Filar and Wirick, 1977). The mucoadhesive and viscosity enhancing properties of chitosan may both contribute to increasing the residence time of the drug formulation in the nasal cavity, thereby increasing the time available for absorption.

Artursson et al. (1994) examined the effect of chitosan on the permeability of Caco-2 monolayers derived from a human intestinal epithelial cell line using C^{14}-labelled mannitol as the model drug. Chitosan resulted in an increase in the rate of mannitol absorption which was shown to be concentration and pH dependent. Mannitol absorption was highest at the lowest pH tested (4.0) and tended to increase with an increase in chitosan concentration reaching a plateau between 0.25% to 0.5% chitosan. The pH-dependency in absorption was attributed to the change in the degree of ionisation of the chitosan molecule hence in its molecular configuration. At lower pH values, the chitosan molecule tends be more ionised, adopting an uncoiled elongated configuration (Filar and Wirick, 1977) resulting in closer contact with the Caco-2 monolayer. A solution of 0.5% chitosan (pH 4.0) applied to the Caco-2 monolayer for a period of 60 minutes was shown to induce changes in the distribution of cytoskeletal filamentous actin which is thought to be directly or indirectly associated with proteins in the tight junction. Thus, it was suggested that chitosan affected the permeability of tight junctions. A recent publication reported that 1.5% chitosan glutamate, increased the permeability of a Caco-2 cell monolayer in terms of a decrease in TER (Borchard et al., 1996). However, 0.5-1.5% chitosan did not alter the permeability to C^{14}-labelled mannitol and FITC-dextran. It was concluded that TER must decrease below a certain threshold value to permit paracellular transport of the marker substances. Mechanisms by which chitosan may act as an absorption enhancer have also been investigated by Dodane et al. (1996). In Caco-2 cell monolayers, chitosan hydrochloride was shown to cause a reversible, dose-dependent increase in membrane permeability indicated by the decrease in TER which was more pronounced when chitosan was applied to the apical side compared to the basolateral side. Changes in TER were observed within 15 minutes after application to the apical side even at the lowest concentration of chitosan tested (0.00125%). At this

lower concentration, chitosan did not modulate TER at the basolateral side. The effect of chitosan on the permeability of the cell monolayer was shown to be reversible with complete recovery within 24 hours. Results indicated that the cells remained viable during the recovery period after treatment with chitosan. Structural alteration of the Caco-2 monolayer, observed using confocal microscopy, showed that the decrease in TER correlated with a constriction of actin which changed from a filamentous- to globular-type at the cell-cell boundaries. Structural changes in the tight junction associated protein ZO-1 were also observed. Following a recovery period of 24 hours, the cell monolayer resembled that of the control. It was suggested that chitosan causes a transient modulation of the permeability and structure of the Caco-2 cell layer.

Exploitation of the use of absorption enhancers or excipients in nasal drug delivery systems requires that full evaluation of their safety in candidate nasal systems is performed. Chitosan is generally considered to be non-toxic having an oral LD_{50} in mice of more than 16 g/kg which is in the same order as that of sugar and salt (Arai et al., 1968). Other research groups have also demonstrated the safety of chitosan after oral administration to animals as previously mentioned (Landes & Bough, 1976, Sugano et al., 1978 and 1980, Nagyvary et al., 1979, Kobayashi et al., 1979, Austin et al., 1981, Jennings et al., 1988, Knapczyk et al., 1989, Kono et al., 1990, Hirano et al., 1990, LeHoux and Grondin, 1993, Deuchi et al.,1995). The oral toxicity of compounds is an important consideration in nasal delivery systems since drug formulations administered to the nasal cavity will ultimately find their way to the gastrointestinal tract. Illum et al. (1994) reported that 60 minutes exposure of rat nasal mucosa to 0.5% chitosan did not cause significant changes in the morphology of the nasal epithelium. Nasal absorption studies in rats reported by Illum et al. (1994) and studies by Artursson et al (1994) and Dodane et al. (1996) using Caco-2 cell monolayers suggest that the effect of chitosan on the permeability of membranes is transient which provides useful indication that chitosan does not cause significant epithelial damage. Aspden et al. (1995a) reported that chitosan caused a reversible reduction in mucociliary transport rate (MTR) in the frog palate model which was attributed to interaction between chitosan and frog palate mucus rather than ciliotoxicity. Aspden et al. (1995b) and Aspden (1996) reported the absence of chitosan toxicity when applied to nasal mucosa. Chitosan glutmate was shown to transiently slow the MTR of graphite particles in the frog palate model and in the human nasal turbinate model. In a study in 10 human volunteers, nasal administration of daily doses of chitosan for a period of 7 days did not cause any change in saccharin clearance times. In nasal in-situ perfusion studies in rats, perfusion of chitosan was reported to induce minimal damage to the nasal membrane compared to that resulting from nasal perfusion of a solution of Laureth-9. Raised concentrations of protein in the nasal perfusate were recorded after perfusion of chitosan in contrast to concentrations after perfusion of a control solution of 0.9% NaCl. However, increased protein concentrations were not accompanied by increased concentrations of the membrane bound enzyme 5'-nucleotidase and the cytosolic enzyme lactate dehydrogenase, which would be expected if membrane damage occurred. The increased protein concentrations were suggested to be due to mucus removal from the nasal membrane after complexation with chitosan and

compensatory increased mucus production. The effect of chitosan on guinea pig nasal cilia beat frequency (CBF) following a once daily nasal dose for 28 days was also reported. Chronic dosing of chitosan had no effect on CBF compared to undosed control animals. Measurement of CBF has been suggested as a valuable tool for evaluating the safety of nasal absorption enhancers (Merkus et al., 1993).

The data would appear to suggest that chitosan is safe as a nasal absorption enhancer although extensive toxicological evaluation would be required before chitosan could find commercial application.

1.8 Project objectives

The objective of this project was to investigate the novel application of chitosan for the nasal delivery of insulin. Utilisation of the nasal route would be a major break-through in the treatment of diabetes mellitus. Insulin is also useful as a model peptide to evaluate the potential of chitosan for the nasal delivery of other peptide drugs. For comparison, nasal chitosan formulations incorporating salmon calcitonin as an alternative peptide were also investigated. Chitosan is commercially available at different degrees of deacetylation, in a number of viscosity (molecular weight) grades and in different salt forms. In this project, a medium viscosity glutamate salt having a degree of deacetylation of about 82% was predominantly used although for comparison other grades were also tested.

Two *in vivo* animal models, an anaesthetised rat model and a conscious sheep model were used to investigate the nasal absorption of insulin or salmon calcitonin from formulations incorporating chitosan. The appropriate nasal or parenteral control formulations were also tested. Preliminary studies were performed to evaluate the efficacy of chitosan as a nasal absorption enhancer by comparing its performance with that of other compounds which had been reported in the literature to enhance the nasal absorption of peptide drugs. Following these preliminary studies, extensive investigations were performed with insulin to evaluate various factors influencing the nasal absorption enhancing efficacy of chitosan. Nasal salmon calcitonin / chitosan formulations were also tested. The project also evaluated potential membrane damaging effects of chitosan and various other compounds using an erythrocyte haemolysis assay. Characterisation of the chitosans employed was also performed using techniques such as sedimentation equilibrium and viscometry.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Introduction

The general materials and methods employed in this project are described in this chapter. Details of the grades of chitosan used and methods of preparation of chitosan solutions and drug formulations are given along with details of the animal models used to investigate intranasal drug absorption, methods of analysis and calculation of results. Additional materials and methods are given in the relevant chapters.

The rat nasal model used was based on that originally described by Hirai et al. (1981a) and modified by Fisher et al. (1985 & 1987). The validity of the use of the in vivo rat nasal model, similar to the model used in this project, was demonstrated by Fisher et al. (1985), Critchley (1989) and Chandler (1993). The sheep model, similar to the one used in this project, has previously been described by Longecker et al. (1987) and Illum et al. (1989). The methods are described in the following sections:

2.2	Preparation of chitosan solutions and drug formulations
2.3	Absorption studies in rats
2.4	Absorption studies in sheep
2.5	Measurement of glucose, insulin or calcium concentrations
2.5	Calculation of results following glucose, insulin or calcium analysis

2.2 Preparation of chitosan solutions and drug formulations

General details of the grades of chitosan used and the methods of preparation of chitosan stock solutions and drug formulations are described along with details of the measurement of formulation pH and osmolality. Insulin was the main drug used during this project although salmon calcitonin (S-CT) was also investigated as an alternative peptide drug.

2.2.1 Grades and properties of the chitosans used

A glutamate salt of a medium viscosity grade of chitosan (MVCSN) was predominantly used in this project. Other grades of chitosan were also evaluated for comparison. Each grade of chitosan used had a similar degree of deacetylation although different viscosity (molecular weight) grades and different salt forms were tested. The grades and properties of the various types of chitosan used are given in Table 2.1.

2.2.2 Preparation of chitosan stock solutions

Chitosan solutions were prepared by weighing the appropriate amount of chitosan into a suitable volumetric flask, adding approximately 8/10ths of the final volume of solvent and stirring overnight, at room temperature, using a magnetic stirrer. The pH of the chitosan solution was measured (refer to Section 2.2.6) and if appropriate adjusted to the required pH with HCl. The solution was made up to the final volume with solvent

which had been preadjusted to the required pH. Chitosan solutions were then filtered through 0.45 μm syringe filters and stored in glass bottles at 4°C prior to use.

Table 2.1. Grades and properties of the chitosans used

	CHARACTERISTICS OF THE VARIOUS GRADES OF CHITOSANS TESTED IN THIS PROJECT				
Chitosan grade	Low viscosity chitosan glutamate	Low viscosity chitosan lactate	Medium viscosity chitosan glutamate	Medium viscosity chitosan hydrochloride	High viscosity chitosan
Synonym	LVCSN	CSN lactate	MVCSN	CSN HCL	HVCSN
Product	Seacure +G	Seacure +110L	Seacure +210G	Seacure +Cl	Seacure 443
Batch number	P390	004-580-07	004-282-10	012-422-01	077-372-05
Nature of material	Spray-dried powder	Spray-dried powder	Spray-dried powder	Spray-dried powder	flaked
Water solubility	Soluble	Soluble	Soluble	Soluble	Insoluble
Salt type	glutamate	lactate	glutamate	hydrochloride	chitosan base
Salt content (% w/w)	55-65	65-75	55-65	80-90	not applicable
Degree of deacetylation (%)	80-85	82	82	81	75
Batch viscosity (ml/g) *	388	-	1010	-	-
Batch viscosity (CPS) **	-	3	7	18	-
Batch viscosity (CPS) ***	-	-	15	-	54

All grades of chitosan were supplied by Pronova Biopolymer A/S, Drammen, Norway

* Intrinsic viscosity
** 0.5% w/v chitosan in phosphate buffer
*** 0.5% chitosan in acetate buffer

2.2.3 Properties of insulin and salmon calcitonin used

Sodium insulin was used throughout the studies. Each batch of insulin used had a specific activity of about 28 IU/mg for pure insulin. However, since the insulin products used also contained salts and water, the purity of each batch was measured to ensure that formulations contained the correct concentration of insulin, thus correct doses were administered. The purity of insulin was determined by spectrophotometry. The absorbance of accurately prepared solutions of insulin product (typically 0.5 to 0.8 mg/ml) were measured at 276 nm using a Hewlett Packard 845A diode array spectrophotometer (Hewlett Packard GmbH, Germany) and matching quartz cuvettes (Philips quartz cuvettes, Unicam Analytical Supplies, Cambridge, UK). From the absorbance readings obtained the purity of the product was calculated with knowledge that at 276 nm the absorbance of 1 mg/ml pure insulin is 1.058. The specific activity of the insulin product was adjusted according to the degree of purity obtained. In all cases the values of purity obtained were in close agreement with values given by the manufacturer. Further details of the insulins used are given in the relevant chapters.

Salmon calcitonin, acetate salt, was used at the specific activity quoted by the maufacturer. Further details are given in the relevant chapters.

2.2.4 Preparation of insulin or salmon calcitonin formulations

Formulations of insulin or salmon calcitonin for testing in animal models were prepared

as described below. Reference solutions for subcutaneous or intramuscular administration and control nasal formulations were prepared by diluting a stock insulin or salmon calcitonin solution to the required concentration with solvent (e.g buffer or NaCl). Nasal test formulations were prepared by mixing appropriate volumes of stock insulin or salmon calcitonin solutions with stock solutions of chitosan or enhancers to achieve the required concentrations. All stock solutions and solvents used in the preparation of nasal formulations were pre-adjusted to the required pH during preparation (further details given in relevant chapters). After preparation the final pH was checked and formulation osmolality measured as described in section 2.2.5.

2.2.5 Measurement of formulation osmolality and pH

Measurements of osmolality and pH were routinely performed during the preparation of chitosan solutions and drug formulations. Osmolality is a measure of the tonicity or osmotic pressure of the solution (Reich et al., 1995). An Osmol is defined as "the weight, in grams, of a solute, existing in a solution as molecules (and/or ions, macromolecules, aggregates, etc), which is osmotically equivalent to a mole of an ideally behaving nonelectrolyte". Osmolality was measured in units of Osmol/kg. Fluids which have the same tonicity as physiological fluids (e.g. 0.9% sodium chloride) are said to be isotonic. Those at a higher or lower tonicity than physiological fluids are referred to as hypertonic and hypotonic, respectively. The pH measures the hydronium ion concentration of a solution and is expressed in terms of the logarithm (log) of the reciprocal of the hydronium ion concentration (Niebergall, 1995). This is written mathematically as follows:

$$pH = \log \frac{1}{[H_3O^+]}$$

Since the log of 1 is zero the above equation can be written:

$$pH = -\log [H_3O^+]$$

2.2.5.1 Measurement of osmolality

The osmolality of selected solution formulations tested either in animal absorption studies or in erythrocyte haemolysis assays were measured using an Osmomat 030 cryoscopic osmometer (Gonotec, Berlin, Germany). The instrument determines the total osmolality of aqueous solutions by comparing measurements of the freezing points of pure water (freezing point 0°C, osmolality 0.000 Osmol/kg) and a standard solution of sodium chloride (freezing point -0.5557°C, osmolality 0.300 Osmol/kg) with that of the test solution. Sample volumes of 50 μl were used. Measurements of single samples were performed automatically and the result displayed on a digital display panel. The osmometer was first calibrated by performing measurements with pure water and 0.300 Osmol/kg sodium chloride standard solution (Gonotec). If necessary the instrument was adjusted to give reproducible readings of 0.000 and 0.300 Osmol/kg for water and sodium chloride calibration solutions respectively. The osmolality of the test solutions was measured, at least in duplicate, to obtain reproducible results.

2.2.5.2 Measurement of pH

The pH of solutions was measured during and/or after formulation preparation using a Corning 240 pH meter (pH meter) from Ciba Corning Diagnostics Ltd, Sudbury, Suffolk, UK. The instrument was calibrated with pH 4.0 and pH 7.0 standard solutions prior to determining formulation pH.

2.3 Absorption studies in rats

Absorption studies in rats were performed in the Biomedical Services Unit (BMSU), QMC, University of Nottingham, UK. In all studies, the rats were under terminal anaesthesia.

2.3.1 Materials

Heparin (sodium salt, Product H-7005), Sigma Chemical Company Poole Dorset.
Pentobarbitone sodium B P injection (Sagatal, 60 mg/ml), May & Baker, Dagenham, Essex, UK.
Sodium chloride, 0.9 % w/v, solution (0.9 % NaCl, Steriflex® 0.9% sodium chloride intravenous infusion B P), Fresenius Health Care Group, Basingstoke, Hants, UK.
Ultrapure water ("Elgastat UHP", Elga, High Wycombe, UK) was used throughout.
Glucose FH/2 tubes (2 ml tubes, containing 2 mg sodium fluoride, 30 I.E. lithium heparin), Sarstedt, Leicester, UK.
All other materials used for the surgical preparation of animals was supplied via Danbiosyst UK Ltd, Nottingham, UK.

2.3.2. The anaesthetised in vivo rat nasal model

2.3.2.1 Study animals and preparation of rats prior to surgical procedure

Male Wistar laboratory rats, weighing approximately 250 g, were obtained from the BMSU or from the Joint Animal Breeding Unit (JABU), School of Agriculture, Nottingham University, UK. All rats were fasted for 16-18 hours prior to the study day but were allowed water *ad libitum*. Food pellets were removed from the cage feeding tray and in addition, since rats tend to be coprophagic (faeces eating) then soiled bedding was also removed from the cages and replaced with fresh bedding. The tail of each rat was uniquely marked with a permanent marker pen. Rats were pre-weighed to ensure that the correct doses of anaesthetic and drug solutions were administered.

2.3.2.2 Implantation of an indwelling needle into the caudal vein of the rat

The caudal (lateral tail) veins, running along the length of the tail, were used for the intravenous administration of anaesthetic and fluid replacement in the rat. To increase blood circulation in the tail and to facilitate implantation of a needle in the caudal vein, rats were placed in a thermostatically controlled box ('hot box', c/o Dept Pharmaceutical Sciences, University of Nottingham, UK), preset at 38 °C, for approximately 10 minutes. The rat was transferred to a restraining cone and a cannula / needle assembly was inserted into the lumen of the caudal vein. The cannula / needle assembly was prepared by the insertion of a syringe needle into a 10 cm length of polyethylene cannula

tubing. The blunt end of the shaft of a second needle was inserted into the free end of the cannula tubing. The cannula / needle assembly was pre-flushed with 0.9 % NaCl prior to use to ensure that there were no blockages or leaks. When the needle was correctly inserted into the vessel, indicated by blood flow along the cannula, anaesthesia was administered as described below. The cannula / needle assembly was secured in place using adhesive plaster.

2.3.2.3 Induction of anaesthesia

Rats were anaesthetised via the indwelling needle implanted into a caudal vein with 60 mg/kg pentobarbitone sodium (Sagatal). The pedal withdrawal reflex was used as an indication of the induction of general anaesthesia in the rats prior to the performance of surgical procedures. Anaesthesia was maintained throughout the experimental period by further injections of Sagatal solution via the indwelling needle in the caudal vein.

2.3.2.4 Tracheal cannulation

Tracheal cannulation of the anaesthetised animals was performed to maintain the patency of the airway throughout the experimental period. During the cannulation, the oesophagus was occluded by tying it to the cannula. This was to prevent any possible drainage of nasally administered solution formulations into the stomach. The anaesthetised rat was placed ventral surface up on a heated table (to maintain the body temperature of the animal). The trachea and underlying oesophagus were exposed by blunt dissection and two cotton ligatures, of approximate length 20 cm, were passed under the oesophagus / trachea. An incision was made in the trachea, between two adjacent cartilagenous rings, and a 4-5 cm length of polyethylene cannula tubing inserted, caudally (towards the lungs), approximately 1-2 cm into the trachea. The cannula was secured in the trachea by tying both ligatures around the cannula, trachea and oesophagus.

2.3.2.5 Carotid artery cannulation

The carotid artery was used as a convenient and easily accessible vessel from which to collect serial blood samples during the experiment. The left common carotid artery, lying in close proximity to the trachea was isolated by gently freeing it from the attached connective tissue and nerve. Three ligatures were passed under the carotid artery. The artery was clamped at the caudal end by attaching a microvessel clip. One of the ligatures was tied at the cranial end of the vessel to prevent the back-flow of blood when the vessel was cut. An incision was made in the vessel and a length of polyethylene cannula tubing of about 20 cm was inserted, caudally into the lumen of the vessel. The cannula was secured in the vessel by using the remaining two ligatures. The free end of the cannula was clamped with artery forceps and the microvessel clip removed. The artery forceps were used to regulate blood flow along the cannula. The cannula was kept patent throughout the experiment by flushing with heparinised saline (0.25 mg/ml heparin in 0.9 % NaCl). The site of operation was then covered by a pad of absorbent paper to protect the position of the cannula and reduce fluid loss from the site.

2.3.2.6 Dose administration

Intranasal dose administration

Intranasal doses were administered at 0.1 ml/kg by instilling the solutions into the right nostril of the rat which was positioned ventral surface up. Thus a dose of 25 μl was administered to a 250 g rat. The doses were administered via a glass 50 or 100 μl microsyringe to which was attached a needle fitted with a 40 mm length of polyethylene cannula tubing. The cannula was inserted to a depth of 0.8 cm into the right nostril of the animal and the dose gently instilled. After dose administration the cannula tubing was carefully withdrawn from the nostril.

Subcutaneous dose administration

Subcutaneous doses were administered at 0.25 ml/kg by injecting the solution into the nape of the neck using a glass Hamilton 100 μl syringe with suitable needle.

Intramuscular dose administration

Intramuscular doses were administered at 0.025 ml/kg by injecting the solution into a gluteal muscle of the hind limb to a depth approximately 5 mm using a glass Hamilton 100 μl syringe with suitable needle. Care was taken to ensure that the needle did not penetrate blood vessels

2.3.2.7 Blood sample collection

All blood samples were collected from the cannulated carotid artery. Prior to the collection of each blood sample, the first 1-2 drops of blood were discarded to prevent contamination of the sample with heparinised saline.

During insulin absorption studies, blood samples of about 0.2 ml (10-12 drops) were collected for blood glucose analysis into glucose FH/2 tubes at 10 and 5 minutes prior to dose administration (-10 and -5 minutes) and at 5, 10, 20, 30, 45, 60, 90, 120, 180 and 240 minutes post-administration. Blood samples were stored at room temperature prior to glucose analysis within approximately 2 hours of collection (refer to Section 2.5). Due to limits in the volumes of blood which could be collected from the rat and due to problems with cross-reactivity of rat plasma with the insulin radioimmunoassay, insulin analysis was not performed.

During salmon calcitonin absorption studies, blood samples of about 0.2 ml were collected into 1.5 ml microcentrifuge tubes containing 0.02 ml of heparinised saline (1 mg/ml heparin in 0.9% NaCl) at 10 and 5 minutes prior to dose administration and at 5, 10, 20, 40, 60, 90, 120, 180, 240 and 300 minutes post-administration. Plasma was separated by centrifugation at 13000 rpm in a MSE Microcentaur centrifuge (Fisons Scientific Equipment, Loughborough, Leicestershire, UK). The plasma was removed (approximately 0.1 ml aliquots) and stored frozen at -20°C in 1.5 ml microcentrifuge tubes prior to calcium analysis (refer to Section 2.5).

After the collection of each blood sample the cannula was flushed through with heparinised saline. Fluid replacement, equivalent to the volume of blood collected, was given to the rats, at suitable time intervals throughout the experiment, in the form of 0.9 % NaCl via the cannulated caudal vein. Following the completion of serial blood sample collection, the rats were killed by an overdose of Sagatal administered via the indwelling needle in the caudal vein and the animals disposed of by incineration.

2.4 Absorption studies in sheep

Absorption studies in sheep were performed in the facilities of the Joint Animal Breeding Unit (JABU), School of Agriculture, University of Nottingham. Studies were performed in conscious sheep. However, the sheep were sedated to facilitate dose administration as detailed below.

2.4.1 Materials

Ketamine hydrochloride (Ketalar®, 100 mg/ml injection,), Parke Davis Medical, Hants., UK.

Heparinised tubes (4 ml, containing 60 IU lithium heparin) and polystyrene tubes (3.5 ml), Sarstedt, Leicester, UK.

All materials for the cannulation or dose administration to sheep were supplied via Danbiosyst UK Ltd, Nottingham, UK.

2.4.2. The conscious in vivo sheep nasal model

2.4.2.1 Study animals

Cross-bred (Suffolk & Texel) male and female sheep weighing in the region of 30-60 kg were used in the studies. The animals were weighed the day prior to dose administration to ensure that the correct doses of ketamine and dose solutions were administered. In the various studies performed in sheep animals were reused allowing at least three weeks between successive studies. The sheep were normally housed outside but were brought indoors and housed in a well-ventilated barn for the duration of each study. On the day of dosing, sheep were enclosed in a pen, in groups of up to 10 animals, to facilitate handling. The animals were not fasted prior to dose administration.

2.4.2.2 Cannulation of the jugular vein

The external jugular veins, running either side of the neck, were used as a convenient and easily accessible site for blood sampling in the sheep. Cannulation of the jugular vein is a non-surgical procedure which does not require anaesthesia of the animals. Jugular vein cannulation was performed the day prior to the actual dosing study day. An area of skin over the position of one of the jugular veins was shaved. The cannula tubing was inserted approximately 15 cm into the lumen of the jugular vein, facilitated by the use of a needle introducer and guidewire which were subsequently removed, and secured in-place with a subcutaneous suture. A bandage was wrapped round the neck of each sheep to protect the cannula. The cannula was kept patent by flushing it with heparinised saline (25 IU/ml heparin in 0.9% NaCl). The cannula remained indwelling in the jugular vein

of each animal for the duration of the study and was removed upon completion of blood sample collection.

2.4.2.3 Dose administration

The sheep were divided into groups of three or four animals and a different dose administered to each group. For the purpose of dose administration, the sheep were sedated with an intravenous dose of ketamine hydrochloride (100 mg/ml injection) at 2.25 mg/kg administered via the jugular vein cannula. This was intended for animal restraint, and also as a counter-measure against the animal sneezing during nasal administration. The anaesthesia lasted for approximately 3 minutes.

Nasal dose administration

Nasal solution formulations were administered at 0.01 ml/kg. The solutions were instilled from a 1 ml syringe attached to a cannula which was inserted into each nostril to a depth of 7-10 cm depending on the weight / size of the animals. The dose solution was divided equally between both nostrils.

Subcutaneous dose administration

Subcutaneous formulations were administered at 0.0476 ml/kg by injecting the solution into the subcutaneous tissue of the flank. To facilitate subcutaneous dose administration an area of skin on the flank was shaved.

2.4.2.4 Blood sampling

All blood samples were collected from the cannulated jugular vein of the sheep. Prior to the collection of each blood sample the initial 2 ml of blood collected was discarded to eliminate traces of heparinised saline.

During insulin absorption studies, blood samples of 4.0 ml were collected at 15 and 5 minutes (-15 and -5 minutes) prior to insulin dose administration and at 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 120, 150, 180 and 240 minutes post-administration. Blood samples were mixed gently in 4 ml heparinised tubes which were kept on crushed ice before plasma separation. The plasma was separated by centrifugation at 4°C and approximately 3000 rpm. Each plasma sample was divided into two aliquots and stored frozen at -20°C or -80°C awaiting glucose or insulin analysis, respectively (refer to Section 2.5).

During salmon calcitonin absorption studies, blood samples were collected pre- and post administration of formulations at -15, -5, 15, 30, 45, 60, 90, 120, 150, 180, 240, 300, 360, 420, 480 and 600 minutes and plasma separated as described above. Plasma was stored frozen at -20°C awaiting calcium analysis (refer to Section 2.5).

2.5 Measurement of glucose, insulin or calcium concentrations

Glucose, insulin or calcium concentrations in blood or plasma were measured in the nasal absorption studies performed in animals as described below.

2.5.1 Measurement of blood or plasma glucose concentrations

Blood or plasma glucose concentrations were measured, in rat and sheep absorption studies respectively, using a YSI Model 23 AM Glucose analyser (glucose analyser) and YSI Syringepet (syringepet) from Yellow Springs Instruments Co. Yellow Springs, Ohio, USA. The glucose analyser used an immobilised glucose oxidase enzyme membrane coupled with a highly glucose specific hydrogen peroxide sensor to measure glucose concentrations. Glucose in the sample making contact with the glucose oxidase membrane and oxygen, supplied by an air supply, is converted to gluconic acid and hydrogen peroxide (H_2O_2). The H_2O_2 is oxidised at a platinum anode and the oxygen produced by this reaction is reduced to water at the silver cathode. The current produced between the anode and cathode is directly proportional to the H_2O_2 and thus glucose concentration in the sample. The measurement of glucose concentrations is automated following the injection of the sample into the instruments sample chamber. Results are displayed digitally on a control panel. Samples of 25 µl were concurrently injected into the sample chamber of the glucose analyser using a syringepet (combination syringe and pipette). The instrument was calibrated before use and after every 10-15 samples using a 10 (± 0.1) mmol/l glucose standard solution. The experimental blood or plasma samples were mixed with a vortex mixer prior to injection. The measured glucose concentrations (mmol/l) were automatically displayed on the control panel of the instrument and were recorded manually.

Basal blood glucose concentrations in rats were typically between 3-4 mmol/l (range 2.8-5.1 mmol/l). Basal plasma glucose concentrations in sheep were typically between 3-4 mmol/l (range 2.8-5.2 mmol/l).

2.5.2 Measurement of insulin concentrations

Insulin concentrations in sheep plasma were measured by radioimmunoassay (RIA) using a double antibody method validated and routinely used by Dr I Gill, Danbiosyst UK Ltd. Some preliminary assays was performed under the supervision of Dr Gill although the bulk of the analysis was performed by Dr Gill. The RIA method relies on the competition between insulin in the standard solution or test (plasma) sample and ^{125}I-insulin (radio-labelled insulin) and a fixed amount of the first antibody raised against insulin in an animal model (guinea pig anti-insulin antibody). Competitive binding occurs between the radio-labelled and unlabelled insulin and the anti-insulin antibody. If the plasma sample contains high concentrations of insulin then the amount of bound unlabelled insulin will be high whereas the amount of bound radio-labelled insulin will be low (the amount of unbound radio-labelled insulin will be high). A second antibody, raised against the first antibody in a second animal model (donkey anti-guinea pig serum), is added followed by polyethylene glycol (PEG) which will precipitate the insulin-antibody complex. Following centrifugation the radioactivity in the supernatant or pellet is measured. The radioactivity in the pellet will be low and radioactivity in the supernatant will be high if plasma concentrations of insulin are high. Insulin standard solutions are used to prepare a standard curve from which the insulin concentrations in the test samples can be read.

Basal insulin concentrations in sheep were generally below 15 mU/l (range 0.0-35.1 mU/l).

2.5.3 Measurement of calcium concentrations

Plasma calcium analysis was performed following studies to investigate the intranasal absorption of salmon calcitonin in rats and sheep. Plasma calcium concentrations were measured at Kingsmill Hospital, Mansfield, Nottinghamshire, UK using an ERIS 6170 selective multitest analyser from Eppendorf (Olympus, Japan). The ERIS tests were developed by E Merck, Darmstadt, Germany. The test for calcium (total calcium) is based on the reaction of calcium ions in the sample with o-cresolphthalein complexone in alkaline solution to form an intense violet colour. The extinction of the colour formed is proportional to the concentration of calcium in the sample. The addition of 8-hydroxyquinoline eliminates interference by magnesium and iron. The ERIS test uses 7 µl of plasma and 70 µl of the reagent (o-cresolphthalein complexone 0.05 mmol/l and 8-hydroxyquinoline 3 mmol/l). Two standard solutions (2.0 and 3.0 mmol/l calcium chloride) are used for the calibration of the instrument. The absorbance of the sample-reagent mixture is calculated automatically as the difference between the measurement at 570 and at 600 nm. Samples (100 µl of rat plasma or 1 ml of sheep plasma which were pre-mixed with a vortex mixer prior to use) were dispensed into the appropriate sized sample cups which were pre-loaded in sequence into cup holders. Once the cup holders were loaded into the instrment, the analysis was fully automated and a printout of the measured calcium concentrations (mmol/l) was obtained.

Basal plasma calcium concentrations in rats and sheep ranged between 1.37-2.35 mmol/l and 2.2-2.6 mmol/l, respectively.

2.6 Calculation of results following glucose, insulin or calcium analysis

2.6.1 Calculation of results following glucose analysis

For each animal blood or plasma glucose concentrations were expressed as percentages of the basal glucose concentration (referred to as blood or plasma glucose concentrations (% of basal)), thus taking into account variation in basal values between animals. The basal glucose concentration (mmol/l) for each animal was calculated as the mean of the two pre-dose values and represented a glucose concentration of 100 %. For each formulation group, the mean blood glucose concentration (% of basal) was calculated, together with the standard deviations (SD), for each time point. The mean glucose concentration (% of basal) versus time curves were plotted for each formulation group.

For each animal, the minimum blood or plasma glucose concentration (% of basal), C_{min} and the time taken to reach the minimum concentration, T_{min}, was determined and mean values of C_{min} and T_{min}, with SD, were calculated for each formulation group. In some instances, the mean values of C_{min} with SD were plotted in the form of a column graph for each formulation group. To show more clearly correlations between formulation

factors such as osmolality, chitosan concentration or transient effect with absorption enhancing efficacy, the decrease in % glucose concentration was also calculated for individual animals by subtracting the value of C_{min} from the basal concentration (100%) and mean values calculated, with SD, for each formulation group. These were plotted against the formulation variables (refer to Chapters 4 and 7).

The area under the blood or plasma glucose concentration (% of basal) versus time curve (AUC) was determined for each animal, from 0-240 minutes (AUC_{0-240}) using a trapezoidal method and a Microsoft Excel programme on a Macintosh computer. For the purpose of determining the AUC, blood glucose concentrations above the basal concentration were taken as 100 %. The AUC_{0-240} was subtracted from the total area to obtain the area over the curve (AOC). Mean values of AOC, with SD, were calculated for each formulation group. The AOC value, representing the total effect of the dose on blood glucose concentrations, gave an indication of the efficacy of the formulation for nasal insulin delivery. In some instances, the mean values of AOC, with SD, were plotted in the form of a column graph for each formulation group.

In selected studies, an indirect estimate of the rate of insulin absorption was obtained by calculating the rate of reduction of glucose concentrations which was obtained from the slope of a plot of glucose concentration versus time over the first 30-60 minutes of the study. The slope of the plot was determined by linear regression of the data using a Cricket Graph programme on a macintosh computer. Some anomalous data points were omitted to improve the curve fit. The correlation coefficient, R^2, for all curves was above 0.9.

In some instances, an estimate of insulin absorption (%) was obtained from the glucose data by calculation of values of pharmacological availability as shown below (Li and Mitra, 1994). This calculation assumed that insulin absorption, i.e. elevation in insulin concentrations, correlated with decreases in glucose concentrations. It was also assumed that the subcutaneous injection of insulin had not saturated the glucose lowering response. It must be pointed out that values of pharmacological availability should be used and quoted with caution.

$$\text{Pharmacological availability (\%)} = \frac{\text{AOC(intranasal) x dose(subcutaneous)}}{\text{AOC(subcutaneous) x dose(intranasal)}} \text{ x } 100$$

For calculation purposes, the mean value of AOC(subcutaneous) was used.

2.6.2 Calculation of results following insulin analysis

For each animal plasma insulin concentrations were determined as mU/l. For each formulation group, the mean plasma insulin concentrations were calculated with SD at each time point. For individual sheep in each group the maximum plasma insulin concentrations (C_{max}) and the times at which C_{max} occurred (T_{max}) were noted and the mean values with SD calculated. For each animal the AUC_{0-240} was determined by the

trapezoidal method, as above, and the mean values of AUC with SD calculated for each formulation group. For each individual animal, the background (basal) plasma insulin concentrations, calculated as an average of the concentrations at -15 and -5 minutes, was subtracted from all subsequent concentrations prior to determining the AUC. Any negative value was assumed as zero. In selected studies, an estimate of the rate of insulin absorption was obtained by calculating the rate of increase of insulin concentrations which was obtained from the slope of a plot of natural log of insulin concentration versus time typically over the first 5-15 minutes of the study. The slope of the plot was determined by linear regression of the data. Anomalous data points were omitted to improve the curve fit. The correlation coefficient, R^2, for all curves was above 0.9.

The bioavailability of insulin from each formulation was calculated relative to the subcutaneous dose as given below.

$$\text{Relative bioavailability (\%)} = \frac{\text{AUC (intranasal) x dose (subcutaneous)}}{\text{AUC (subcutaneous) x dose (intranasal)}} \text{ x } 100$$

Values of bioavailability were calculated for individual sheep in each nasal formulation group prior to determining mean values (± SD). The mean value of AUC following subcutaneous administration was used for calculation purposes

2.6.3 Calculation of results following calcium analysis

Values of mean plasma calcium concentration (% of basal), C_{min}, T_{min} and $AUC_{0\text{-}600}$ were determined according to the method used to calculate results following glucose analysis (Section 2.6.1). Mean plasma calcium concentration (% of basal) versus time curves and mean values of C_{min} and AOC (± SD) were plotted for each formulation group. In some instances an estimate of salmon calcitonin (S-CT) absorption was obtained from the calcium data by calculation of values of pharmacological availability as shown below. This calculation assumed that S-CT absorption, i.e. elevation in S-CT concentrations, correlated with decreases in glucose concentrations and that the calcium lowering response following dose administration was not saturated.

$$\text{Pharmacological availability (\%)} = \frac{\text{AOC(intranasal) x dose(intramuscular)}}{\text{AOC(intramuscular) x dose(intranasal)}} \text{ x } 100$$

For calculation purposes, the mean values of AOC(intramuscular) was used.

2.6.4 Statistical analysis of glucose, calcium or insulin data

Statistical analysis of glucose (values of C_{min}, T_{min}, AOC and rates of reduction of glucose concentrations) calcium (values of C_{min}, T_{min}, AOC) and insulin (values of C_{max}, T_{max}, AUC and rates of increase of insulin concentrations) data was performed to determine significant differences between the various formulation groups tested. For each of the parameters tested, significant differences were evaluated by comparing the mean values in each formulation group. A one-way analysis of variance (one-way

ANOVA) was the statistical test of choice when comparing the mean data from three or more formulation groups. When comparison was made between the mean values from only two formulation groups, an unpaired Student's t-test was used. Statistics were performed with an Instat 2.03 programme on a Macintosh computer. A brief review of the ANOVA and the unpaired Student's t-test is given below. Reference should be made to Linton and Gallo (1975) and Bolton (1995) for mathematical details of the statistical tests.

The one-way ANOVA can be used to evaluate the significance between the mean values of three or more groups. The ANOVA makes the assumptions that the groups are randomly sampled from larger populations having a normal (Gaussian) distribution, that groups are independent of each other and that the standard deviations of the populations are equal. The ANOVA compares the variability between groups with the variability within groups by testing the null hypothesis that the means of the groups are equal. If the null hypothesis is rejected then the means of at least two of the groups are not equal (i.e. are significantly different). The statistic used to test the null hypothesis in the ANOVA is the F value which is a ratio of the variation between groups to that within the group. Calculated values of the F statistic are compared to tabulated values at a certain probability or P level. All P values were evaluated at a 95% confidence interval, i.e. for samples randomly selected from larger populations there is a 95% certainty that the true population means lies within the 95% confidence interval. At this level there is a 5% chance of randomly obtaining sample means which are significantly different from the population mean. Thus, the 5% value is the probability of mistakenly rejecting the null hypothesis. The Instat programme automatically calculates the F values and reports the P values. At a 95% confidence interval, results were interpreted as statistically significance if ($P<0.05$) and not significant if ($P>0.05$). Lower values of P indicate greater significance. The Instat programme interpreted a value of ($P<0.01$) as 'very significant' and a value of ($P<0.001$) as 'extremely significant'.

Rejection of the null hypothesis in the ANOVA indicates that there is significant difference between the means of the groups under consideration. However, it does not identify the source of the significant differences. Thus, if there were significant differences in the ANOVA ($P<0.05$), a Tukey-Kramer multiple comparisons test (post-test) was performed following the ANOVA to identify significant differences between the individual groups tested. In this test the probability of rejecting a true null hypothesis (i.e. the significant differences observed are really due to chance) is low (referred to as a Type I error). The Tukey-Kramer multiple comparisons test compares the mean values of two groups at a time (similar to the student's t-test) and identifies the 'q' statistic. The Tukey-Kramer multiple comparisons test was pre-selected to be performed automatically by the Instat programme if ($P<0.05$) in the ANOVA test. Calculated values of q were compared to tabulated values at a 95% confidence interval. Thus, there was significant difference between groups if ($P<0.05$).

When there were only two study groups, statistical comparison was made using an

unpaired student's t-test. The unpaired student's t-test compares the mean values of two data groups with the null hypothesis that the means of the two columns are equal. The t statistic was calculated and compared to tabulated values at a 95% confidence interval. Thus, P values obtained were significant if ($P<0.05$). The use of the t-test to compare mean values from two groups of data is the most elementary of statistical comparisons. If multiple t-tests are performed, i.e. there are more than two data groups requiring statistical comparison, the risk of falsely rejecting the null hypothesis is increased since the 5% chance of falsely rejecting the null hypothesis (at a 5% significance level) in each t-test is multiplied depending on the number of comparisons made. Hence for multiple comparisons the ANOVA should be used.

In each statistical test, either the raw data or mean data with standard deviation (SD) was entered in the Instat programme and results calculated automatically.

CHAPTER 3

PRELIMINARY INVESTIGATIONS OF THE EFFICACY OF CHITOSAN AND VARIOUS OTHER COMPOUNDS IN ENHANCING THE INTRANASAL ABSORPTION OF INSULIN IN THE RAT MODEL

3.1 General introduction

In this chapter the preliminary investigations of the efficacy of chitosan and various other compounds in enhancing the intranasal absorption of insulin in the rat model are reported. An initial study investigated the effect of a low viscosity glutamate salt of chitosan (LVCSN) at two concentrations (0.1% and 0.5%) and at two values of pH (~4 and ~ 7) on the nasal absorption of insulin. This study was apparently the first ever study performed to investigate the nasal absorption of chitosan. As a control an insulin solution (pH ~ 7) without absorption enhancers was administered nasally. In a second study, the efficacy of medium viscosity chitosan glutamate (MVCSN) was compared to that of various other compounds which had been shown in the literature to promote peptide drug absorption. The compounds investigated were: a bile salt, glycodeoxyxholate (GDC); a surfactant, polyoxyethylene-9-lauryl ether (Laureth-9); and a lysophospholipid, lysophosphatidylcholine (LPC). As controls, buffer solution and an insulin control solution were administered nasally. For reference, a subcutaneous dose of insulin was administered.

In the absorption studies, formulations were administered intranasally or subcutaneously to rats. Blood samples were collected pre- and post administration of the insulin formulations and blood glucose concentrations were measured. Insulin absorption was assessed, indirectly, from the decrease in blood glucose concentrations following insulin dose administration.

The studies are reported in the following Sections:

3.2 Effect of low viscosity chitosan, at pH ~ 4 or pH ~ 7, on the intranasal absorption of insulin in the rat

3.3 Efficacy of medium viscosity chitosan and various other compounds in enhancing the intranasal absorption of insulin in the rat

Materials and methods used were as described in Chapter 2 / Appendix 1. Additional details are given in the relevant sections.

3.2 Effect of low viscosity chitosan, at pH ~ 4 or pH ~ 7, on the intranasal absorption of insulin in the rat

3.2.1 Aims and objectives

In a preliminary investigation in an anaesthetised rat model, the effect of a water soluble low viscosity grade of chitosan glutamate (LVCSN) on the nasal absorption of insulin from liquid formulations was investigated. Insulin doses were administered nasally to

rats at 8 IU/kg. LVCSN was evaluated in liquid formulations at two concentrations; 0.1% and 0.5% w/v, expressed as the concentration of chitosan glutamate salt, and at two values of pH: pH ~ 4 or pH ~ 7. At pH ~4, the insulin and LVCSN were in solution. However, at pH ~ 7, a suspension was formed (refer to Section 3.2.3). As a control, a nasal insulin solution without LVCSN, at pH ~ 7, was administered.

3.2.2 Outline of study

3.2.2.1 Materials

Insulin (Semi-synthetic human sodium insulin, specific activity of pure insulin 28 IU/mg), Nordisk, Gentofte, Denmark. Using spectrophotometry, the purity of the material was determined to be 86.5%. Thus, the specific activity of the insulin was equivalent to 24.22 IU/mg and this value was used for all subsequent calculations.
Low viscosity chitosan glutamate (LVCSN), Pronova Biopolymer A/S, Drammen, Norway (refer to Table 2.1).
All other reagents were of analytical grade.

3.2.2.2 Preparation of insulin formulations

Insulin formulations were prepared freshly on the morning of each study day. Each formulation contained 80 IU/ml insulin (sufficient material to administer a dose of 8 IU/kg insulin in a volume of 0.1 ml/kg). The formulations containing chitosan contained either 0.1% (1 mg/ml) or 0.5% (5 mg/ml) LVCSN, expressed as the concentration of chitosan glutamate salt. The formulations are listed below. Each formulation was prepared in 14.65 mM phosphate buffer of pH 7.4. Formulations prepared at pH ~ 7 were prepared directly in phosphate buffer. To the formulations at pH ~ 4 was added HCl, during formulation preparation. Suspensions of insulin / LVCSN were obtained at pH ~ 7 whereas at pH ~ 4 the insulin and LVCSN were in solution. Each formulation was vortex mixed immediately prior to nasal administration to rats.

Table 3.1. Outline composition of formulations

Formulation No.	Outline composition
1	Solution: 80 IU/ml insulin, pH~7 (pH 7.4) (control solution)
2	Suspension: 80 IU/ml insulin with 0.1% LVCSN, pH~7 (pH 7.1)
3	Suspension: 80 IU/ml insulin with 0.5% LVCSN, pH~7 (pH 6.6)
4	Solution: 80 IU/ml insulin with 0.1% LVCSN, pH~4 (pH 3.6)
5	Solution: 80 IU/ml insulin with 0.5% LVCSN, pH~4 (pH 4.4)

* Concentration of LVCSN expressed as the concentration of chitosan glutamate

3.2.2.3 Absorption study in the rat model

Each rat was dosed with 8 IU/kg insulin at a volume of 0.1 ml/kg. A summary of the dose groups is given in Table 3.2. In this study there was a slight deviation from the times of blood sample collection given in Chapter 2 (additional samples at 15 and 40 minutes, omitted samples at 30 and 45 minutes). Values of C_{min}, T_{min} and AOC were

not determined in this study since only two rats survived for the full duration of the study after administration of an insulin solution containing 0.1% LVCSN at pH ~ 4 and all rats dosed with insulin solution containing 0.5% LVCSN at pH ~ 4 died prematurely probably as a result of severe hypoglycaemia. Thus, meaningful comparison of the different dose groups was limited.

3.2.3 Results and Discussion

Results are given in Table 3.3 and Figure 3.1. In the study the reduction in blood glucose concentrations was used as an indirect assessment of insulin absorption. The control solution of insulin (pH ~7) dosed at 8 IU/kg, resulted in substantial reductions in blood glucose concentrations in rats following nasal administration with mean blood glucose concentrations decreasing to approximately 48% of the basal concentration 60 minutes after dose administration (Table 3.3, Figure 3.1). These results were in contrast to the results published by Chandler et al (1991b) who administered nasal doses of 8 IU/kg insulin (from a solution prepared in pH 7.3 buffer) to male rats of a similar species, strain and weight as those used in this study. Chandler et al delivered a dose volume of 20 μl approximately 0.5 cm into the right nostril of the rat and assessed absorption, indirectly from blood glucose concentrations, over a 60 minute period, finding that insulin was not absorbed. The reasons for the discrepancy in the results between the two studies is not known. However, in this study the dose was delivered 0.8 cm into the nostril of the rat compared to about 0.5 cm in the study by Chandler et al. In the pioneering studies of Hirai et al. (1981c) the nasal administration of a control solution of insulin (10 U/kg) was shown to lower blood glucose concentrations to less than 95% of basal values within 1 hour of dosing with maximum hypoglycaemia of about 90% at 2 hours. Glucose concentrations did not return to basal values until 3-4 hours after dose administration.

Based on the dimensions of cross sections of casts of the rat nasal cavity reported by Schreider (1986), the length of the nasal cavity of a 250g rat was 2.3 cm of which the external nares occupied the anterior 0.5 cm. Thus, it is possible that in the study by Chandler et al (1991b), the nasal dose was delivered to the posterior region of the external nares compared to dose delivery to the maxilloturbinate and nasoturbinate regions of the nasal cavity in this study. Lower absorption of compounds would be expected from the external nares since the epithelium is likely to be stratified squamous which may in-part be keratinised and will thus present a more robust barrier to absorption than the respiratory epithelium in the nasal turbinate regions. Also, the mucosa of the nares will be less vascular than that of the the respiratory region.

Nasal administration of a suspension of insulin (8 IU/kg) with 0.5% LVCSN (pH ~ 7) or solutions of insulin (8 IU/kg) with 0.1% or 0.5% LVCSN at pH 4, resulted in decreases in blood glucose concentrations which were more pronounced than those resulting from the insulin control solution (Table 3.3, Figure 3.1). However, the suspension of insulin with 0.1% LVCSN at pH ~ 7 appeared to be less effective at promoting insulin absorption than the control insulin solution. The solution containing 0.5% LVCSN at pH

~4 was the most effective formulation and its administration resulted in the premature death of all animals in the group probably due to severe hypoglycaemia. At 60 minutes following dose administration the mean blood glucose concentration had dropped to about 16% of the basal concentration although at this time glucose concentrations were probably still continuing to decrease in three out of four animals in the group which died prior to collecting the 90 minute blood sample. The premature death of all animals in the group was preceded by twitching, muscle spasms, salivation and respiratory depression which indicate symtoms associated with severe hypoglycaemia. Of the other dose groups, decreases in mean blood glucose concentrations (% of basal concentration) were as follows: 61% (0.1% LVCSN suspension pH ~7), 27% (0.5% LVCSN suspension pH ~7), 44% (0.1% LVCSN suspension pH ~4) compared to 48% for the control insulin solution (Table 3.3, Figure 3.1).

In this preliminary study in rats, LVCSN enhanced the absorption of insulin. The effect of LVCSN was more pronounced at pH ~ 4 than at pH ~ 7. This was expected in view of the fact that at pH 4 the insulin and LVCSN were in solution whereas at pH 7 the insulin and chitosan were in the form of a course suspension due to the formation of a complex. Chitosan can adsorb proteins from aqueous solution and maximum adsorption is reported to occur at the isoelectric point of the protein (Roberts, 1992). Furthermore, at a given pH, the degree of adsorption will decrease with an increase in the ionic concentration of the medium. Errington (1993) investigated complex formation between insulin, having an isoelectric point of about 5.30 to 5.35 (The Merck Index, 1996), and medium viscosity chitosan glutamate (Seacure +210) at a pH of about 7. The complex was reported to be insoluble and consist of approximately 12 insulin molecules to every chitosan molecule. However, it was pointed out that this value represents an empirical average since the polydispersity of the chitosan sample, possible crosslinking and aggregation of complex particles will influence the values of the insulin:chitosan binding ratios obtained. The suspension was reported to become 'clear to the naked eye' at a pH of about 4.8. Complex formation was attributed to the aggregation of chitosan and insulin due to the interaction of the negative carboxyl groups of insulin, at neutral or weakly acidic pH, and the positively charged amino groups of chitosan and insulin. At acid pH, the amino groups of chitosan and insulin will be strongly charged. However, the charge of the carboxyl groups will tend to diminish as pH is lowered which will reduce aggregation. Electrostatic repulsion between the positively charged amino groups of insulin and chitosan will also prevent aggregation. The amount of insulin which is available for absorption will be reduced by the formation of an insoluble complex which probably accounted for the reduced insulin absorption observed following the nasal administration of these formulations to rats. Furthermore, the absorption enhancing properties of chitosan are likely to be affected by complex formation since this will decrease the amount of chitosan in solution and will dramatically influence the viscosity of the formulation. Since there is a fixed binding ratio between insulin and chitosan, then increasing the formulation concentration of either insulin or chitosan would be expected to improve nasal absorption since this should increase the concentrations of the respective component which is non-complexed.

The concentration of chitosan influenced the absorption enhancing efficacy and formulations containing 0.5% chitosan (as glutamate salt) either as solutions or suspensions, were more effective than those containing 0.1% LVCSN. In view of the unexpected absorption of insulin from the control insulin solution and to fully evaluate the absorption enhancing efficacy of the chitosan formulations it was decided that a lower dose of insulin (4 IU/kg) would be used in subsequent nasal absorption studies in rats. Based on the results of a preliminary nasal absorption study in sheep (refer to Chapter 6), showing that medium viscosity chitosan glutamate (MVCSN) was more effective than low viscosity chitosan glutamate (LVCSN) in promoting the intranasal absorption of insulin, it was decided to focus absorption studies in the rat on the evaluation of MVCSN.

Table 3.2. Summary of formulations and dose groups in the study to investigate the effect of LVCSN at pH ~4 or pH ~7 on the intranasal absorption of insulin in rats

Formulation or Group No.	Insulin (IU/kg)	LVCSN (mg/kg)	Dose volume (ml/kg)
1. Insulin control solution	8.0	-	0.1
2. Insulin + 0.1% LVCSN pH~7 (suspension)	8.0	0.1	0.1
3. Insulin + 0.5% LVCSN pH~7 (suspension)	8.0	0.5	0.1
4. Insulin + 0.1% LVCSN pH~4 (solution)	8.0	0.1	0.1
5. Insulin + 0.5% LVCSN pH~4 (solution)	8.0	0.5	0.1

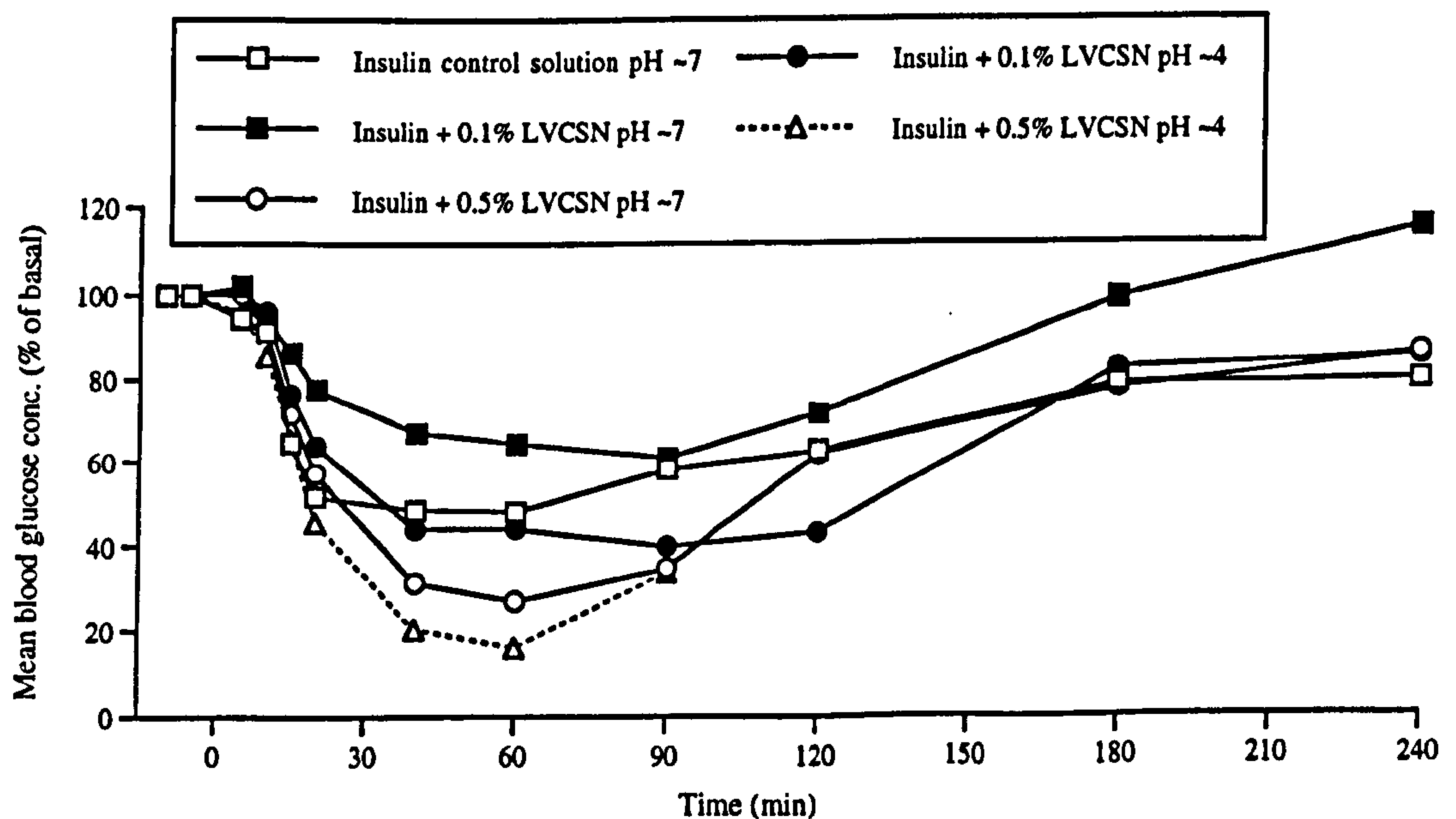
* Dose of LVCSN expressed as the dose of chitosan glutamate.

Table 3.3. Summary of mean blood glucose concentrations following the intranasal administration of 8 IU/kg insulin with or without LVCSN at pH ~4 or pH ~7 in rats

Time before or after dosing (min)	Mean ± SD (n=3-4) blood glucose concentration (% of basal)									
	Insulin control (pH ~7)		Insulin/ 0.1% LVCSN (pH ~7)		Insulin / 0.5% LVCSN (pH ~7)		Insulin / 0.1% LVCSN (pH ~4)		Insulin / 0.5% LVCSN (pH ~4)	
		SD		SD		SD		SD		SD
-10	100.0	0.00	100.0	0.00	100.0	0.00	100.0	-	100	0.00
-5	100.0	0.00	100.0	0.00	100.0	0.00	100.0	-	100	0.00
5	94.5	3.85	102.0	3.27	100.2	3.06	100.5	-	95.8	8.49
10	91.4	5.50	94.3	5.17	91.8	5.13	96.1	-	85.6	7.79
15	64.6	1.27	86.4	6.15	71.6	16.31	76.5	-	65.4	13.41
20	51.8	7.15	77.6	10.60	57.3	15.82	63.8	-	45.6	14.75
40	48.6	13.99	67.2	15.56	31.4	3.84	44.1	-	20.5	10.67
60	48.2	15.45	64.6	8.02	27.1	7.14	44.2	-	16.1	11.41
90	58.3	13.46	61.1	12.96	34.7	14.28	39.9	-	33.7	-
120	62.8	20.05	72.0	24.08	62.0	8.87	43.2	-	*	-
180	79.2	30.74	99.1	31.46	78.0	-	82.7	-	*	-
240	79.6	21.43	115.0	32.29	86.1	-	85.3	-	*	-

* All animals in this formulation group died prematurely

Figure 3.1. Blood glucose concentration versus time profiles following the intranasal administration of 8 IU/kg insulin with or without LVCSN at pH ~4 or pH ~7 in rats



3.3 Efficacy of chitosan and various other compounds in enhancing the intranasal absorption of insulin in the rat

3.3.1 Aims and objectives

The absorption enhancing efficacy of medium viscosity chitosan glutamate (MVCSN) was compared to that of various other compounds which had been shown in the literature to promote peptide drug absorption. The compounds investigated were: a bile salt; glycodeoxyxholate (GDC), a surfactant; polyoxyethylene-9-lauryl ether (Laureth-9) and a lysophospholipid; lysophosphatidylcholine (LPC). As controls, buffer solution and an insulin control solution were administered nasally. For reference, a subcutaneous dose of insulin was administered.

3.3.2 Study outline

3.3.2.1 Materials

Insulin (Semi-synthetic human sodium insulin, specific activity of pure insulin 28 IU/mg), Nordisk, Gentofte, Denmark. Using spectrophotometry the purity of the material was determined to be 78%. Thus, the specific activity of the insulin was equivalent to 21.84 IU/mg and this value was used for all subsequent calculations.
Medium viscosity chitosan glutamate (MVCSN), Pronova Biopolymer A/S, Drammen, Norway (refer to Table 2.1).
Glycodeoxycholate, sodium salt (GDC, No. G-3258), Polyoxyethylene-9-lauryl ether (Laureth-9, No. P-9641) and Lysophosphatidylcholine (LPC, No. L-4219) from Sigma Chemical Company Limited, Poole, Dorset, UK.

3.3.2.2 Preparation of solution formulations

Solution formulations were prepared for nasal (Formulations 1-6) and subcutaneous (Formulation 7) administration to rats as outlined in Table 3.4. In each of the nasal formulations, with the exception of Formulation 1 which did not contain insulin (buffer solution used), the insulin concentration was 40 IU/ml (sufficient to administer a dose of 4 IU/kg insulin in a dose volume of 0.1 ml/kg). The subcutaneous formulation contained 4 IU/ml insulin (sufficient to administer a dose of 1 IU/kg insulin in a dose volume of 0.25 ml/kg). With the exception of Formulation 3, the nasal formulations were prepared in 14.65 mM phosphate buffer of pH 7.3-7.4. Formulation 3 was prepared in 0.1125 % NaCl and adjusted to pH 4 during formulation preparation by the addition of 1 M HCl. The subcutaneous solution (Formulation 7) was prepared in 0.9 % NaCl. Each formulation was freshly prepared on the day of the study.

3.3.2.3 Absorption study in the rat model

With the exception of rats dosed with buffer control solution, each rat was dosed nasally with 4 IU/kg insulin at a volume of 0.1 ml/kg. The subcutaneous dose was administered at 1 IU/kg at a volume of 0.25 ml/kg. A summary of the dose groups is given in Table 3.5.

Table 3.4. Outline composition of formulations

Formulation No.	Outline composition
1	Buffer control solution, pH 7.4
2	40 IU/ml insulin control solution, pH 7.4
3	40 IU/ml insulin with 0.5% (5 mg/ml) MVCSN, pH 4
4	40 IU/ml insulin with 0.9% (9 mg/ml) GDC, pH 7.4
5	40 IU/ml insulin with 0.9% (9 mg/ml) Laureth-9, pH 7.4
6	40 IU/ml insulin with 0.5% (5 mg/ml) LPC, pH 7.4
7	4 IU/ml insulin subcutaneous reference solution

* Concentration of MVCSN expressed as the concentration of chitosan glutamate

3.3.3 Results and Discussion

Results are given in Tables 3.6-3.7 and Figures 3.2-3.3. There was no reduction in mean blood glucose concentrations following the nasal administration of the control buffer solution (Table 3.6, Figure 3.2). Blood glucose concentrations were actually shown to increase over the experimental period which may be due to the affects on normal physiology by the use of anaesthetic, the surgical procedure or blood sample collection. In contrast, each of the nasal formulations containing insulin resulted in a reduction in mean blood glucose concentrations following administration although the extent and duration of the hypoglycaemia was variable between the formulation groups (Table 3.6, Figure 3.2). The control insulin solution resulted in a small decrease in blood glucose concentrations. The hypoglycaemia after dosing the insulin control solution tended to be of relatively short duration and glucose concentrations had returned to basal values between 45-60 minutes. The coadministration of insulin with either MVCSN or the absorption enhancers; GDC, Laureth-9 or LPC, resulted in more substantial and prolonged decreases in blood glucose concentrations compared to the insulin control solution. Following subcutaneous insulin administration (1 IU/kg), the hypoglycaemia was sustained and gluocse concentrations had not recovered to basal values by the end of the study period. Insulin was also apparently absorbed nasally at a faster rate following the administration of the MVCSN or enhancer formulations compared to the control insulin solution as indicated by the lower rates of decrease of glucose concentrations for the control solution (Table 3.7). However, after dosing the nasal formulations, the rates of reduction of glucose concentrations were marginally lower than those following subcutaneous dose administration.

Lower reductions in glucose concentrations, indicated by higher values of C_{min}, and AOC (hence pharmacological availability) were observed (Table 3.7, Figure 3.3) following the nasal administration of the buffer and insulin control solutions (values of C_{min} ~ 97% and 86% and AOC 53 and 1172 % glucose.min, respectively). Values of C_{min} and AOC show that LPC was apparently the most effective nasal absorption enhancer in terms of the extent and duration of the hypoglycaemia which ensued

following dose administration. The magnitude of the hypoglycaemia (C_{min}) following the nasal administration of 4 IU/kg insulin with LPC (~ 32%) was similar to that of a subcutaneous dose of 1 IU/kg insulin (~ 29%) although the subcutaneous dose resulted in more prolonged hypoglycaemia as indicated by the greater value of AOC for the latter (8680 and 13379 % glucose.min, respectively). Formulations containing either chitosan or GDC were slightly less effective than the LPC formulation in lowering blood glucose concentrations. The chitosan and GDC formulations lowered blood glucose concentrations to a similar extent (values of C_{min} ~ 43% and 45%, respectively) although the hypoglycaemia was more sustained following the administration of the chitosan formulation (values of AOC 7386 and 4556 % glucose.min, respectively). Laureth-9 was generally the least effective of the potential absorption enhancers in lowering blood glucose concentrations (C_{min} ~ 55%, AOC 3661 % glucose.min) which was surprising considering the severe damage caused to the rat nasal membrane by 1% Laureth-9 in the studies by Chandler et al (1991).

Based on the mean values of C_{min}, the effectiveness of the formulations in promoting insulin absorption were ranked in decreasing order of: LPC > chitosan > GDC > Laureth-9 >> insulin control > buffer control. However, there was no significant difference between the performance of the formulations which contained either LPC, chitosan, GDC or Laureth-9 when they were compared with a Tukey-Kramer multiple comparisons test although each of these formulations was significantly different from the control insulin solution Values of AOC followed a similar trend to the values of C_{min} and were ranked in decreasing order: LPC > chitosan >> GDC > Laureth-9 >> insulin control > buffer control. Values of AOC indicate the overall extent and duration of the hypoglycaemia and considering the inter-animal variations show that the performance of the LPC formulation was only marginally better than the chitosan formulation. However, despite similar values of C_{min}, the chitosan formulation was apparently much more effective than the GDC formulation due to the sustained hypoglycaemia. Comparison of the values of AOC using a Tukey-Kramer multiple comparisons test, showed that there was no significant difference between the LPC and chitosan formulations but that each of these formulations was significantly different from the GDC, Laureth-9 and insulin control solutions. The coadministration of either MVCSN or LPC with insulin signifcantly increase values of Tmin compared to the control insulin formulation. For LPC, values of Tmin were also statistically different from the GDC and Laureth-9 dose groups.

Assuming that after insulin dose administration the lowering of blood glucose concentrations correlates with the absorption of insulin (elevation in blood insulin concentrations), then calculation of values of pharmacological availability (refer to Section 2.6) provided an estimation of the % absorption of insulin administered nasally compared to the subcutaneous route. Estimated absorption was only about 2% for the insulin control solution compared to values of approximately 16%, 14%, 8% or 7% following coadministration of insulin with LPC, MVCSN, GDC or Laureth-9 respectively. Thus, compared to the insulin control solution, nasal insulin absorption was increased ~8-fold by coadministration with LPC, ~7-fold with MVCSN, ~4-fold

with GDC and ~3-fold with Laureth-9.

The results obtained following the nasal administration of a formulation of insulin (dosed at 4 IU/kg) with 0.5% medium viscosity chitosan glutamate are in agreement with those reported by Aspden (1996) in terms of values of C_{min} although there are discrepancies between values of T_{min} and AOC obtained in the respective studies. After nasally administering the formulations, Aspden reported mean values of C_{min}, T_{min} and AOC of approximately 37%, 120 minutes and 10670 % glucose.min, respectively compared to values of about 43%, 47 minutes and 7386 % glucose.min in this study. The values of C_{min} are in close agreement. However, there is less agreement between values of T_{min} and AOC. The reasons for this are unclear but, as in the studies of Chandler et al. (1991b) previously mentioned, Aspden administered the nasal dose solution only 0.5 cm into the rat nasal cavity compared to 0.8 cm in this study. Thus, it is possible that in the studies of Aspden, the viscous chitosan solution was delivered to the posterior of the external nares hence a longer time was required for chitosan to exert its effect on the more robust nasal tissue in this region. Alternatively, slow spread and drainage of the chitosan formulation in the rat nasal cavity from the external nares to the respiratory regions, from where absorption can readily occur, may also account for the higher value of T_{min} observed by Aspden. This would also explain the higher values of AOC observed. Aspden (1996) also showed that there was very little nasal absorption of insulin from a control solution (C_{min} 96% compared to 86% in this study) which again may be due to deposition and poor absorption of dose from the posterior of the external nares.

The study demonstrated that insulin was not effectively absorbed via the nasal route without coadministration with absorption enhancing compounds. The compounds investigated ranked in decreasing order of absorption enhancing potency as follows: LPC > chitosan >> GDC > Laureth-9. The results are extremely encouraging since MVCSN, reported to be non-damaging to the nasal mucosa (Illum et al., 1994, Aspden, 1996) ranked almost as well as LPC and better than either GDC or Laureth-9 which have each been shown in various studies to damage the integrity of the nasal membrane (refer to Section 1.6.2).

Table 3.5. Summary of formulations and dose groups in the study to investigate the efficacy of chitosan and various other compounds in enhancing the intranasal absorption of insulin in rats

Formulation or Group No.	Insulin (IU/kg)	Enhancer (mg/kg)	Dose volume (ml/kg)
1. Buffer control solution	-	-	0.1
2. Insulin control solution	4.0	-	0.1
3. Insulin + 0.5% MVCSN	4.0	0.5	0.1
4. Insulin + 0.9% GDC	4.0	0.9	0.1
5. Insulin + 0.9% Laureth-9	4.0	0.9	0.1
6. Insulin + 0.5% LPC	4.0	0.5	0.1
7. Subcutaneous insulin reference solution	1.0	-	0.25

* Dose of MVCSN expressed as the dose of chitosan glutamate.

Table 3.6. Summary of blood glucose concentrations following the intranasal (4 IU/kg) or subcutaneous (1 IU/kg) administration of insulin with or without MVCSN or various other compounds in rats

Time before or after dosing (min)	Mean ± SD (n=3-5) blood glucose concentration (% of basal)													
	buffer control		Insulin control		Insulin/MVCSN		Insulin/GDC		Insulin/Laureth-9		Insulin/LPC		Subcut. insulin	
		SD		SD		SD		SD		SD		SD		SD
-10	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
-5	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
5	100.1	5.70	99.6	3.78	98.0	4.40	95.9	8.93	95.9	5.53	97.3	5.35	91.9	7.66
10	103.7	5.59	95.0	5.69	87.4	4.32	75.0	7.30	75.9	5.73	66.3	7.70	67.2	11.33
20	105.4	8.21	86.5	11.97	58.9	2.80	57.1	9.15	58.4	12.38	59.3	8.28	45.1	3.22
30	108.8	17.32	91.1	17.64	47.5	6.34	49.2	11.66	57.7	20.20	50.8	4.05	44.3	10.97
45	107.8	7.35	97.0	16.76	44.6	7.97	46.9	16.26	64.5	15.98	41.5	10.06	39.1	4.68
60	111.2	8.28	100.2	16.05	47.7	9.13	55.5	15.26	71.2	11.98	34.3	6.11	33.2	4.00
90	117.7	8.92	98.8	11.43	55.5	4.48	67.0	16.95	78.5	7.67	41.0	13.17	41.0	14.16
120	119.8	14.03	107.7	15.89	64.0	4.85	93.1	18.10	85.8	6.22	53.2	10.30	33.3	7.25
180	121.6	10.04	124.4	14.70	80.3	5.51	116.4	17.90	104.9	15.36	81.8	4.97	40.9	13.19
240	129.5	4.85	117.6	29.55	102.3	8.38	139.1	21.39	133.1	8.53	102.2	7.42	63.0	8.33

Table 3.7. Summary of pharmacokinetic parameters determined from glucose data following the intranasal (4 IU/kg) or subcutaneous (1 IU/kg) administration of insulin with or without MVCSN or various other compounds in rats

Formulation or Group number	No. rats in group	Mean ± SD				
		Cmin (% glucose)	Tmin (min)	AOC (% glucose.min)	Rate of decrease of glucose conc. (%/min)	Pharmacological availability * (%)
1. Buffer control	3	96.5 (± 3.09)	6 (± 5.2)	53 (± 65.2)	-	-
2. Insulin control	5	85.9 (± 11.12)	20 (± 7.2)	1172 (± 1017.32)	0.44 (± 0.350)	2.1 (± 1.89)
3. Insulin / MVCSN	4	43.3 (± 7.46)	47 (± 9.4)	7386 (± 748.2)	1.58 (± 0.134)	13.8 (± 1.37)
4. Insulin / GDC	4	45.4 (± 14.59)	38 (± 8.7)	4556 (± 1800.3)	1.53 (± 0.341)	8.5 (± 3.39)
5. Insulin / Laureth-9	5	54.9 (± 17.66)	24 (± 0.0)	3661 (± 1601.7)	1.83 (± 0.707)	6.8 (± 3.04)
6. Insulin / LPC	4	31.8 (± 6.18)	64 (± 18.9)	8680 (± 837.1)	1.64 (± 0.960)	16.2 (± 1.59)
7. Subcut. insulin	3	28.9 (± 0.95)	110 (± 45.8)	13379 (± 1396.0)	2.13 (± 0.156)	100.0

* Relative to subcutaneous

Tests of statistical significance (nasal dose groups)

One-way ANOVA

Sig. dif. in values of Cmin, Tmin, AOC (P<0.001) and rate of decrease of glucose conc. (P<0.05) between the formulation groups.

Tukey-Kramer Multiple Comparisons Test following ANOVA

Cmin: sig. dif. between groups:1vs3, 1vs4, 1vs5, 1vs6, 2vs3, 2vs4, 2vs6 (P<0.001) and 2vs5 (P<0.01)

Tmin: sig. dif. between groups: 1vs6, 2vs6 (P<0.001), 1vs3, 5vs6 (P<0.01) and 1vs4, 2vs3, 4vs6 (P<0.05)

AOC: sig. dif. between groups:1vs3, 1vs6, 2vs3, 2vs6, 5vs6 (P<0.001), 1vs4, 2vs4, 3vs5, 4vs6 (P<0.01) and 1vs5, 3vs4 (P<0.05)

Rate of decrease of glucose conc.: sig. dif. between groups: 2vs5 and 2vs6 (P<0.05).

No sig. dif. (p>0.05) in values of Cmin, Tmin, AOC or rate of decrease of glucose conc. between other groups.

Figure 3.2. Blood glucose concentrations versus time profiles following the intranasal (4 IU/kg) or subcutaneous (1 IU/kg) administration of insulin with or without MVCSN or various other compounds in rats

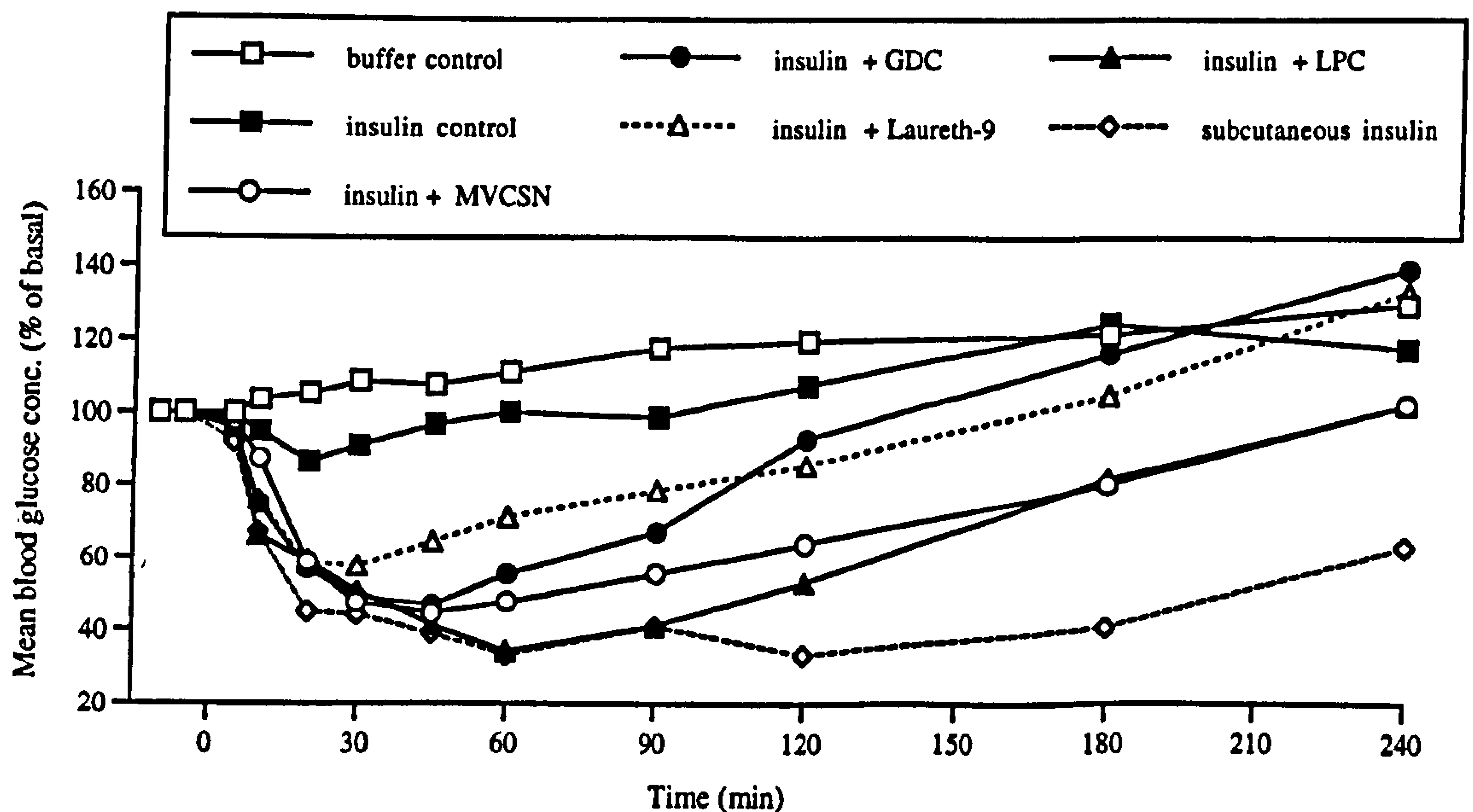
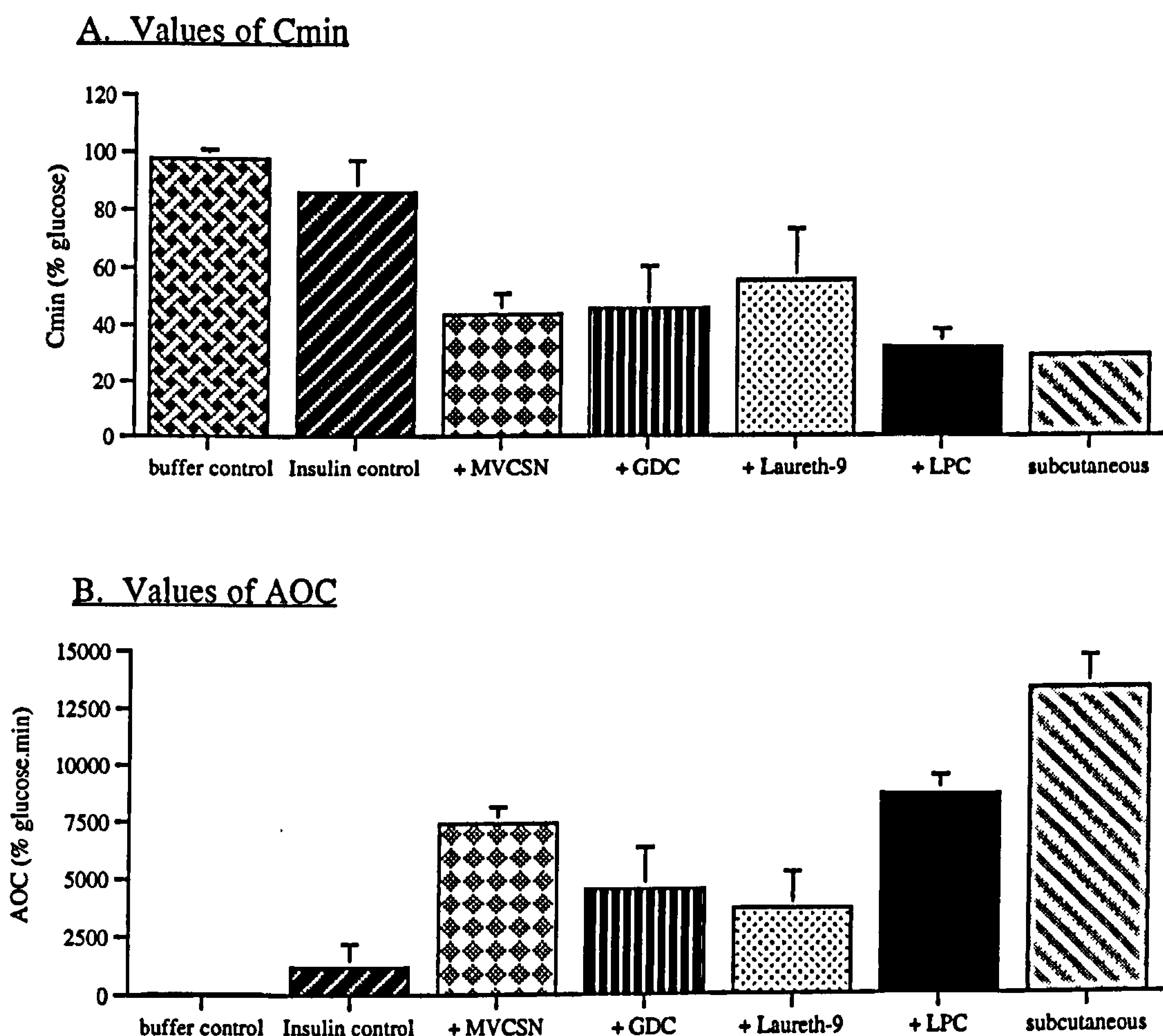


Figure 3.3. Values of pharmacokinetic parameters determined from glucose data following the intranasal (4 IU/kg) or subcutaneous (1 IU/kg) administration of insulin with or without MVCSN or various other compounds in rats



3.4 Conclusions

The studies reported in this chapter have demonstrated that a relatively high dose of insulin (8 IU/kg) was substantially absorbed nasally in the anaesthetised rat model even in the absence of absorption promoting compounds although coadministration of a low viscosity chitosan glutamate (LVCSN) was shown to further improve nasal absorption. The efficacy of LVCSN was improved at acid pH and at higher concentrations.

At a lower dose of insulin (4 IU/kg), insulin was not effectively absorbed via the nasal route without coadministration with absorption enhancing compounds. Medium viscosity chitosan glutamate (MVCSN) at 0.5% and at pH 4 was highly effective in enhancing insulin absorption ranking almost as well as LPC and better than either GDC or Laureth-9. A comparison of the performance of LVCSN and MVCSN is difficult since different concentrations of insulin were used in the respective studies. The results of the preliminary investigations of nasal delivery systems based on chitosan are encouraging in view of the reported low toxicity (Illum et al., 1994, Aspden, 1996) of chitosan and thus it was concluded that chitosan would be further evaluated as an enhancer for nasal delivery.

CHAPTER 4

INVESTIGATION OF FACTORS INFLUENCING THE NASAL ABSORPTION ENHANCING EFFICACY OF CHITOSAN IN THE RAT

4.1 General Introduction

In preliminary investigations, medium viscosity chitosan glutamate (MVCSN) was shown to effectively enhance the intranasal absorption of insulin in the anaesthetised rat model. In this chapter, investigations of a number of formulation factors which may influence the absorption enhancing efficacy of chitosan are reported along with additional studies which were performed to further elucidate the potential use of chitosan in nasal delivery systems.

The tonicity of nasal formulations is an important consideration since this may influence the influx or efflux of water in the nasal cavity. Water transport through the numerous glands in the nasal cavity has been reported to influence nasal absorption through solvent drag (Kotani et al., 1983). Morimoto et al. (1985) reported that water absorption from a gel base applied nasally could improved nasal drug absorption through intracellular channels. Tonicity will also influence the potential irritant action of nasal formulations and to prevent or limit nasal irritancy formulations should be isotonic to physiological fluids. The tonicity of drug formulations has been reported to influence drug absorption (Ohwaki et al, 1987). Furthermore, in formulations incorporating MVCSN, ionic concentration will influence the conformation of chitosan in solution and hence the formulation viscosity. Pennington et al. (1988) suggested that the therapeutic efficacy of nasal preparations may be increased by increasing formulation viscosity since this will reduce the rate of clearance of the formulation from the nasal cavity. Thus, formulation viscosity may be an important factor for the absorption enhancing efficacy of chitosan. Thus, the effects of tonicity (adjusted using sodium chloride) on the nasal absorption of insulin in formulations with and without MVCSN was investigated.

The effect of changing the concentration of chitosan in the nasal insulin formulation was also investigated. Chitosan concentration will influence both the viscosity (Filar and Wirick, 1977) and bioadhesive (Lehr et al., 1992) properties of the formulation as well as obviously determining the number of molecules of chitosan available to interact with the nasal mucosa. In Chapter 3, it was shown that the absorption enhancing efficacy of LVCSN was improved by increasing its concentration in a formulation with insulin from 0.1% to 0.5%.

When developing nasal formulations containing absorption enhancers or excipients, it is a prerequisite that these compounds do not cause serious damage to the nasal mucosa or result in prolonged impairment of the normal physiology and function of the nasal cavity. Thus, the enhancer should have a transient effect in the nasal cavity following administration (Illum et al., 1994). This is especially important for potential chronic nasal medication which may require once or multiple daily administrations. Many

compounds which have been demonstrated in the literature to enhance nasal peptide absorption have also been shown to cause severe damage to the nasal membrane which may also be the principal mode of action for these compounds. A study was performed to evaluate whether the effect of chitosan on nasal insulin absorption was transient which would provide a useful indication of the effect of chitosan on the nasal mucosal barrier.

In previous studies reported in this project, low and medium viscosity grades of chitosan glutamate were tested. Since chitosan is commercially available in numerous grades and salt forms then investigation of the use of chitosan as a nasal absorption enhancer should focus on optimising the grade of chitosan employed. A study was performed to compare the absorption enhancing efficacy of four different grades of chitosan in the rat model.

An important criteria for the selection of candidate nasal delivery systems is the reproducibility and predictability of intrasubject and intersubject drug absorption. Unreproducible and unpreditable nasal absorption are likely to prevent the application of the nasal delivery system on a commercial basis. Thus, a study was perfomed to investigate the inter-animal variations in nasal insulin absorption following nasal insulin/ MVCSN dose administration to different groups of rats on different study days.

In the nasal absorption studies, the various formulations were administered intranasally to rats. Blood samples were collected pre- and post administration of the insulin formulations and blood glucose concentrations measured. Insulin absorption was assessed, indirectly, from the decrease in blood glucose concentrations following insulin dose administration. Materials and methods used were as described in Chapter 2 / Appendix 1. Additional details are given in the relevant sections.

The studies are reported in the following sections:

4.2. Effect of tonicity on intranasal insulin absorption of insulin from solution formulations with and without chitosan in rats

4.3. Effect of different concentrations of chitosan on the intranasal absorption of insulin in rats

4.4. Investigation of the transient effect of chitosan on the intranasal absorption of insulin in the rat

4.5 Comparison of the efficacy of different grades of chitosan in enhancing the nasal absorption of insulin in the rat

4.6 The reproducibility of insulin absorption from a formulation containing chitosan in the rat

4.2 Effect of tonicity on intranasal insulin absorption from solution formulations with and without chitosan in rats

4.2.1 Aims and objectives

In studies in rats, the effect of osmolality on the intranasal absorption of insulin (4 IU/kg)

from solution formulations (pH 4.0) both with or without 0.5% w/v MVCSN was investigated. Sodium chloride (NaCl) was used to increase the osmolality of the insulin and insulin / MVCSN solutions. Nasal formulations were prepared for testing in rats containing various concentrations of NaCl (0.1125%, 0.225%, 0.45%, 0.9% and 1.35%) and the osmolality of each formulation was measured using a cryoscopic osmometer prior to the study.

4.2.2 Study outline

4.2.2.1 Materials.

Insulin (Semi-synthetic human sodium insulin, specific activity of pure insulin 28 IU/mg), Novo-Nordisk, Gentofte, Denmark. Using spectrophotometry the purity of the material was determined to be 84.4%. Thus, the specific activity of the insulin was equivalent to 23.63 IU/mg and this value was used for all subsequent calculations.
Medium viscosity chitosan glutamate (MVCSN), Pronova Biopolymer A/S, Drammen, Norway (refer to Table 2.1).

4.2.2.2 Preparation of insulin formulations.

Five nasal insulin-MVCSN solutions (Formulations 1-5) and five nasal insulin solutions (Formulations 6-10) were prepared for testing in rats. An outline composition of each of the formulations is given in Table 4.1. Formulations were prepared freshly on the morning of the study. Each formulation contained 40 IU/ml insulin and where appropriate 0.5%w/v (5 mg/ml) MVCSN. Formulations were prepared in aqueous media containing the appropriate concentration of sodium chloride (NaCl) as follows: 0.1125%, 0.225%, 0.45%, 0.9% and 1.35%. All formulations were adjusted to pH 4.0 during preparation using HCl. The osmolality of the final formulations was measured as an indication of the tonicity of the formulations and values are given in Table 4.1.

Table 4.1. Outline composition of formulations

Formulation No.	Outline composition	Osmolality (Osmol/kg)
1	Insulin + MVCSN in 0.1125% NaCl, pH 4	0.070
2	Insulin + MVCSN in 0.225% NaCl, pH 4	0.106
3	Insulin + MVCSN in 0.45% NaCl, pH 4	0.183
4	Insulin + MVCSN in 0.9% NaCl, pH 4	0.329
5	Insulin + MVCSN in 1.35% NaCl, pH 4	0.467
6	Insulin in 0.1125% NaCl, pH 4	0.051
7	Insulin in 0.225% NaCl, pH 4	0.088
8	Insulin in 0.45% NaCl, pH 4	0.172
9	Insulin in 0.9% NaCl, pH 4	0.335
10	Insulin in 1.35% NaCl, pH 4	0.477

* Concentration of MVCSN expressed as the concentration of chitosan glutamate

4.2.2.3 Absorption study in the rat model

Each rat was dosed with 4 IU/kg insulin in a volume of 0.1 ml/kg. From Formulations

1-5, MVCSN was dosed at 0.5 mg/kg expressed as the dose of chitosan glutamate.

4.2.3 Results and Discussion

The results are shown in Tables 4.2-4.4 and Figures 4.1-4.3). The results show that insulin was absorbed from each of the insulin / MVCSN and insulin solution formulations administered nasally to rats. As expected from previous studies, nasal insulin absorption was improved by coadministration with 0.5% MVCSN. Increasing the osmolality of the insulin solution formulations (without chitosan) (Formulations 6-10), over the range 0.051-0.477 osmol/kg, did not have an overall marked effect on the degree of insulin absorption (Tables 4.3-4.4, Figures 4.2-4.3). Mean blood glucose concentrations decreased to about 72-84% of basal values 30-90 minutes after dosing (Table 4.3, Figure 4.2). Values of the pharmacokinetic parameters determined from glucose data showed that decreases in blood glucose concentrations ranged from approximately 23-33%, values of T_{min} from 42-90 minutes and values of AOC from 746-3275 % gluocse.min (Table 4.4, Figure 4.3). The reason for the much lower value of AOC observed for Formulation 6 (insulin in 0.1125% NaCl) was not known. However, one of the three animals in this group died prematurely and hence an AOC value was not calculated for this animal.

Following the administration of the insulin / MVCSN formulations (Formulations 1-5), increasing formulation osmolality over the range 0.070-0.329 osmol/kg (Formulations 1-4) had very little effect on the degree of absorption enhancement (Tables 4.2, 4.4, Figures 4.1, 4.3). For Formulations 1-4, mean blood glucose concentrations decreased to about 36-45% of basal values 45-60 after dose administration (Table 4.2, Figure 4.1). There was very little difference in pharmacokinetic values for Formulation Groups 1-4 with decreases in blood glucose concentrations ranging from approximately 57-65%, values of T_{min} from 47-60 minutes and values of AOC from 6668-7893 % glucose.min (Table 4.4, Figure 4.3). However, an insulin / MVCSN solution at 0.467 osmol/kg (Formulation 5) further improved nasal insulin absorption with mean blood glucose concentrations decreasing to about 20% of basal values at 60 mninutes. Following the nasal administration of Formulation 5, decreases in blood glucose concentrations and values of AOC were greater than those following administration of the other insulin / MVCSN formulations (approximately 19% and 12520 % gluocse.min, respectively).

A one-way ANOVA (with a Tukey-Kramer multiple comparisons test) showed that there were significant differences in values of AOC between the insulin / MVCSN formulation groups and that the AOCs were significantly higher in animals dosed with Formulation 5 compared to those dosed with Formulations 1, 3 and 4 (Table 4.4). However, there were no significant differences in values of C_{min} (and hence in the % decrease in glucose concentrations) between the insulin / MVCSN formulation groups. With the exception of animals dosed with Formulation 5, inter-animal variations in values of C_{min} in each dose group tended to be high and probably accounted for the lack of significant differences. There were no statistically significant differences between the performance of the control insulin formulations (without chitosan) in terms of their pharmacokinetic parameters

(Table 4.4).

Ohwaki et al (1987) reported that maximal absorption of the hormone secretin, administered nasally in rats, was observed at a sodium chloride solution molarity of 0.462. This corresponds to a sodium chloride concentration of about 2.9% which will be hypertonic to physiological fluids. It was suggested that enhanced absorption was due to the interaction of sodium chloride with the nasal epithelium since microscopic examination of rat nasal mucosa exposed to 0.462 M sodium chloride showed shrinkage of the epithelial cells. However, structural changes in the rat nasal epithelium were not observed in tissue exposed to 0.924 M sorbitol, having almost the same osmolarity as 0.462 M sodium chloride. The absorption of secretin was shown to decrease as the molar concentration of sorbitol increased. These observations were suggested to be due to the stronger action of the ionic species sodium chloride on the nasal epithelial cells than sorbitol.

In this study, the maximum concentration of sodium chloride used was 1.35% which was less than half the concentration shown by Ohwaki et al (1987) to cause shrinkage of epithelial cells. Although a hypertonic medium containing 1.35% sodium choride may have some effect on the nasal epithelium, it was shown in this study that the structural integrity of the membrane was not breached since improved absorption of insulin alone was not observed following nasal administration of insulin in 1.35% sodium chloride. The marked improvement in insulin absorption following the administration of a formulation of insulin and MVCSN in 1.35% sodium chloride appears to be due to the combined effects of MVCSN and 1.35% sodium chloride on the structural integrity of the epithelial membrane.

The viscosity of chitosan solutions is strongly influenced by the ionic concentration of the medium. The apparent viscosity of chitosan solutions has been shown to decrease dramatically with an increase in sodium chloride concentration (refer to Section 10.5). Although the viscosities of the chitosan formulations were not measured, based on the results presented in Section 10.5, it is likely that the formulation containing 0.1125% NaCl was marginally more viscous than the other formulations, yet this does not appear to have significantly influenced the degree of nasal absorption enhancement.

Despite the apparent improved absorption of insulin by increasing the osmolality of the insulin / MVCSN formulation, this should not be considered as a feasible option for improving formulation efficacy. The nasal administration of strongly hyper- or hypotonic formulations are likely to cause irritation in the nasal cavity and may also cause cumulative damage to the nasal mucosa with chronic application. Furthermore, a hypertonic nasal formulation (adjusted with NaCl) was shown to improve the absorption enhancing efficacy of chitosan probably through a combined action on the nasal membrane. However, this potentially introduces an experimetal variable since the effect of NaCl on nasal absorption may not be consistent when other factors such as the concentration or grade of chitosan are altered. Thus, it was decided that in future nasal

absorption studies in the rat, formulations would be prepared in 0.9% NaCl.

Table 4.2. Summary of mean blood glucose concentrations following the intranasal administration of insulin / MVCSN formulations of different tonicity in rats

Time before or after dosing (min)	Mean ± SD (n=3-4) blood glucose concentration (% of basal) 0.1125% NaCl	SD	0.225% NaCl	SD	0.45% NaCl	SD	0.9% NaCl	SD	1.35% NaCl	SD
-10	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
-5	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
5	98.0	4.40	99.3	5.38	96.4	4.36	94.0	6.14	93.4	1.26
10	87.4	4.32	85.8	10.32	86.2	3.04	84.0	6.27	86.7	4.57
20	58.9	2.80	67.3	5.07	60.9	14.27	67.2	8.35	63.8	5.73
30	47.5	6.34	55.7	12.81	44.6	13.49	51.5	18.83	44.6	10.07
45	44.6	7.97	45.1	8.11	36.0	12.76	40.9	20.18	26.0	5.01
60	47.7	9.13	40.8	9.05	40.2	13.16	40.6	19.10	19.9	3.06
90	55.5	4.48	49.4	10.60	54.4	15.30	52.2	20.81	24.5	4.12
120	64.0	4.85	58.1	11.17	64.9	14.59	69.5	14.12	37.0	3.56
180	80.3	5.51	84.1	11.69	82.2	17.10	91.4	12.07	55.9	14.98
240	102.3	8.38	90.2	18.45	111.5	18.62	118.7	12.25	77.5	13.94

Table 4.3. Summary of mean blood glucose concentrations following the intranasal administration of insulin formulations (without MVCSN) of different tonicity in rats

Time before or after dosing (min)	Mean ± SD (n=3-5) blood glucose concentration (% of basal) 0.1125% NaCl	SD	0.225% NaCl	SD	0.45% NaCl	SD	0.9% NaCl	SD	1.35% NaCl	SD
-10	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
-5	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
5	97.0	5.12	87.9	5.42	91.9	7.27	100.5	6.34	94.1	5.45
10	90.6	5.85	90.0	4.08	87.5	10.10	93.3	8.36	90.1	3.04
20	85.4	8.33	92.1	4.31	85.4	13.74	86.9	9.91	83.0	10.03
30	83.6	8.84	92.6	1.89	84.2	15.23	82.3	10.93	76.8	16.26
45	85.0	18.81	90.6	6.13	92.2	14.66	77.8	13.79	75.9	17.43
60	77.8	21.39	83.7	8.04	99.3	23.84	72.7	17.63	79.3	22.33
90	82.3	21.07	74.1	8.07	103.1	31.43	71.9	21.87	78.4	20.09
120	102.8	-	79.0	12.30	101.9	27.10	84.9	19.14	84.1	14.00
180	125.3	-	103.5	20.07	96.8	5.82	110.8	28.49	109.5	25.53
240	121.0	-	120.2	14.76	110.7	0.49	123.2	16.96	130.7	20.13

Table 4.4. Summary of pharmacokinetic parameters determined from glucose data following the intranasal administration of formulations of different tonicity containing insulin with and without MVCSN in rats

Formulation or Group number	No. rats in group	Mean ± SD Cmin (% glucose)	Decrease in % glucose conc.	Tmin (min)	AOC (% glucose. min)
1. Insulin / MVCSN / 0.1125% NaCl	4	43.3 (± 7.46)	56.7 (± 7.46)	47 (± 9.4)	7385.8 (± 748.23)
2. Insulin / MVCSN / 0.225% NaCl	3	40.8 (± 9.08)	59.2 (± 9.08)	60 (± 0.0)	7892.7 (± 1788.00)
3. Insulin / MVCSN / 0.45% NaCl	4	35.3 (± 13.13)	64.7 (± 13.13)	47 (± 9.4)	7622.5 (± 2465.69)
4. Insulin / MVCSN / 0.9% NaCl	4	38.7 (± 19.24)	61.3 (± 19.24)	54 (± 7.2)	6667.7 (± 2789.52)
5. Insulin / MVCSN / 1.35% NaCl	4	19.2 (± 2.25)	80.8 (± 2.25)	66 (± 16.6)	12520.0 (± 1573.97)
6. Insulin / 0.1125% NaCl	3	76.9 (± 20.53)	23.1 (± 20.53)	42 (± 16.1)	746.2 (24.96)
7. Insulin / 0.225% NaCl	4	73.4 (± 7.26)	26.6 (± 7.26)	90 (± 21.2)	2872.8 (± 1377.34)
8. Insulin / 0.45% NaCl	4	79.2 (± 10.45)	20.9 (± 10.45)	43 (± 32.6)	1733.5 (± 823.86)
9. Insulin / 0.9% NaCl	5	67.4 (± 17.91)	32.6 (± 17.91)	64 (± 34.2)	3273.6 (± 1975.64)
10. Insulin / 1.35% NaCl	5	70.6 (± 16.11)	29.4 (± 16.11)	49 (± 25.7)	3275.1 (± 2883.49)

Tests of statistical significance

One-way ANOVA
Formulation Groups 1-5: Sig. dif. ($P<0.01$) in values of AOC. No sig. dif. ($P>0.05$) in values of Cmin and Tmin.
Formulation Groups 6-10: No sig. dif. ($P>0.05$) in values of Cmin, Tmin and AOC.

Tukey-Kramer Multiple Comparison Test following ANOVA
AOC (groups 1-5): Sig dif. between Groups 1 vs 5, 3 vs 5 ($P<0.05$) and 4 vs 5 ($P<0.01$).
No sig. dif. ($P>0.05$) between all other formulation groups.

Figure 4.1. Blood glucose versus time profiles following the intranasal administration of insulin / MVCSN formulations of different tonicity in rats

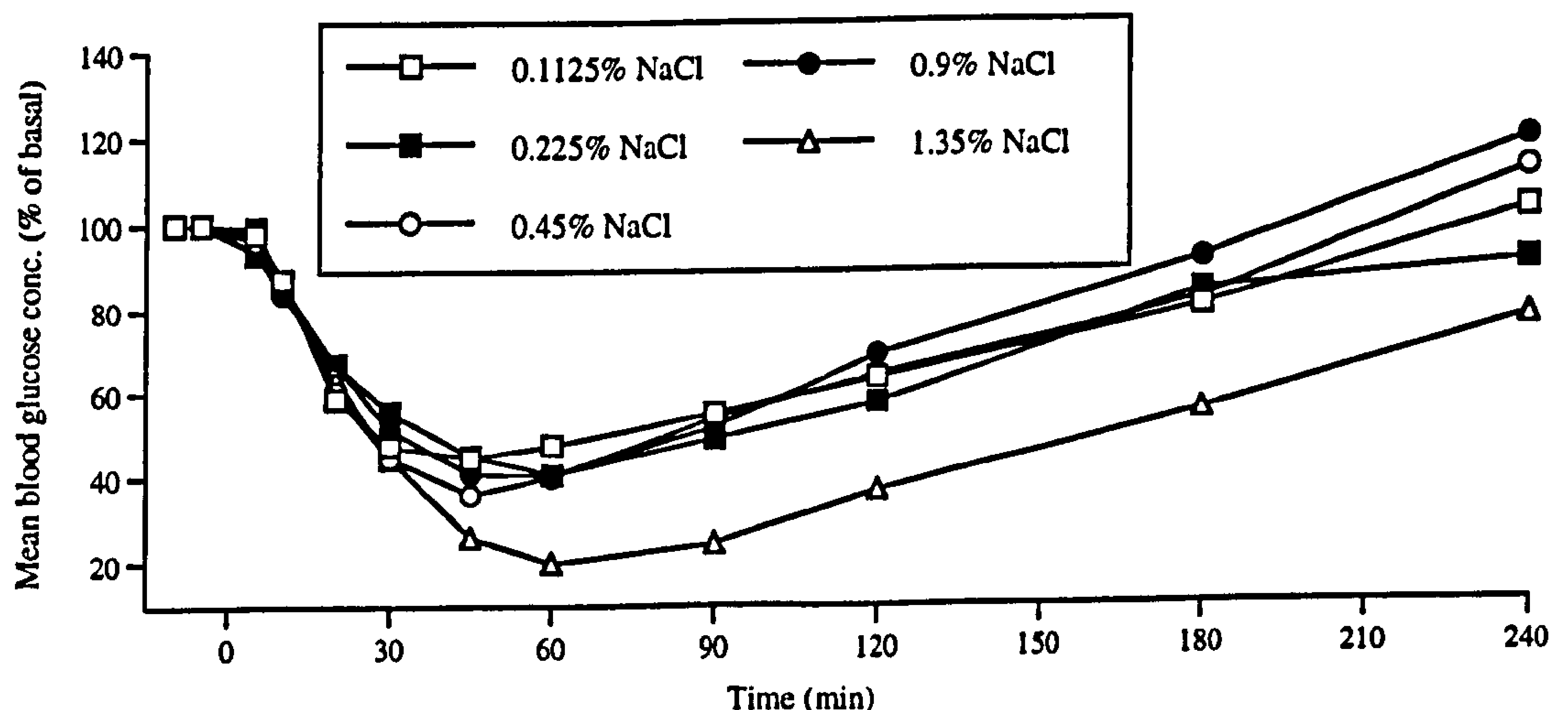


Figure 4.2. Blood glucose versus time profiles following the intranasal administration of insulin / MVCSN or insulin formulations of different tonicity in rats

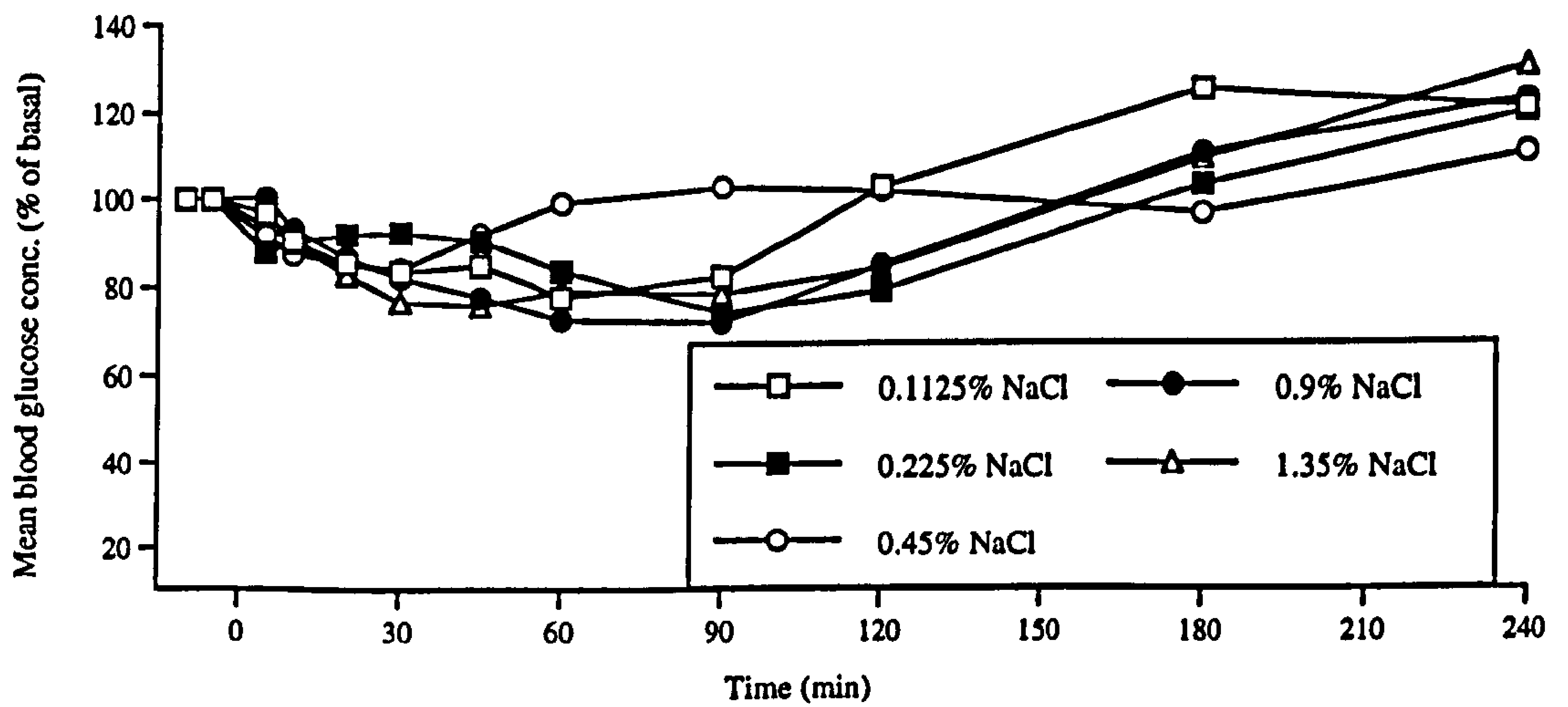
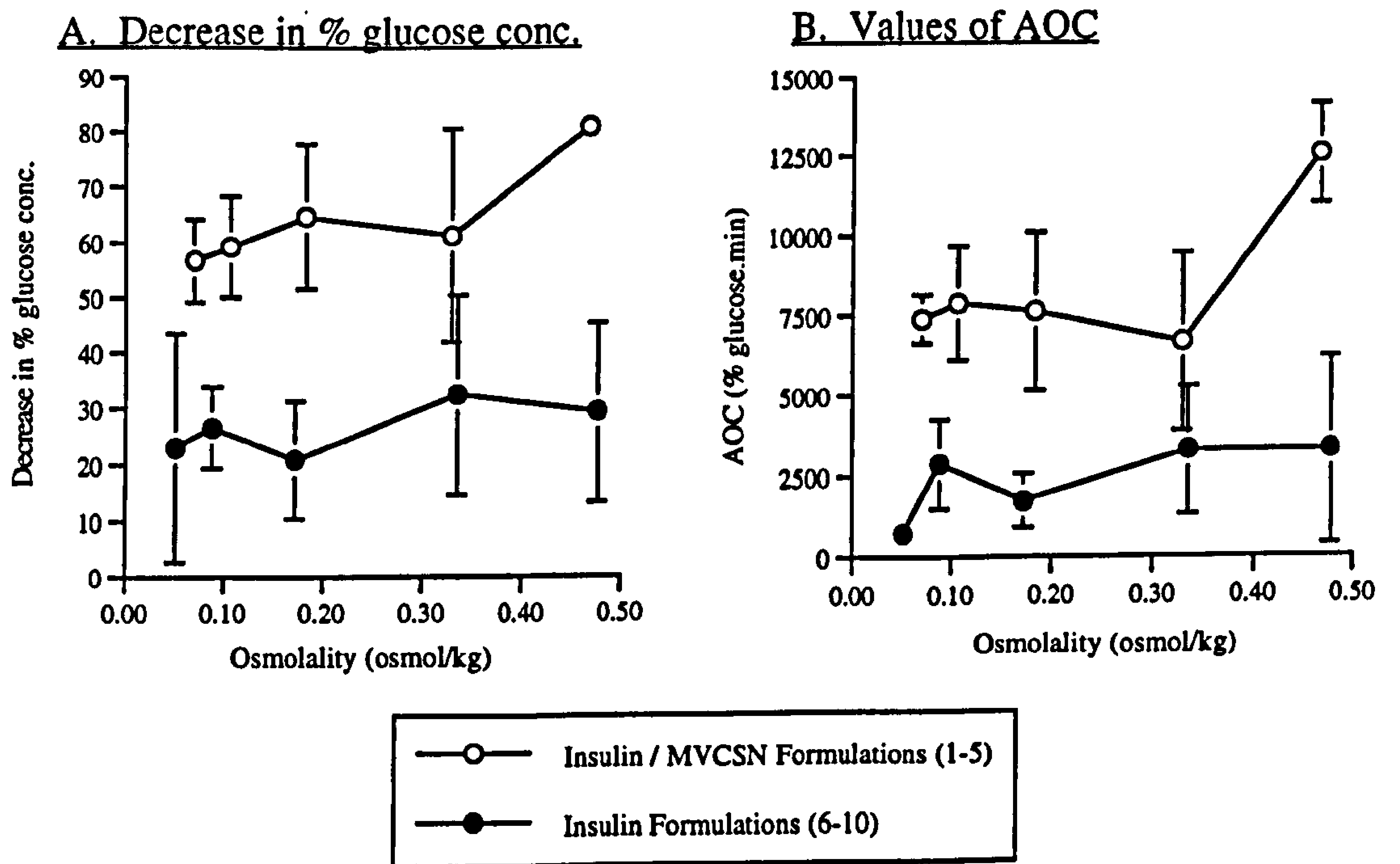


Figure 4.3. Selected pharmacokinetic parameters determined from glucose data following the intranasal administration of formulations of different tonicity containing insulin with and without MVCSN in rats



4.3 Effect of different concentrations of chitosan on the intranasal absorption of insulin in rats

4.3.1 Aims and objectives

Formulations for nasal administration should contain minimal concentrations of enhancer whilst retaining absorption enhancing efficacy so that possible irritant and toxicological effects or effects on the normal nasal physiology are prevented or at least limited. The aim of this study in rats was to investigate the effect of different concentrations of chitosan, ranging from 0 to 1.25%, on the intranasal absorption of insulin with a view to selecting a concentration of chitosan for further evaluation.

4.3.2 Study outline

4.3.2.1 Materials.

Additional materials used were as given in Section 4.2.

4.3.2.2 Preparation of insulin formulations.

Solution formulations (Formulations 1-9) at pH 4.0 were prepared freshly on the morning of the study in 0.9% NaCl for nasal administration to rats. Each formulation contained 40 IU/ml insulin with different concentrations (% w/v) of MVCSN, expressed as the concentration of chitosan glutamate, as follows: 0% (Formulation 1), 0.1% (Formulation 2), 0.2% (Formulation 3), 0.3% (Formulation 4), 0.4% (Formulation 5), 0.5% (Formulation 6), 0.75% (Formulation 7), 1.0% (Formulation 8) and 1.25% (Formulation 9) (0, 1.0, 2.0, 3.0, 4.0, 5.0, 7.5, 10.0 and 12.5 mg/ml, respectively). A summary of the formulations and dose groups is given in Table 4.5.

4.3.2.3 Absorption study in the rat model

Each rat was dosed with 4 IU/kg insulin in a volume of 0.1 ml/kg. The nasal doses of MVCSN are given in Table 4.5

4.3.3 Results and Discussion

The nasal absorption of insulin was apparently increased by increasing the formulation concentration of MVCSN (Table 4.6-4.7, Figures 4.3-4.4). However, the results showed that maximal insulin absorption was attained at a MVCSN concentration of 0.5% (based on values of AOC) or 0.75% (based on values of C_{min} or decrease in % glucose concentrations) (Table 4.7, Figure 4.4). Thus, maximal absorption enhancing efficacy was observed in the range 0.5-0.75% MVCSN and outside these concentrations there was a decrease in the degree of absorption enhancement attained. Mean blood glucose concentrations after administering insulin formulations containing 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1.0 and 1.25 % MVCSN decreased to approximately 85%, 59%, 45%, 44%, 40%, 34%, 53% and 57% of basal values, respectvely (20-120 minutes after dose administration) (Table 4.6, Figure 4.3). Pharmacokinetic parameters (Table 4.7, Figure 4.4) showed that decreases in glucose concentrations after administration of the nasal insulin formulations ranged from about 33% with 0% MVCSN to a maximum of 70%

with 0.75% MVCSN. At concentrations above 0.75% MVCSN, decreases in glucose concentrations were gradually reduced reaching to about 45% at a concentrations of 1.25% MVCSN. Values of T_{min} ranged between 45-98 minutes although fluctuations in these values did not appear to follow a particular trend. Maximum values of AOC (~ 9251 % glucose.min), hence pharmacological availability (~ 17%) were observed following the nasal administration of a formulation containing 0.5% MVCSN. As expected, minimum values of AOC (~ 3274 % glucose.min) were observed after dosing the nasal control formulation (0% MVCSN). The lowest rate of decrease of glucose concentrations was observed after administering the nasal formulation containing 0.1% MVCSN with a maximum rate at 0.5% MVCSN. However, with the exception of the formulation containing 0.1% MVCSN, there was little difference between the formulations tested.

Statistical comparison of the dose groups using a one-way ANOVA showed that there were significant differences in values of C_{min} and AOC but not in values T_{min} or rate of decrease of glucose concentrations. The Tukey-Kramer multiple comparisons test following ANOVA showed that values of C_{min} after dosing formulations containing 0.2%, 0.5% and 0.75% MVCSN were significantly higher than values obtained after dosing a control solution (0% MVCSN). Values of AOC were shown to be significantly higher than those after administering the control solution. However, despite the apparent increased efficacy, in terms of values of C_{min} or AOC, of formulations containing 0.5% or 0.75% MVCSN, values obtained for these dose groups were not significantly different from those of the other formulation groups. Large inter-animal variations in the data probably accounted for the lack of significant differences between these formulation groups.

The apparent increase in nasal absorption enhancing efficacy with increasing chitosan concentration may be attributed to a number of factors. Chitosan is a viscosity enhancing agent and hence, an increase in chitosan concentration will be accompanied by an increase in formulation viscosity (refer to Section 10.5) which may reduce the clearance of the formulation from the nasal cavity. Increasing the residence time of the formulation in the nasal cavity and hence increase in the contact time between the drug and the nasal mucosa may consequently enhance drug absorption (Pennington et al., 1988, Illum, 1992). In the anaesthetised rat model, nasal mucociliary clearance mechanisms may be prevented or impaired (Mayor and Illum, 1994) and hence clearance of formulation from the nasal cavity by this route will probably not be as important as in conscious animals. However, spread and drainage of the formulation from the site of deposition in the nasal cavity, either posteriorly towards pharynx or anteriorly towards the nostrils may also decrease the residence time of the formulation in the main part of the nasal cavity where absorption is most likely to occur. The decrease in the efficacy of the formulation containing 1.0% and 1.25% MVCSN may be due to the viscosity characteristics of the formulation. Formulation viscosity increases with increase in chitosan concentration. The viscosity of the formulation may prevent or reduce the spread of the formulation from the site of deposition in the nasal cavity and hence reduce the surface area over

which contact between the formulation and the nasal cavity is made. Bioadhesive properties of chitosan (Lehr et al., 1992) may also contribute towards the absorption enhancing efficacy of chitosan. Interaction of the chitosan with the nasal mucosa, for example due to the interaction of the positively charged chitosan with the negatively charged surface of the nasal epithelium, will reduced the clearance of the formulation from the nasal cavity either by mucociliary clearance or by spread and subsequent drainage from the nasal cavity.

Increase in chitosan concentration may increase the interaction of the formulation with the nasal membrane. The decreased effectiveness of the formulation containing 1% MVCSN may be due to inhibition of spread of the formulation in the nasal cavity reducing the surface area of the nasal mucosa over which the formulation is deposited. Furthermore, the release of insulin from relatively more viscous solutions may be slowed down which would also tend to reduce insulin absorption (Morimoto et al., 1985). Both the bioadhesive and viscosity enhancing properties of chitosan may increase the residence time of formulations in the nasal cavity although it is difficult to separate the importance of each of these properties. The direct action of chitosan on nasal epithelial tight junctions may be another factor leading to increased insulin absorption with increase in chitosan concentration (Arturrson et al., 1994). Thus, as chitosan concentration increases so too does the effect on tight junction. This could be in the degree of opening of the tight junctions or in the number of junctions opened or a combination of the two.

Table 4.5. Summary of formulations and dose groups in the study to investigate the effect of MVCSN concentration on the intranasal absorption of insulin in the rat.

Formulation or Group No.	Insulin (IU/kg)	MVCSN (mg/kg)	Dose volume (ml/kg)
1. Insulin + 0% MVCSN	4.0	0.0	0.1
2. Insulin + 0.1% MVCSN	4.0	0.1	0.1
3. Insulin + 0.2% MVCSN	4.0	0.2	0.1
4. Insulin + 0.3% MVCSN	4.0	0.3	0.1
5. Insulin + 0.4% MVCSN	4.0	0.4	0.1
6. Insulin + 0.5% MVCSN	4.0	0.5	0.1
7. Insulin + 0.75% MVCSN	4.0	0.75	0.1
8. Insulin + 1.0% MVCSN	4.0	1.0	0.1
8. Insulin + 1.25% MVCSN	4.0	1.25	0.1

* Dose of MVCSN expressed as the dose of chitosan glutamate

Table 4.6. Summary of mean blood glucose concentrations following the intranasal administration of insulin formulations containing different concentrations of MVCSN in rats

Time before or after dosing (min)	Mean ± SD (n=3-5) blood glucose concentration (% of basal)																	
	0% MVCSN		0.1% MVCSN		0.2% MVCSN		0.3% MVCSN		0.4% MVCSN		0.5% MVCSN		0.75% MVCSN		1.0% MVCSN		1.25% MVCSN	
		SD		SD		SD		SD		SD		SD		SD		SD		SD
-10	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100	0.00
-5	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100	0.00
5	95.1	6.34	93.7	10.01	96.8	1.64	99.6	2.43	101.8	3.13	97.6	3.65	98.6	3.80	94.6	3.96	96.7	4.35
10	85.2	8.36	94.7	15.76	87.9	2.51	93.3	4.95	92.7	6.88	95.3	3.47	94.4	4.98	99.1	3.42	90.5	10.27
20	85.2	9.91	74.7	7.68	63.6	2.36	73.4	6.61	73.9	7.93	71.4	4.20	76.8	8.02	77.1	10.33	70.7	8.11
30	85.2	10.93	70.2	6.52	53.9	9.53	59.0	10.80	62.1	12.77	55.7	8.84	59.2	10.50	66.1	15.85	64.6	9.2
45	88.5	13.79	66.9	13.18	44.9	8.64	50.9	12.24	47.3	11.36	43.1	6.46	48.0	11.16	62.5	13.06	63.1	4.21
60	88.5	17.63	61.4	12.54	44.7	13.77	44.4	12.56	43.9	15.50	42.3	9.53	33.8	11.21	53.2	13.17	57.1	5.68
90	101.6	21.87	58.7	13.20	51.0	16.74	53.1	14.85	49.7	21.64	39.5	9.86	34.1	4.07	53.0	11.96	60.2	4.99
120	114.8	19.14	58.5	12.36	56.6	10.78	71.6	29.51	58.8	20.62	45.1	9.65	53.9	8.57	56.8	8.16	70	13.23
180	160.7	28.49	72.9	8.56	91.2	24.21	98.3	23.51	80.9	8.78	74.3	15.32	75.2	10.49	77.8	13.88	93.1	9.95
240	150.8	16.96	87.9	9.74	109.3	18.83	127.0	26.37	97.2	17.94	95.3	26.13	99.3	17.04	100.6	17.92	117.1	11.34

Table 4.7. Summary of pharmacokinetic parameters determined from glucose data following the intranasal administration of insulin formulations containing different concentrations of MVCSN in rats

		Mean ± SD					
Formulation or Group number	No. rats in group	Cmin (% glucose)	Decrease in glucose conc. (% glucose)	Tmin (min)	AOC (% glucose. min)	Rate of decrease of glucose conc. (%/min)	Pharmacological availability * (%)
1. 0% MVCSN	5	67.4 (± 17.91)	32.6 (17.91)	64 (± 34.2)	3274 (± 1975.6)	-	6.1 (± 3.69)
2. 0.1% MVCSN	4	53.9 (± 10.16)	46.2 (± 10.16)	98 (± 28.7)	7203 (± 1660.6)	1.12 (± 0.572)	13.5 (± 3.10)
3. 0.2% MVCSN	4	40.7 (± 9.50)	59.4 (± 9.50)	60 (± 21.2)	7505 (± 2079.8)	1.72 (± 0.542)	14.0 (± 3.89)
4. 0.3% MVCSN	4	43.6 (± 13.04)	56.4 (± 13.04)	68 (± 15.0)	5753 (± 2913.5)	1.40 (± 0.296)	10.8 (±5.44)
5. 0.4% MVCSN	4	42.5 (± 14.60)	57.5 (± 14.60)	64 (± 18.87)	7610 (± 2961.0)	1.37 (± 0.222)	14.2 (± 5.53)
6. 0.5% MVCSN	4	37.0 (± 7.97)	63.0 (± 7.97)	73 (± 15.5)	9251 (± 1945.2)	1.87 (± 0.664)	17.3 (± 3.63)
7. 0.75% MVCSN	4	30.2 (± 4.64)	69.9 (± 4.64)	71 (± 14.4)	8805 (± 1515.1)	1.46 (± 0.544)	16.5 (± 2.83)
8. 1.0% MVCSN	3	47.6 (± 8.55)	52.4 (± 8.55)	75 (± 26.0)	7179 (± 1212.6)	1.42 (± 0.944)	13.4 (± 2.27)
9. 1.25% MVCSN	4	55.3 (± 2.26)	44.7 (± 2.26)	45 (± 17.3)	5383 (± 840.7)	1.50 (± 0.464)	10.1 (± 1.57)

* Relative to subcutaneous

Tests of statistical significance

One-way ANOVA

Sig. dif. ($P<0.01$) in values of Cmin. No sig. dif. in values of Tmin and AOC.

Tukey-Kramer Multiple Comparison Test following ANOVA

Cmin: Sig. dif. between groups 1 vs 3, 1 vs 6 ($P<0.05$) and 1 vs 7 ($P<0.01$). No sig dif ($P>0.05$) between all other groups.

Figure 4.4. Blood glucose versus time profiles following the intranasal administration of insulin formulations containing different concentrations of MVCSN in rats

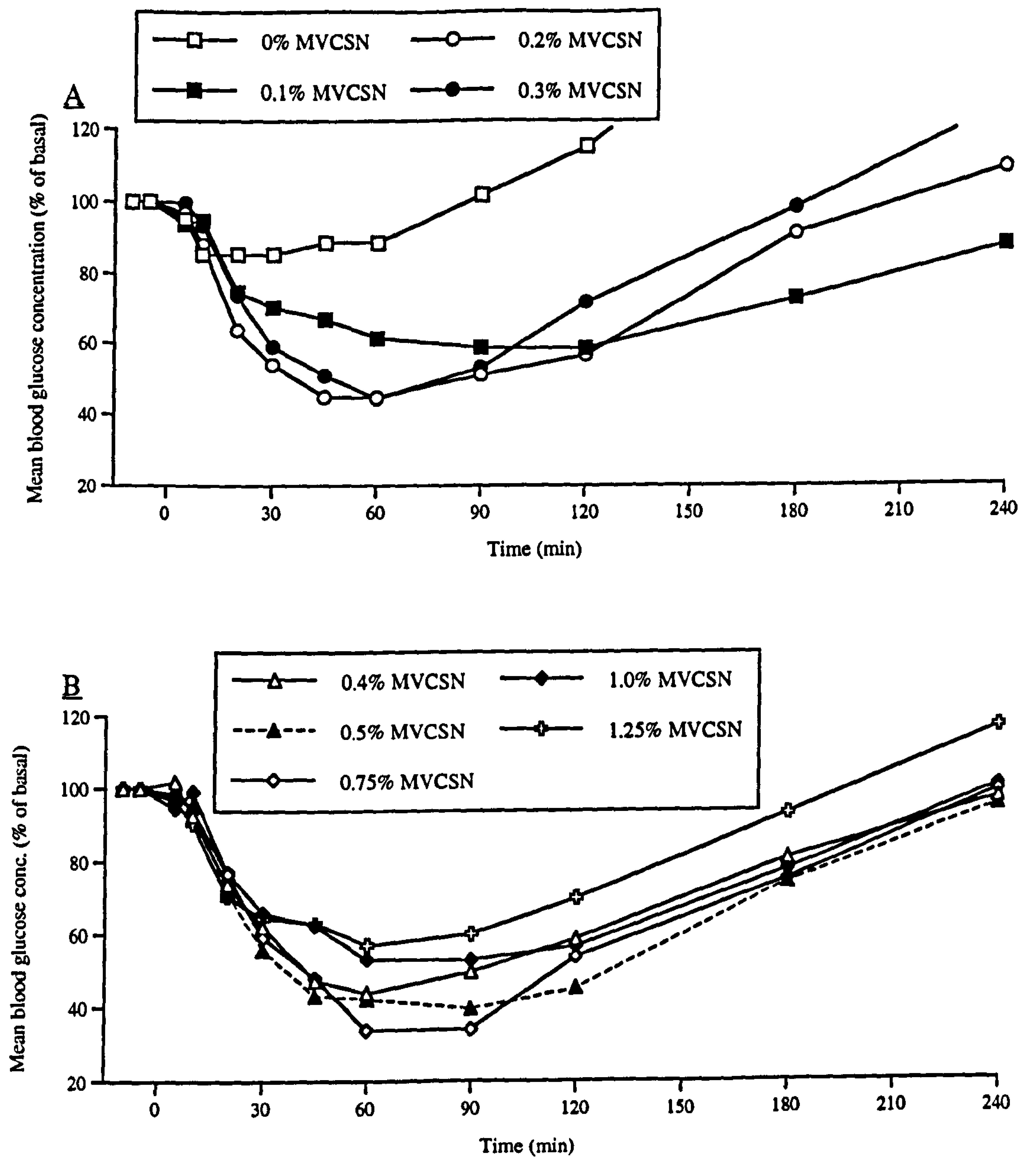
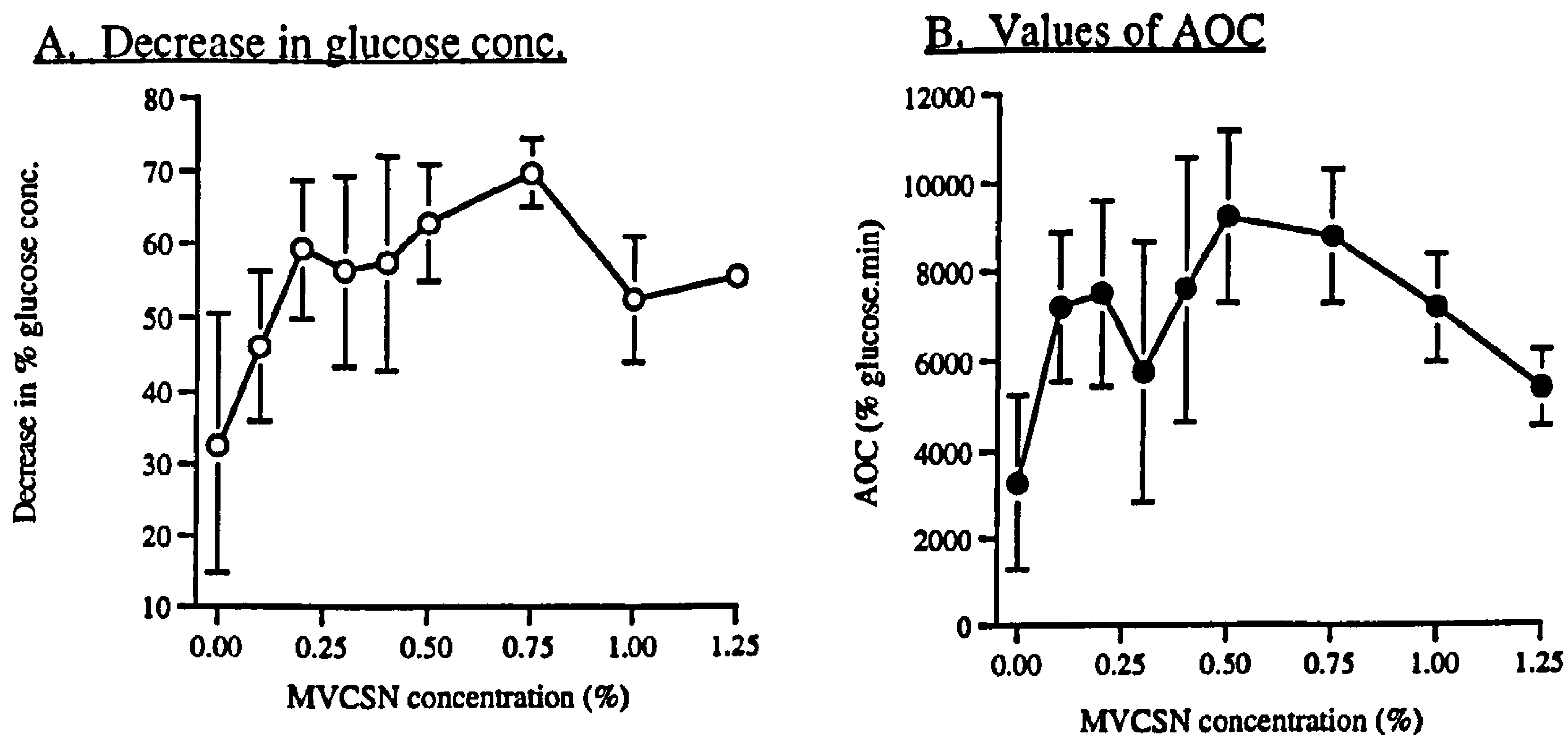


Figure 4.5. Selected pharmacokinetic parameters determined from glucose data following the intranasal administration of insulin formulations containing different concentrations of MVCSN in rats



4.4 Investigation of the transient effect of chitosan on the intranasal absorption of insulin in the rat

4.4.1 Aims and objectives

Many compounds which have been demonstrated in the literature to enhance nasal peptide absorption have also been shown to have severe membrane damaging properties which may be the principal mode of action for these compounds. Clearly, severe damage to the nasal mucosa would be unacceptable in nasal delivery systems particularly for chronic application. The aim of this study was to evaluate whether chitosan had a transient effect on the nasal insulin absorption in rats. This would be a useful indication of any damaging effects to the nasal mucosa caused by chitosan and may also give a further clue to its mechanism of absorption enhancement. Chitosan has been reported to cause no histological damage to the rat nasal mucosa following exposure for 60 minutes (Illum et al., 1994). In the study, chitosan was administered to the nasal cavity of the rat and at certain fixed time intervals after chitosan administration, up to 60 minutes, the animal was dosed with a solution of insulin and absorption assessed.

4.4.2 Study outline

4.4.2.1 Materials.

Additional materials used were as given in Section 4.2.

4.4.2.2 Preparation of insulin and chitosan formulations.

Solutions of 40 IU/ml insulin and 0.5% (5 mg/ml) MVCSN were prepared separately at pH 4 in 0.9% NaCl for nasal administration to rats. A summary of the formulation groups is given in Table 4.7.

4.4.2.3 Absorption study in the rat model

The 0.5% MVCSN solution was instilled into the nasal cavity of rats at 0.1 ml/kg. After a pre-determined time interval, a dose of 4 IU/kg insulin was administered into the same nostril (at 0.1 ml/kg from the 40 IU/kg solution) and insulin absorption assesssed. The time intervals between chitosan and insulin administrations were: 15, 30, 45 and 60 minutes. The results were compared to previous results obtained following the nasal administration of a solution of 40 IU/kg insulin with 0.5% MVCSN (administered at 0.1 ml/kg). The nasal dose of MVCSN was 0.5 mg/kg expressed as the dose of chitosan glutamate.

4.4.3 Results and Discussion

The results indicate that the effect of chitosan on the nasal absorption of insulin was transient. Following the nasal administration of insulin solution at 15, 30, 45 and 60 minutes after MVCSN administration, mean blood glucose concentrations decreased to approximately 61%, 52%, 75% and 82% of basal values, respectively, compared to about 41% when insulin and MVCSN were administered together (0 minutes) in the same formulation (Table 4.8, Figure 4.6). Values of the pharmacokinetic parameters showed that following the administration of insulin from 0 to 60 minutes post administration of MVCSN solution, values of % decrease in glucose concentrations and AOC were reduced from 63% to 22% and from 9251 to 2191 % glucose.min, respectively (Table 4.9, Figure 4.7). The values of AOC and decrease in glucose concentrations at 15 minutes post-administration appeared anomalously low compared to values at 0 and 30 minutes. Significant differences between the dose groups in terms of values of C_{min} and AOC were shown with a one-way ANOVA (Table 4.9). However, the Tukey-Kramer multiple comparisons test showed that there were no significant differences in the values obtained when insulin was administered at 0, 15 and 30 minutes post-administration of MVCSN. Thus, the apparently anomalous values obtained at 15 minutes post administration were not significantly different from values obtained at 0 and 30 minutes.

It appears that the absorption enhancing effect of chitosan on the rat nasal membrane is transient and lasts about 30 minutes. The reduction in absorption when chitosan and insulin were administered at a 15 minute interval was probably due to some other factor rather than due to the recovery of the nasal membrane from the effects of chitosan pre-treatment. Previous studies in the rat have shown that the nasal absorption of insulin tends to occur within 5-10 minutes of insulin-chitosan dose administration. This suggests that the effect of chitosan on the nasal membrane is fairly rapid and occurs prior to or during this 5-10 minute period. Thus, the reduction at 15 minutes cannot be explained by an insufficient time allowed for chitosan to effect the nasal membrane. One possibility for the reduced absorption at 15 minutes is that the relatively viscous chitosan dose had not had sufficient time to spread and drain or be cleared from the site of deposition in the nasal cavity. Thus, insulin solution delivered at the same site as the pre-dosed chitosan solution in the nasal cavity may be partially displaced anteriorly or posteriorly in the nasal cavity by chitosan solution remaining at the site of deposition.

Consequently, the insulin solution will not be deposited over the exact same areas of the nasal cavity which have been affected by chitosan pre-treatment although insulin must be deposited on some regions which have been affected by chitosan since at 15 minutes absorption was greater than that previously observed for a control insulin solution. Similarly, this may contribute to the slight reduction in absorption observed at 30 minutes. A greater spread of chitosan solution in the nasal cavity after 30 minutes pre-treatment, thus providing a larger surface area available for absorption may also contribute to the results obtained. It appears that after a 30 minute period, the effects of chitosan on the nasal membrane are reversed and the absorptive properties of the membrane are reduced.

Hirai et al. (1981c) investigated the recovery of the nasal mucosa from the induced hyperabsorptive state by the surfactants sodium glycocholate and Laureth-9. In the study it was demonstrated that as the time interval between administration of surfactant and insulin solution increased, nasal absorption of insulin decreased. Thus, it was demonstrated that the effect of the surfactants on nasal absorption and hence on the nasal mucosa was transient. After administering sodium glycocholate, the time taken for insulin absorption to decrease to values similar to those obtained without surfactant was between 2-4 hours compared to between 6-24 hours for Laureth-9. In this study, following pre-dosing chitosan solution, nasal insulin absorption had decreased to values similar to those obtained without chitosan between 30-45 minutes.

The results appear encouraging since they indicate that in the rat model, chitosan does not appear to cause serious damage to the nasal membrane otherwise the absorption enhancing capacity of the nasal membrane would probaby be retained for a much longer period of time than the 30 minutes demonstrated in this study. However, it would have been beneficial to include a control formulation such as Laureth-9 or LPC, which have both been reporpted in the literature (refer to Chapter 1) to have severe membrane disrupting properties, to allow direct comparison with chitosan.

Table 4.7. Summary of formulations and dose groups in the study investigating the transient effect of MVCSN on the intranasal absorption of insulin in rats

Formulation or Group No. Time interval between insulin and MVCSN administrations	Insulin (IU/kg)	MVCSN (mg/kg)	Dose volume (ml/kg)
1. 0 (control) **	4.0	0.5	0.1 (coadministered)
2. 15 minutes	4.0	0.5	0.1 each
3. 30 minutes	4.0	0.5	0.1 each
4. 45 minutes	4.0	0.5	0.1 each
5. 60 minutes	4.0	0.5	0.1 each

* Dose of MVCSN expressed as dose of chitosan glutamate

Table 4.8. Summary of mean blood glucose concentrations following the intranasal administration of insulin solution at various time intervals after nasally administering MVCSN solution in rats

Time before or after dosing (min)	Mean ± SD (n=4-6) blood glucose concentration (% of basal)									
	0 minutes post		15 min post		30 min post		45 min post		60 min post	
		SD		SD		SD		SD		SD
-10	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
-5	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
5	94.0	6.14	95.3	9.22	97.5	7.88	97.5	2.76	99.5	7.35
10	84.0	6.27	86.2	8.39	81.6	3.29	93.2	4.06	92.7	5.79
20	67.2	8.35	72.4	13.75	64.3	4.84	82.3	7.02	88.9	5.13
30	51.5	18.83	69.2	11.77	62.2	11.33	74.9	6.36	81.7	8.88
45	40.9	20.18	61.3	13.62	51.3	12.90	84.7	14.30	81.6	8.95
60	40.6	19.10	60.8	19.36	52.4	12.94	92.5	10.53	84.9	12.93
90	52.2	20.81	65.8	16.41	55.9	15.23	99.1	6.25	88.7	16.47
120	69.5	14.12	76.4	18.33	53.9	15.56	112.8	3.93	92.7	17.81
180	91.4	12.07	92.4	14.56	76.9	9.94	140.5	10.14	108.2	16.04
240	118.7	12.25	112.7	16.60	98.3	11.52	173.1	9.47	124.7	15.78

Table 4.9. Summary of pharmacokinetic parameters determined from glucose data following the intranasal administration of insulin solution at various time intervals after nasally administering MVCSN solution in rats

Formulation or Group number	No. rats in group	Mean ± SD			
		Cmin (% glucose)	Decrease in % glucose conc.	Tmin (min)	AOC (% glucose. min)
1. 0 min post	4	37.0 (± 7.97)	63.0 (± 7.97)	73 (± 15.5)	9250.8 (± 1945.20)
2. 15 min post	5	54.9 (± 16.70)	45.1 (± 16.70)	60 (± 18.4)	5249.2 (± 2561.56)
3. 30 min post	5	45.2 (± 12.84)	54.8 (± 12.84)	59 (± 36.3)	7845.7 (± 2015.49)
4. 45 min post	4	73.5 (± 8.08)	26.5 (± 8.08)	33 (± 8.7)	1052.0 (± 620.64)
5. 60 min post	6	78.4 (± 9.62)	21.6 (± 9.62)	50 (± 22.6)	2190.7 (± 1734.05)

Tests of statistical significance

One-way ANOVA
Sig. dif. ($P<0.001$) in values of Cmin and AOC.
No sig. dif. ($P>0.05$) in values of Tmin.

Tukey-Kramer Multiple Comparisons Test following ANOVA
Cmin: Sig. dif. between groups 1 vs 5 ($P<0.001$), 1 vs 4, 3 vs 5 ($P<0.01$) and 2 vs 5, 3 vs 4 ($P<0.01$).
AOC: Sig. dif. between groups 1 vs 4, 1 vs 5, 3 vs 4 ($P<0.001$), 3 vs 5 ($P<0.01$) and 2 vs 4 ($P<0.05$).
No sig. dif. ($P>0.05$) between the other formulation groups.

Figure 4.6. Blood glucose versus time profiles following the intranasal administration of insulin solution at various time intervals after nasally administering MVCSN solution in rats

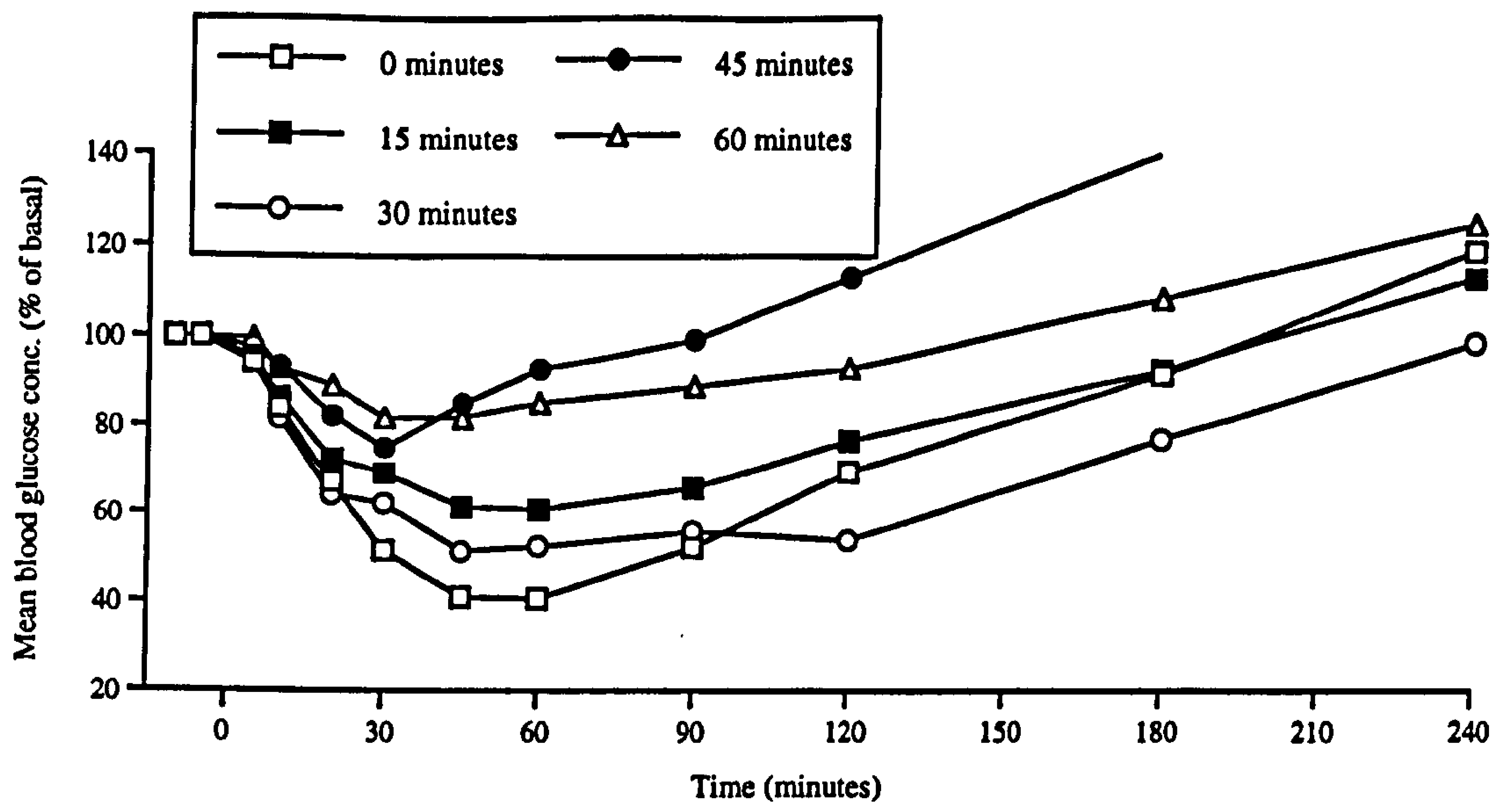
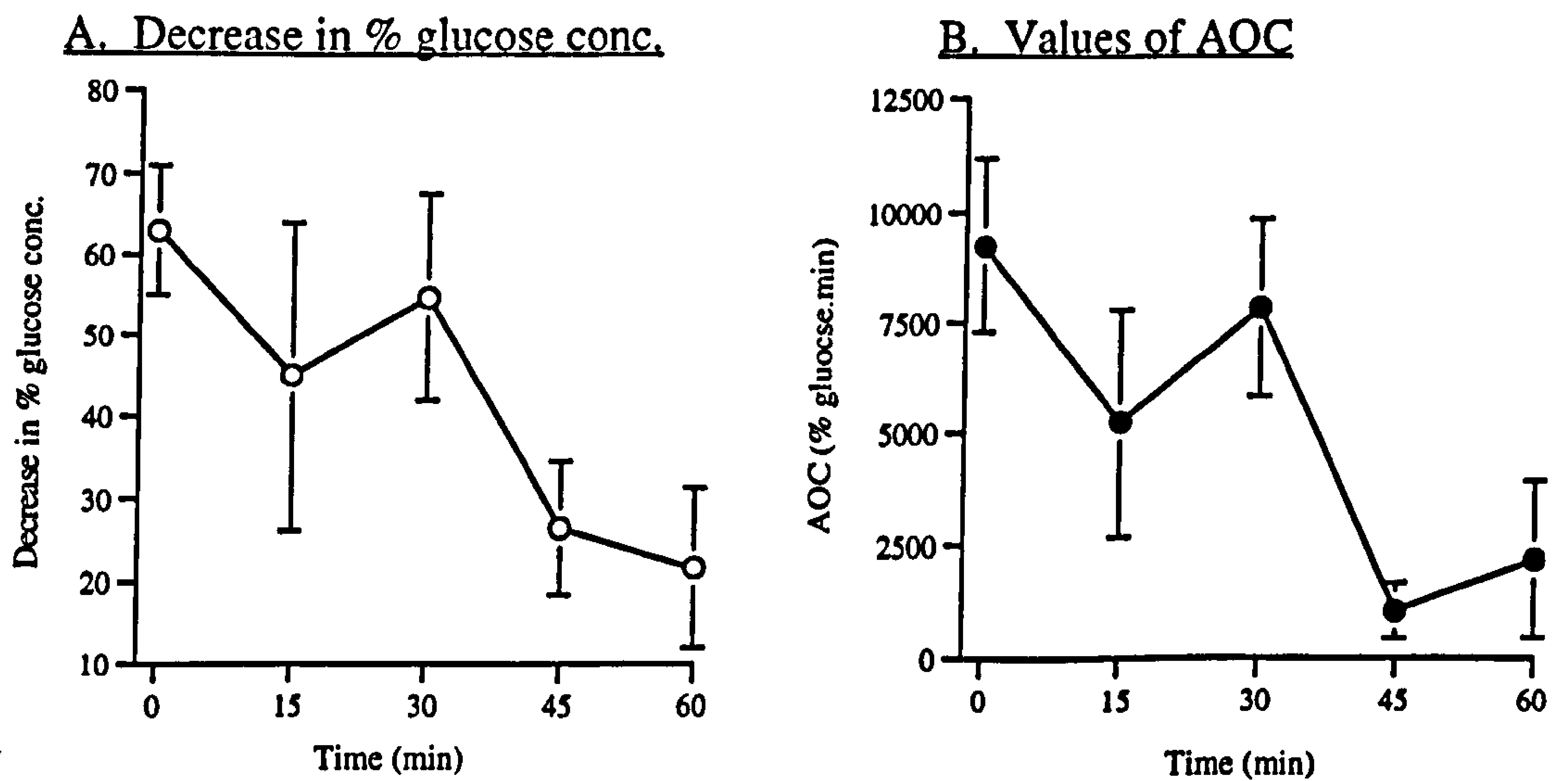


Figure 4.7. Selected pharmacokinetic parameters determined from glucose data following the intranasal administration of insulin solution at various time intervals after nasally administering MVCSN solution in rats



4.5 Comparison of the efficacy of different grades of chitosan in enhancing the nasal absorption of insulin in the rat

4.5.1 Aims and objectives

The grade of chitosan employed may influence its absorption enhancing efficacy and thus should be fully evaluated when optimising systems for nasal drug delivery. Previously, low and medium viscosity grades of chitosan glutamate were shown to enhance the intranasal absorption of insulin in the rat model. In this study the efficacy of three different grades of chitosan in enhancing the nasal absorption of insulin (4 IU/kg) were compared. The grades investigated were low viscosity chitosan lactate (CSN lactate) medium viscosity chitosan glutamate (MVCSN) and high viscosity chitosan (HVCSN) which each had a similar degree of deacetylation (refer to Table 2.1). Since HVCSN was not water soluble but was readily soluble in dilute acid media to allow direct comparison of the grades, all formulations (pH 4) were prepared in dilute acid. As a control, an insulin solution (pH 4) in dilute acid was nasally administered to rats.

4.5.2 Study outline

4.5.2.1 Materials.

Insulin (Semi-synthetic human sodium insulin, specific activity of pure insulin 28 IU/mg) Novo Biolabs, Novo-Nordisk, Denmark was used. Using spectrophotometry the purity of the material was determined to be 78.0%. Thus, the specific activity of the insulin was equivalent to 21.84 IU/mg and this value was used for all subsequent calculations.
Low viscosity chitosan lactate (CSN lactate), medium viscosity chitosan glutamate (MVCSN) and high viscosity chitosan (HVCSN), Pronova Biopolymer A/S, Drammen, Norway (refer to Table 2.1).

4.5.2.2 Preparation of insulin formulations.

Four nasal formulations each containing 40 IU/ml insulin were prepared for testing in rats. The outline composition of the formulations is given in Table 4.10. One formulation was a control solution of insulin (no chitosan) and the remaining three formulations contained 0.5% of the appropriate grade of chitosan, expressed as the concentration of chitosan salt or with HVCSN as the concentration of the material supplied. The insulin control solution (Formulation 1) was prepared in water and adjusted to pH 4.0 with 10 M acetic acid. The chitosan formulations (Formulations 2-4) were prepared in 1% acetic acid and adjusted to pH 4.0 by the addition of 10 M acetic acid or 4 M sodium hydroxide.

4.5.2.3 Absorption study in the rat model

Nasal doses of 4 IU/kg insulin were administered to rats in a volume of 0.1 ml/kg. Where appropriate, chitosan was dosed at 0.5 mg/kg. The dose of CSN lactate and MVCSN was expressed as the dose of chitosan salt. The dose of HVCSN was expressed as the dose of chitosan base.

Table 4.10. Outline composition of formulations

Formulation No.	Outline composition
1	40 IU/ml insulin control solution, pH 4
2	40 IU/ml insulin with 0.5% CSN lactate, pH 4
3	40 IU/ml insulin with 0.5% MVCSN, pH 4
4	40 IU/ml insulin with 0.5% HVCSN, pH 4

* Concentration of CSN lactate and MVCSN expressed as the concentration chitosan salt.
* Concentration of HVCSN expressed as the concentration of chitosan base.

4.5.3 Results and Discussion

Insulin was absorbed from each of the nasal formulations tested in rats (Tables 4.11-4.12, Figures . Insulin, dosed at 4 IU/kg, was absorbed from the control insulin solution at pH 4. However, insulin absorption was improved by coadministration with the different grades of chitosan. CSN lacate and MVCSN, at a concentration of 0.5%, were equally effective in promoting nasal insulin absorption and these appeared to perform marginally better than the HVCSN. Values of C_{min} for CSN lactate and MVCSN were about 34% compared to about 45% for HVCSN and 67% for the control insulin solution. Values of AOC for CSN lactate, MVCSN and HVCSN were approximately 12249, 11046 and 9092 % gluocse.min, respectively, compared to about 3415 % gluocse.min for the insulin control solution.

A one-way ANOVA showed that there were significant differences in values of C_{min} and AOC between the formulation groups. Further comparison of the formulation groups with a Tukey-Kramer multiple comparisons test showed that the values of C_{min} were significantly lower and values of AOC significantly higher in animals dosed with each of the chitosan formulations than values obtained after administration of the control insulin solution. However, there were no significant differences between CSN lacate, MVCSN or HVCSN in terms of values of C_{min} or AOC.

Based on the results in rats, the low, medium and high viscosity grades of chitosan investigated in this study appeared to be effective for the intranasal delivery of insulin. The HVCSN was shown to be marginally less effective than LVCSN or HVCSN at concentrations of 0.5% which was speculated to be due to the increased viscosity of the formulation thereby reducing the spread of the formulation in the nasal cavity and hence reducing the absorptive area and also through inhibiting the release of insulin. The efficacy of the lactate (CSN lactate) and glutamate (MVCSN) salts of chitosan was interesting in view of the much lower content of chitosan in these grades compared to the high viscosity grade (HVCSN). The salt content of CSN lactate and MVCSN is between 65-75% and 55-65%, respectively. Thus, the absorption enhancing efficacy of chitosan may be attributed to the complex interaction of a number of formulation factors such as the molecular weight / viscosity grade of chitosan and salt form used, buffer composition and fromulation concentrations of chitosan and drug. Aspden (1996) investigated a

number of different grades of chitosan, mainly hydrochloride salts, having different degrees of deacetylation and different molecular weights. However, no apparent difference between the grades in enhancing the nasal absorption of insulin in rat and sheep models was reported.

Table 4.11. Summary of mean blood glucose concentrations following the intranasal administration of insulin with different grades of chitosan in rats

	Mean ± SD (n=4)							
Time before or after dosing (min)	Insulin control	SD	Insulin / CSN lactate	SD	Insulin / MVCSN	SD	Insulin / HVCSN	SD
-10	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
-5	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
5	111.3	9.88	96.2	5.92	100.9	4.42	103.2	9.56
10	94.8	7.02	87.4	7.53	87.2	11.49	90.0	12.82
20	98.3	12.53	67.8	16.73	55.4	4.72	71.7	6.21
30	81.4	8.28	52.4	14.02	50.0	2.61	59.2	6.96
45	77.4	16.71	44.5	13.61	39.1	8.67	48.5	4.73
60	71.8	5.30	43.4	12.80	34.5	6.65	48.5	6.61
90	72.0	15.88	34.4	5.44	40.5	3.77	46.0	4.69
120	78.6	14.25	39.8	4.95	38.1	6.59	52.9	13.74
180	103.7	16.60	44.9	12.04	54.2	16.80	64.9	16.25
240	110.0	11.30	62.3	15.70	79.1	29.62	88.1	13.98

Table 4.12. Summary of pharmacokinetic parameters determined from glucose data following the intranasal administration of insulin with different grades of chitosan in rats

	Mean ± SD (n=4)		
Formulation or Group No.	Cmin (% glucose)	Tmin (min)	AOC (% glucose. min)
1. Insulin control	66.8 (±10.85)	68 (± 19.4)	3415.0 (± 1982.92)
2. Insulin / CSN lactate	33.7 (±5.98) *	90 (± 36.7)	12248.5 (± 1888.78) *
3. Insulin / MVCSN	33.7 (± 6.76) *	79 (± 28.4)	11046.2 (± 1400.83) *
4. Insulin / HVCSN	44.7 (± 3.54) **	101 (± 22.5)	9091.5 (± 2152.44) **

Tests of statistical significance

One-way ANOVA
Sig. dif. ($p<0.001$) in values of Cmax and AOC between the formulation groups.
No sig. dif. ($p>0.05$) in values of Tmin.

Tukey-Kramer Multiple Comparisons Test following ANOVA
* Values significantly different ($p<0.001$) to those in Formulation Group 1.
** Values significantly different ($p<0.01$) to those in Formulation Group 1.
No sig. dif. ($p>0.05$) in values of Cmáx or AOC between the other groups.

Figure 4.8. Blood glucose versus time profiles following the the intranasal administration of insulin with different grades of chitosan in rats

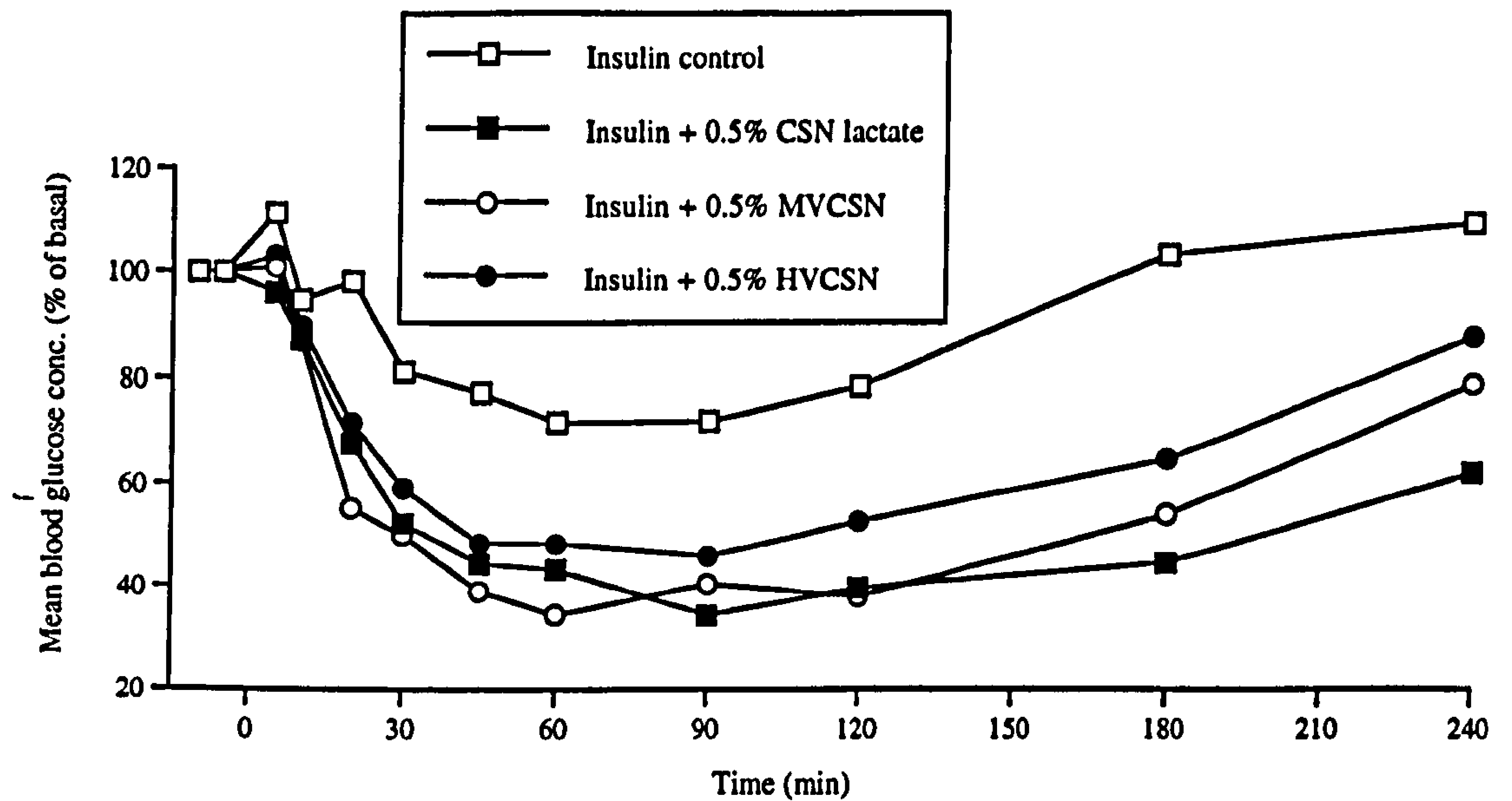
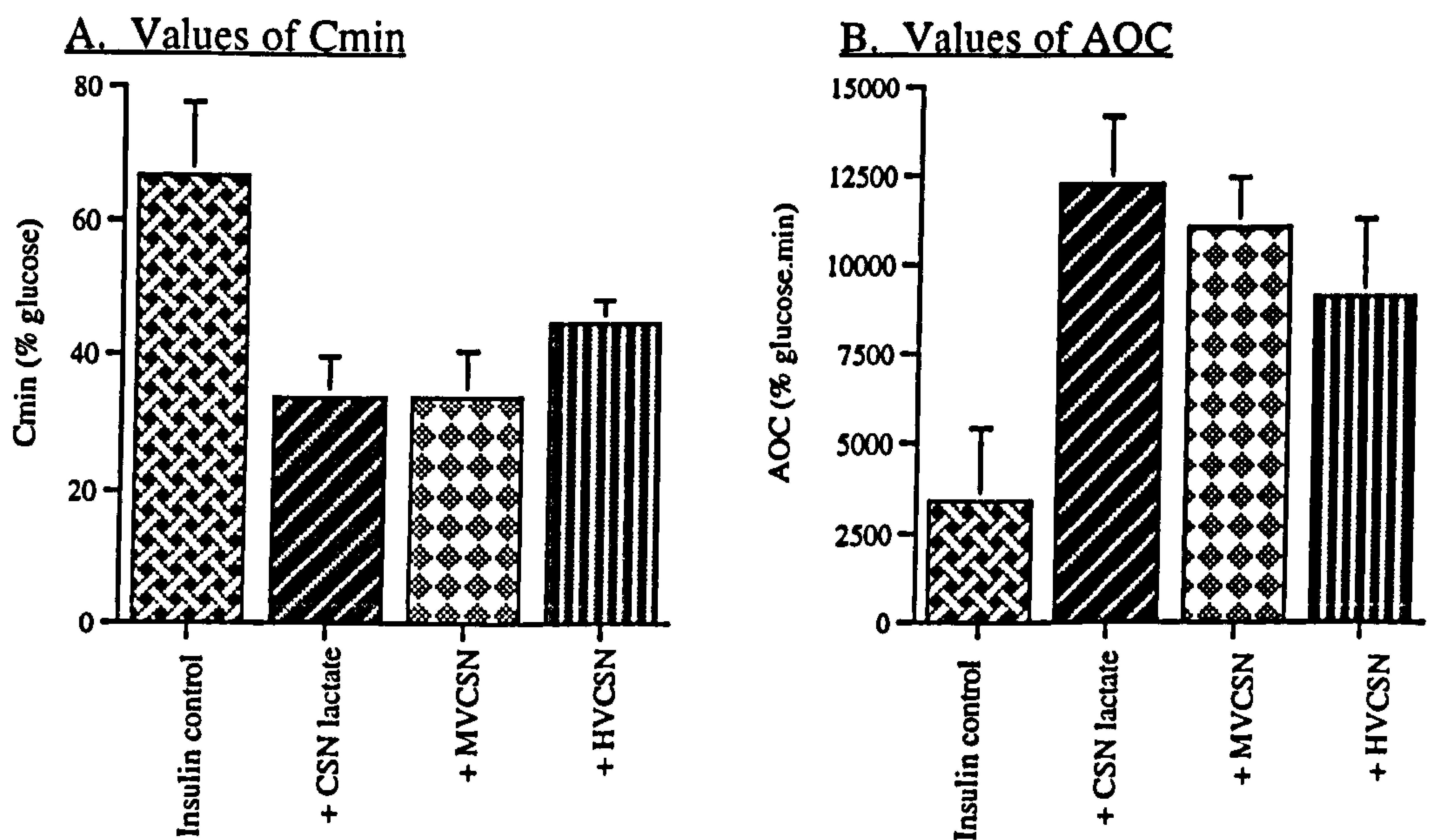


Figure 4.9. Selected pharmacokinetic parameters determined from glucose data following the intranasal administration of insulin with different grades of chitosan in rats



4.6 The reproducibility of insulin absorption from a formulation containing chitosan in the rat

4.6.1 Aims and objectives

In the development of nasal peptide and protein delivery systems for therapeutic application, an important criteria for the selection of candidate systems is the reproducibility and predictability in drug absorption. Intrasubject and intersubject variation in drug absorption are factors which may prevent the exploitation of nasal formulations commercially. The results presented in this section were not obtained from a study specifically designed to investigate intra- and inter-animal variations, hence reproducibility, in insulin absorption. The results were collated from studies in which a formulation of 40 IU/ml insulin with 0.5% MVCSN was dosed to different groups of rats on different study days. To investigate this further, the reproducibility in insulin absorption was compared in groups of rats which were dosed on different occasions with a formulation of insulin and MVCSN.

4.6.2 Outline of study

Materials and methods are as given in Section 4.2. A formulation of 40 IU/ml insulin with 0.5% MVCSN at pH 4.0, was prepared on three separate occasions and dosed to groups of rats on different study days. The doses of insulin and MVCSN administered on each study day were 4.0 IU/kg and 0.5 mg/kg, respectively (Dose volume was 0.1 ml/kg).

4.6.3 Results and Discussion

The results indicate that nasal insulin absorption (i.e. the lowering of blood glucose concentrations) was reproducible in the groups of rats which were dosed on different study days. Similar blood glucose versus time profiles were obtained for each group of rats (Table 4.13, Figure 4.10). On study days 1, 2 and 3, values of C_{min} were approximately 37%, 41% and 40%, respectively (corresponding values of T_{min} were 73, 68 and 75 minutes, respectively) (Table 4.14, Figure 4.11). Values of AOC were about 9251, 7061 and 8717 % glucose.min, respectively. There were no significant differences in values of C_{min}, T_{min} and AOC between the groups (Table 4.14). Calculation of values of pharmacological availability showed that for each of the three studies insulin absorption was about 17% (± 4.0), 13% (± 3.3) and 16% (± 2.5), respectively. These values are similar to the values reported previously for chitosan (Chapter 3). These results are encouraging showing that MVCSN consistently and reproducibly enhanced insulin absorption in the rat and that between-day variations in insulin absorption were low. The results also demonstrate the validity of the rat in intranasal absorption studies as a model for initial screening of candidate enhancers and show that the day-to-day variations in experimental conditions were negligible.

Table 4.11. Summary of mean blood glucose concentrations following the intranasal administration of an insulin / MVCSN formulation to rats on different occasions

Time before or after dosing (min)	Mean ± SD (n=3-4) plasma glucose conc. (% of basal)					
	Insulin / MVCSN Day 1	SD	Insulin / MVCSN Day 2	SD	Insulin / MVCSN Day 3	SD
-10	100.0	0.00	100.0	0.00	100.0	0.00
-5	100.0	0.00	100.0	0.00	100.0	0.00
5	97.6	3.65	99.0	2.87	96.7	3.10
10	95.3	3.47	92.0	2.04	90.8	4.44
20	71.4	4.20	72.6	4.36	68.8	7.09
30	55.7	8.84	62.1	6.27	57.1	9.87
45	43.1	6.46	44.0	11.65	45.3	16.05
60	42.3	9.53	46.1	12.52	47.7	13.98
90	39.5	9.86	44.8	13.64	41.9	11.45
120	45.1	9.65	62.0	11.59	50.0	10.20
180	74.3	15.32	86.2	9.91	73.8	2.48
240	95.3	26.13	108.6	3.88	94.3	9.18

Table 4.12. Summary of pharmacokinetic parameters determined from glucose data following the intranasal administration of an insulin / MVCSN formulation to rats on different occasions

Formulation or Group number	No. rats in group	Mean ± SD			
		Cmin (% glucose)	Tmin (min)	AOC (% glucose. min)	Pharmacological availability *
1. Insulin / MVCSN Day 1	4	37.0 (± 7.97)	73 (± 15.5)	9250.8 (± 1945.20)	17.0 (± 4.04)
2. Insulin / MVCSN Day 2	4	41.3 (± 12.33)	68 (± 26.0)	7061.4 (± 1762.04)	13.2 (± 3.30)
3. Insulin / MVCSN Day 3	3	39.8 (± 13.00)	75 (± 26.0)	8716.7 (± 1325.94)	16.3 (± 2.50)

* Relative to subcutaneous

Tests of statistical significance

One-way ANOVA
No sig. dif. (P>0.05) in values of Cmin, Tmin and AOC.

Figure 4.8. Blood glucose versus time profiles following the intranasal administration of an insulin / MVCSN formulation to rats on different occasions

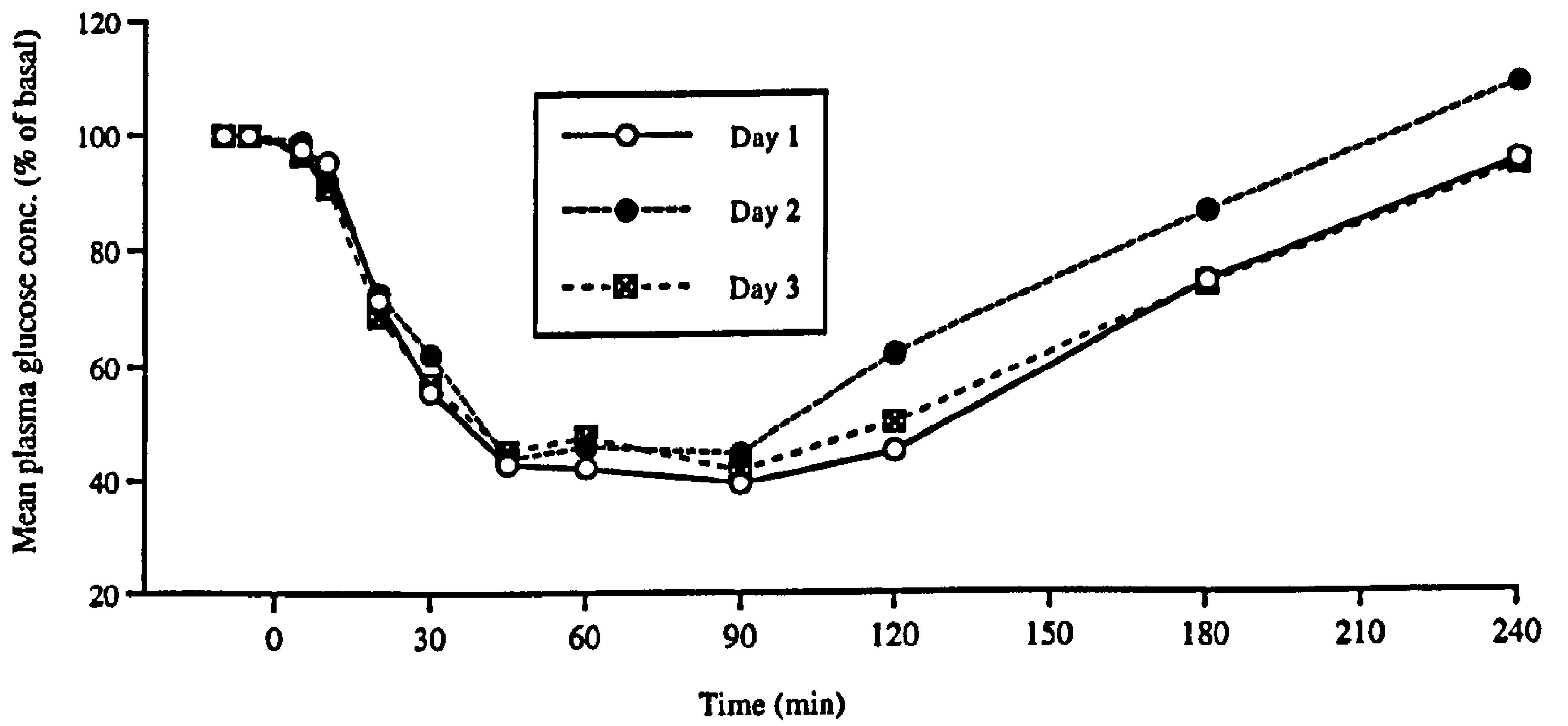
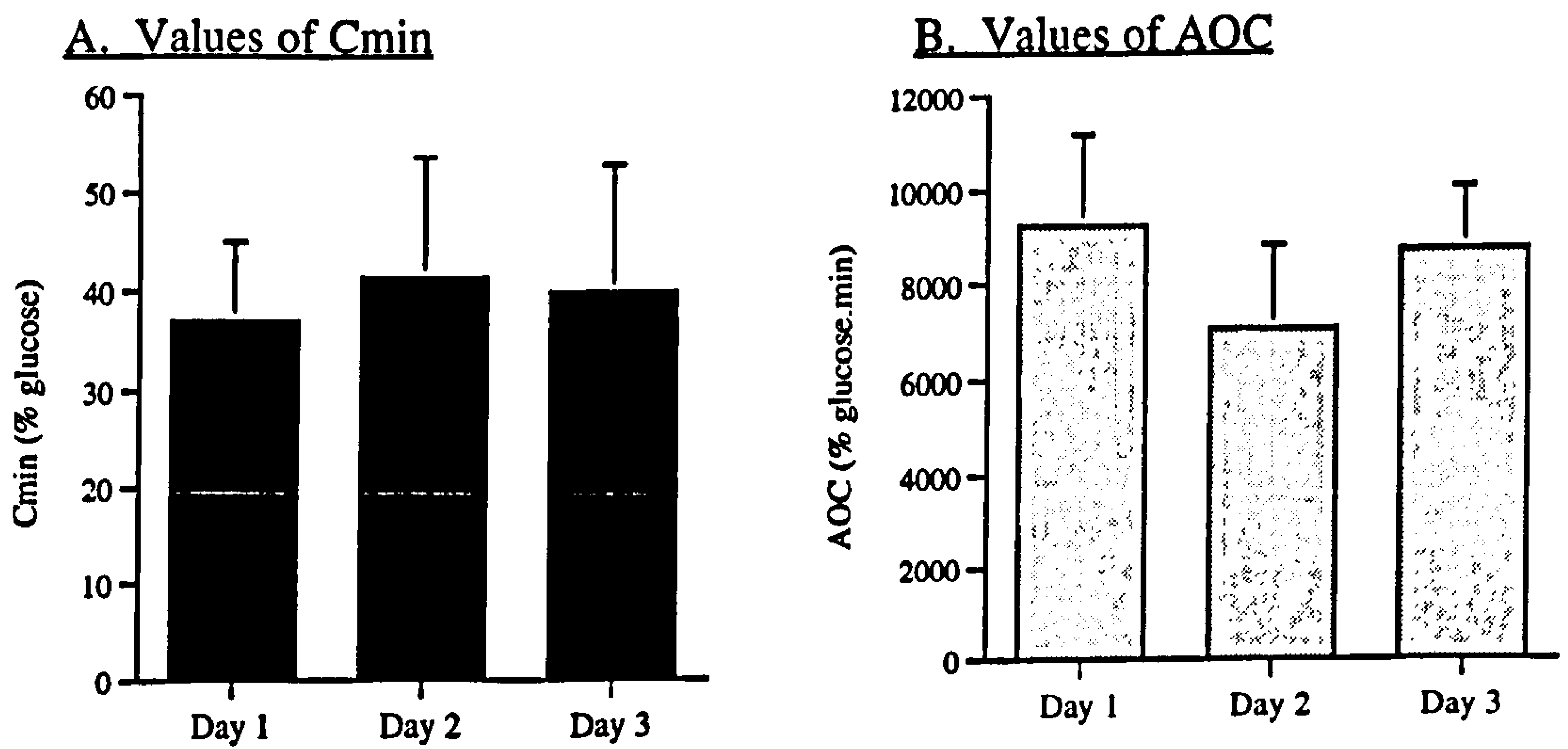


Figure 4.9. Selected pharmacokinetic parameters determined from glucose data following the intranasal administration of an insulin / MVCSN formulation to rats on different occasions



4.7 Conclusions

Reported in this chapter are the results of various studies in rats which have further investigated the efficacy of chitosan (MVCSN) as a nasal absorption enhancer for insulin. It was shown that hypotonic and isotonic formulations (0.070-0.329 osmol/kg) containing 0.5% MVCSN were equally effective in promoting the nasal absorption of insulin. However, absorption enhancing efficacy was improved by administering a hypertonic formulation (0.467 osmol/kg) containing 0.5% MVCSN which was attributed to the combined effects of MVCSN and sodium chloride (used as the tonicity enhancing agent) on the structural integrity of the epithelial membrane. Despite the apparent improvement in absorption by increasing the osmolality of the insulin / MVCSN formulation, it was considered that this would not be a feasible option for improving absorption enhancement due to the likelihood of nasal irritation and potential toxicity with therapeutic use. Furthermore, since hypotonic solutions may also be irritant following nasal administration, it was decided that isotonic formulations of insulin and MVCSN would be used, wherever possible, in future studies.

The nasal absorption of insulin was apparently improved by increasing the formulation concentration of MVCSN. The optimum concentration of MVCSN was between 0.5% and 0.75% (above or below which concentrations there was a decrease in the degree of absorption enhancement). This may be due to the optimal viscosity / bioadhesive properties of the formulation (with optimal interaction of the MVCSN with the nasal membrane). Since there was no apparent difference in the efficacy of formulations containing 0.5% or 0.75% MVCSN, then clearly the use of 0.5% chitosan would reduce potential adverse reactions and increase the acceptability of chitosan in commercial nasal preparations. It was decided that in future investigations in rats, formulations containing 0.5% chitosan would be further investigated. Insulin absorption from isotonic nasal formulations containing 0.5% MVCSN was shown to be reproducible in groups of rats dosed on different study days. Furthermore interanimal variations in each study group and between the groups were low. Insulin absorption, relative to subcutaneous absorption, was estimated to be between 13-17% based on decreases in blood glucose concentrations. The absorption enhancing effect of MVCSN was shown to be transient, lasting about 30 minutes, which was encouraging since it indicated that MVCSN did not appear to cause serious damage to the nasal membrane.

In a study comparing different grades of chitosan (0.5% concentrations) CSN lactate and MVCSN were equally effective in promoting nasal insulin absorption and these appeared to perform marginally better than HVCSN which may be attributable to the increased viscosity of the HVCSN formulation. Further comparison of the efficacy of the different grades of chitosan should be investigated in the sheep model since the rat does not appear to be a sufficiently sensitive model to allow subtle differences between the performance of nasal formulations to be evaluated. However, the rat model remains a useful model for the preliminary screening of candidate systems.

CHAPTER 5

THE EFFECT OF CHITOSAN ON THE INTRANASAL ABSORPTION OF SALMON CALCITONIN IN THE RAT

5.1 Introduction

In Chapters 3 and 4, chitosan was demonstrated to significantly enhance the intranasal absorption of insulin in the rat. Thus, chitosan may act as an absorption enhancer for other peptides applied nasally. Salmon calcitonin (S-CT) was selected as an alternative model peptide since it is readily available commercially and its absorption could be monitored indirectly from the lowering of plasma calcium concentrations.

Salmon calcitonin is a synthetically manufactured peptide hormone composed of 32 aminoacids and has a molecular weight of about 3500 (Azria, 1989). Human calcitonin and S-CT differ in 16 of the 32 aminoacids although the potency, in terms of its hypocalcaemic effect, of S-CT is much higher (4000-6000 IU/mg) than that of human calcitonin (100-200 IU/mg). In humans, endogenous calcitonin is secreted mainly in the thyroid parafollicular (C) cells. Its main physiological role is in the regulation of calcium homoestasis acting principally on the bone by protecting against bone resorption. Therapeutically, calcitonin can be used to treat a variety of diseases characterised by high bone turnover such as Paget's disease of bone and osteoporosis. Osteoporosis can be a major concern in postmenopausal women, since sex hormones such as oestrogen play a vital role in the maintenance of bone mass, and in the elderly (Raisz, 1995). In osteoporosis, los of bone mass and microarchitectural deterioration of the bone structure leads to an increase in the incidence of fractures. Calcitonin therapy is usually by intramuscular or subcutaneous injection although some have been administered nasally or rectally (Reynolds, 1996).

This study investigated the effect of chitosan (MVCSN) on the intranasal absorption of salmon calcitonin (S-CT) in the rat. As a control, a buffer solution (without S-CT) was administered nasally. For reference, S-CT was administered by intramuscular injection. Blood samples were collected pre- and post administration of the formulations and plasma separated. The nasal absorption of S-CT was assessed, indirectly, by measurement of plasma calcium concentrations.

5.2. Study outline

Materials and methods used were as described in Chapter 2 / Appendix 1. Additional details are given below.

5.2.1 Materials

Salmon calcitonin acetate salt (S-CT) (S-CT.4AcOH.13$H_2$0, Bioactivity = 4730 IU/mg), Bachem Feinchemikalien AG, Switzerland.

Dimethyldichlorosilane solution, FSA Laboratory Supplies, Fisons plc, Loughborough, Leicestershire, UK.

MSE Microcentaur centrifuge from Fisons Scientific Equipment, Fisons plc.
All other materials are as given in Section 4.2.

5.2.2 Preparation of S-CT solutions

To avoid adsorption losses of S-CT, all glassware (except Hamilton syringes used for nasal dose administration) that came into contact with S-CT formulations was pre-treated with dimethyldichlorosilane solution, a silicone water-repellent coating agent. Glassware was soaked in dimethyldichlorosilane solution for a minimum of 1 hour and then dried under a fume hood and then in a drying cabinet. All glassware was then washed thoroughly prior to use. Where contact between S-CT solutions and plastics (e.g. pipette tips, dosing cannula) was unavoidable, the plasticware was pre-flushed with some of the S-CT solution. The glass Hamilton syringe was also pre-flushed prior to dose administration.

Three nasal solutions (Formulations 1-3) were prepared for testing in rats as detailed below. Each formulations was prepared in 50 mM acetate buffer solution at pH 4.0. The buffer solution was administered as a negative control (Formulation 1). The nasal S-CT solutions (Formulations 2 and 3) contained 10 IU/ml S-CT (sufficient to administer a dose of 1 IU/kg in a volume of 0.1 ml/kg). A solution for intramuscular injection (Formulation 4) was prepared in PBS, pH 7.4. The intramuscular solution contained 20 IU/ml S-CT (sufficient to administer a dose of 0.5 IU/kg S-CT in a volume of 0.025 ml/kg).

Table 5.1. Outline composition of formulations

Formulation No.	Outline composition
1	Buffer control solution, pH 4
2	10 IU/ml S-CT control solution, pH 4
3	10 IU/ml S-CT with 0.5% (5 mg/ml) MVCSN, pH 4
4	20 IU/ml Intramuscular reference solution, pH 7.4

* Concentration of MVCSN was expressed as the concentration of chitosan glutamate

5.2.3 Absorption study in the rat model

The rats were dosed nasally at 0.1 ml/kg and intramuscularly at 0.025 ml/kg. The nasal dose of S-CT from formulations 2 and 3 was 1 IU/kg. The intramuscular dose of S-CT was 0.5 IU/kg. A summary of the dose groups is given in Table 3.1.

Blood samples of approximately 200 μl were collected from the cannulated carotid artery, pre- and post administration of the formulations and plasma separated by centrifugation at 13000 rpm for 10 minutes in a Microcentaur centrifuge. The plasma was collected (approximately 100 μl) and plasma calcium concentrations determined.

5.3 Results and Discussion

The results as shown in Tables 5.2-5.3 and Figures 5.1-5.3. The results show that MVCSN significantly improved the nasal absorption of S-CT in the rat, as indicated indirectly from the lowering of plasma calcium concentrations. A Tukey-Kramer multiple comparisons test following one-way ANOVA showed that the values of Cmin were significantly lower and values of AOC significantly higher in animals dosed with the S-CT / MVCSN formulation compared to those dosed with the buffer control solution or the S-CT control solution. Despite apparent absorption from the control nasal solution of S-CT, values of Cmin and AOC were not significantly different from those of the buffer control group.

After administering the buffer control solution basal calcium concentrations were observed to fluctuate in rats over the experimental period. Furthermore, there was considerable interanimal variation in the degree of fluctuation of calcium concentrations over the experimental period. With the assumption that the lowering of plasma calcium concentrations correlated with the absorption of S-CT (elevation in plasma S-CT concentrations), calculation of the values of pharmacological availability provided an estimate of the % absorption of S-CT administered nasally compared to the intramuscular route. However, Pontiroli et al. (1985) showed that plasma calcium concentrations were lowered to similar concentrations after intravenous and nasal administration of human calcitonin to healthy volunteers despite plasma calcitonin concentrations being higher after intravenous administration. Furthermore, the nasal administration of a higher dose of S-CT increased absorption although the hypocalcaemic response was not improved. Chierichetti et al. (1985) in a study in human volunteers, used changes in plasma concentrations of cAMP rather than the hypocalcaemic response to evaluate the absorption of calcitonin after intramuscular injection. Increases in plasma cAMP concentrations were shown to correlate well with increases in plasma calcitonin concentrations. In normal subjects, cAMP stimulation is suggested to be a more sensitive test for evaluating calcitonin absorption than its capacity to lower calcium concentrations (Gennari et al., 1990). Thus, the calcium data may be useful for comparing the efficacy of different nasal formulations, particularly when comparing the performance of enhancer formulations with those of a control, although these may not reliably mirror the overall bioavailability of the calcitonin.

The values of pharmacological availability obtained after nasal administration of control S-CT and S-CT / MVCSN (16% and 63%, respectively) showed that absorption was increased almost 4-fold by the incorporation of MVCSN. Morimoto et al. (1985) reported that 10 U/kg [$Asu^{1,7}$]-eel calcitonin was not absorbed nasally from a control solution (determined from the hypocalcaemic response). Coadministration of 0.1% polyacrylic gel was shown to significantly improve nasal absorption of the calcitonin. Richardson et al. (1992) showed that a dose of 40 IU/kg S-CT (no enhancer) was absorbed nasally with plasma calcium concentrations decreasing to approximately 85% of basal values. Although the nasal absorption of the calcitonins have been demonstrated in the literature, few studies have reported the bioavailability of calcitonin, probably

reflecting the difficulty in determining plasma or serum calcitonin concentrations. Reginster et al. (1987) reported that in humans, based on measurements of plasma calcium concentration, the nasal absorption of S-CT (using a commercially available nasal spray formulation of S-CT developed by Sandoz, Basle, Switzerland) relative to an intramuscular injection was 40%. The bioavailability of a nasally administered synthetic analogue of eel calcitonin (Elcatonin), relative to the intramuscular route, was shown to be about 39% in dogs (Manzoni et al., 1989). In a study in patients with Paget's disease of bone, the bioequivalence of nasal S-CT (in a solution with NaCl and albumin to minimise adsorption to plastic syringes) relative to a subcutaneous injection was reported tobe 7.5 % (O'Doherty et al., 1990).

Determination of S-CT concentrations would be advantageous when comparing the efficacy of nasal formulations. However, measurement of calcium concentrations would appear to be useful for screening purposes. This study has shown that the coadministration of MVCSN significantly increased the nasal absorption of S-CT in the rat.

5.4 Conclusions

The nasal absorption of S-CT, administered at 1 IU/kg, was significantly improved in rats by the incorporation of 0.5% MVCSN in a solution formulation. Compared to a control solution of S-CT, nasal absorption relative to the intramuscular route was improved from 16% to 63% by coadministration with MVCSN. These results demonstrate that MVCSN may be useful for the nasal delivery of other peptide and protein drugs

Table 5.1. Summary of formulations and dose groups in the study to investigate the intranasal absorption of S-CT with and without chitosan relativc to intramuscular absorption in rats

Formulation or Group No.	S-CT (IU/kg)	MVCSN (mg/kg)	Dose volume (ml/kg)
1. Buffer control solution	-	-	0.1
2. S-CT control solution	1.0	-	0.1
3. S-CT / MVCSN solution	1.0	0.5	0.1
4. Intramuscular S-CT	0.5	-	0.025

* Dose of MVCSN expressed as dose of chitosan glutamate

Table 5.2. Summary of plasma calcium concentrations following the intranasal administration of S-CT with and without chitosan or the intramuscular administration of S-CT in rats

	Mean ± SD (n=4) plasma calcium concentration (% of basal)							
Time before or after dosing (min)	Buffer control	SD	S-CT control	SD	S-CT / 0.5% MVCSN	SD	S-CT intramuscular	SD
-10	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
-5	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
5	99.4	6.26	101.1	10.24	97.5	1.84	102.1	5.12
10	101.3	6.19	99.6	10.31	89.4	2.00	92.8	5.33
20	106.5	10.77	94.4	10.69	84.8	1.77	91.5	4.78
40	101.7	3.45	88.5	8.01	74.0	13.37	88.6	7.30
60	98.6	1.51	90.3	4.78	80.2	1.81	82.8	5.93
90	100.7	3.24	100.6	14.15	79.3	4.49	83.0	10.27
120	103.4	4.04	99.4	10.07	81.3	5.47	89.7	16.69
180	104.2	4.58	103.3	7.46	93.9	9.88	92.3	9.68
240	107.1	6.38	113.9	10.19	106.5	17.35	101.9	7.70
300	103.1	12.65	117.6	15.19	103.9	6.76	106.3	20.67

Table 5.3. Summary of pharmacokinetic parameters following the intranasal administration of S-CT with and without chitosan or the intramuscular administration of S-CT in rats

	Mean ± SD (n=4)			
Formulation or Group number	Cmin (% calcium)	Tmin (min)	AOC (% calcium. min)	Pharmacological availability (%) *
1. Buffer control	92.8 (±4.72)	8 (± 2.9)	267.6 (± 339.11)	-
2. 1 IU/kg S-CT nasal control	86.4 (±5.64)	40 (± 16.3)	1085.6 (± 895.17)	16 (± 17.9)
3. 1 IU/kg S-CT / 0.5% MVCSN	70.4 (± 10.99) *	58 (± 23.6)	3423.0 (± 1206.69) **	63 (± 24.2)
4. 0.5 IU/kg S-CT intramuscular	78.1 (± 5.66)	78 (± 35.0)	2763.6 (± 1589.67)	100

* Relative to intramuscular

Tests of statistical significance (nasal dose groups)

One-way ANOVA

Sig. diff. ($p<0.01$) in values of Cmin and AOC between the formulation groups (Tukey-Kramer Multiple Comparisons Test Performed). No sig. diff. ($p>0.05$) in values of Tmin

Tukey-Kramer Multiple Comparisons Test

Cmin: * Sig. diff. to group 1 ($P<0.01$) and group 2 ($P<0.05$)

AOC: ** sig diff. to group 1 ($P<0.01$) and group 2 ($P<0.05$).

No sig. diff. ($p>0.05$) in Cmin or AOC between groups 1 & 2

Figure 5.1. Plasma calcium concentration versus time profiles following the intranasal administration of S-CT with and without chitosan or the intramuscular administration of S-CT in rats

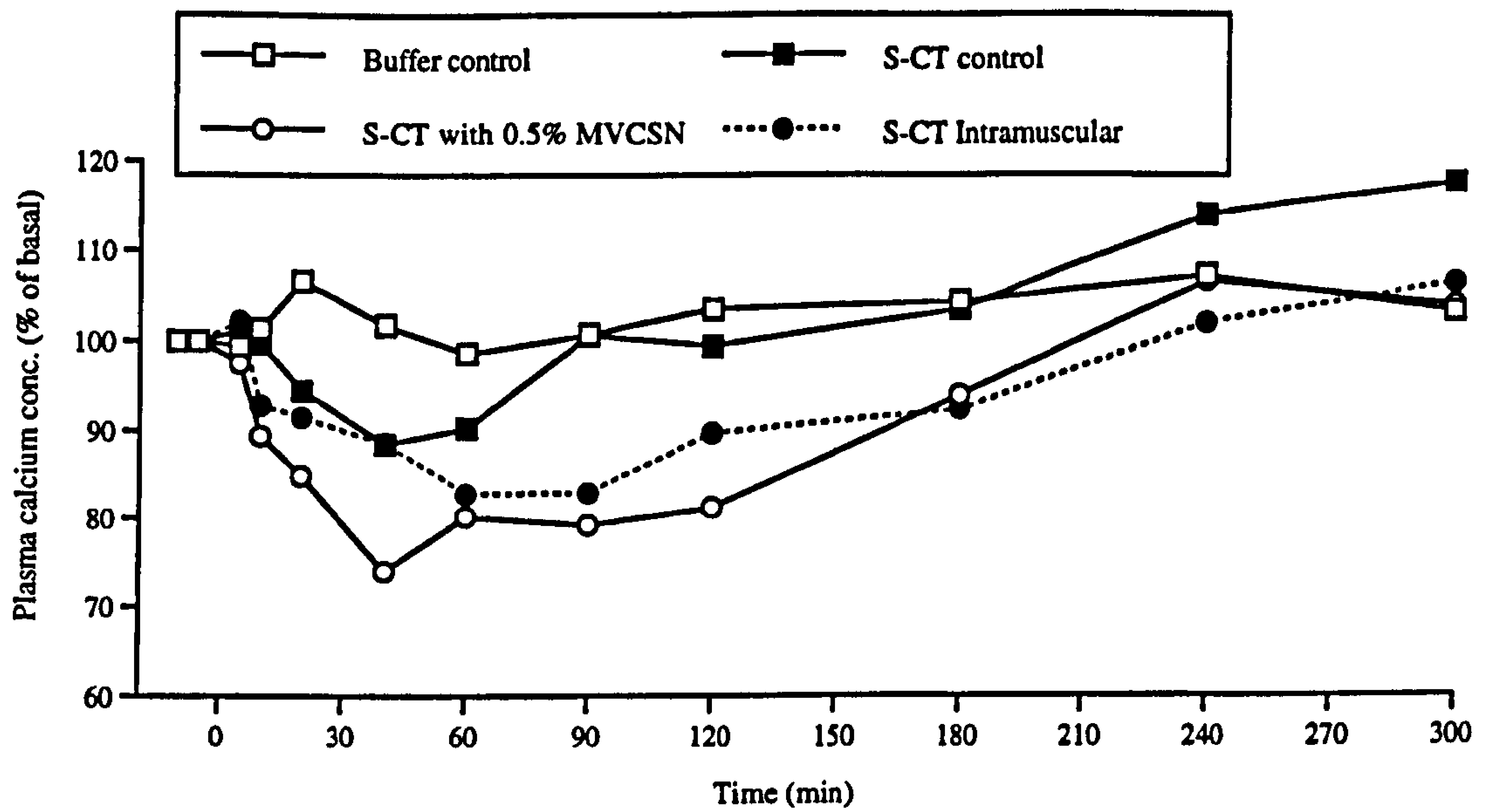
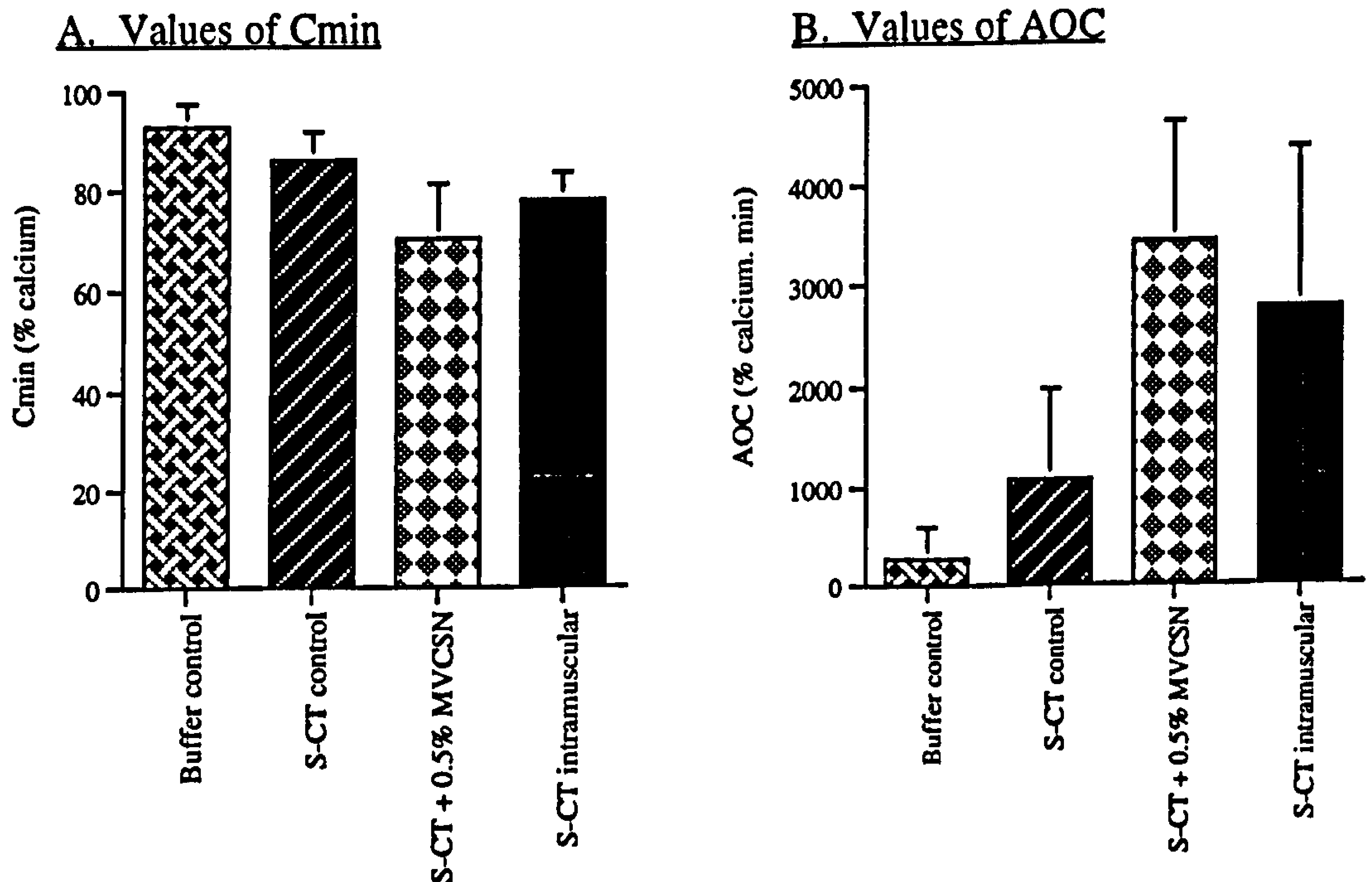


Figure 5.2. Selected pharmacokinetic parameters following the intranasal administration of S-CT with and without chitosan or the intramuscular administration of S-CT in rats



CHAPTER 6

PRELIMINARY INVESTIGATION OF THE EFFICACY OF CHITOSAN AS A NASAL ABSORPTION ENHANCER FOR INSULIN IN THE SHEEP

6.1 Introduction

In this chapter a preliminary study investigating the efficacy of chitosan in enhancing the intranasal absorption of insulin in the sheep model is reported. In the study the performance of low and medium viscosity chitosan glutamate (LVCSN & MVCSN, respectively) were compared to that of lysophosphatidylcholine (LPC) which was shown to be marginally more effective than MVCSN in promoting the nasal absorption of insulin in rats. A nasal solution of insulin was administered without absorption enhancers as a control. Since subcutaneous insulin is used in standard preparations for the treatment of diabetes mellitus, a subcutaneous dose of insulin was administered for reference purposes.

In the absorption studies, formulations were administered intranasally or subcutaneously to sheep. Blood samples were collected pre- and post administration of the insulin formulations, plasma separated and plasma glucose and insulin concentrations measured. Insulin absorption was assessed, indirectly, from the decrease in blood glucose concentrations and directly from the elevation in plasma insulin concentrations following insulin dose administration.

6.2 Materials & Methods

Materials and materials used were as described in Chapter 2 / Appendix 1. Additional details are given below.

6.2.1.1 Materials

Insulin (specific activity 24.22 IU/mg) was used as given in Section 3.2. Additional materials used were as given in Section 3.3.

6.2.1.2 Preparation of insulin formulations

Solution formulations were prepared for nasal (Formulations 1-6) and subcutaneous (Formulation 7) administration to sheep as listed below. Each nasal solution contained 200 IU/ml insulin (sufficient to administer a dose of 2 IU/kg insulin in a volume of 0.01 ml/kg). The solution for subcutaneous administration contained 4.2 IU/ml insulin (sufficient to administer a dose of 0.2 IU/kg insulin in a volume of 0.0476 ml/kg). All formulations were prepared in 14.65 mM phosphate buffer of pH 7.4. Formulations containing chitosan (Formulations 2-5) were adjusted to pH 4.0 during formulation preparation by the addition of HCl. Each formulation was freshly prepared on the day of the study.

Table 6.1. Outline composition of formulations

Formulation No.	Outline composition
1	200 IU/ml insulin control solution, pH 7.4
2	200 IU/ml insulin with 0.1% (1 mg/ml) LVCSN, pH 4
3	200 IU/ml insulin with 0.5% (5 mg/ml) LVCSN, pH 4
4	200 IU/ml insulin with 0.1% (1 mg/ml) MVCSN, pH 4
5	200 IU/ml insulin with 0.5% (5 mg/ml) MVCSN, pH 4
6	200 IU/ml insulin with 0.5% (5 mg/ml) LPC, pH 7.4
7	4.2 IU/ml insulin subcutaneous reference solution, pH 7.4

* Concentration of LVCSN / MVCSN expressed as the concentration of chitosan glutamate

6.2.1.3 Absorption study in the sheep model

Sheep were dosed nasally or subcutaneously with the appropriate insulin formulation. A summary of the dose groups is given in Table 6.2. Nasal doses of 2 IU/kg insulin were administered at a volume of 0.01 ml/kg. Each nasal dose was divided between both nostrils. The subcutaneous dose of 0.2 IU/kg was administered at 0.0476 ml/kg by injection into the flank. Insulin analysis was not performed on plasma samples collected from sheep dosed with 0.1% LVCSN or 0.5% LVCSN.

6.3 Results & Discussion

Results are presented in Tables 6 2-6.5 and Figures 6.1-6.3. Following subcutaneous insulin administration to sheep, sustained reductions of plasma glucose concentrations (Table 6.2, Figure 6.1) and elevations of plasma insulin concentrations (Table 6.3, Figure 6.2) were observed and these values had not returned to basal values by the end of the study period. The insulin pharmacokinetic data of the subcutaneous dose group (Table 6.5, Figure 6.3) showed that the values of C_{max} and AUC (mean (± SD) of approximately 79 mU/l (± 26) and 9205 mU/l.min (± 2288), respectively) were in agreement with values reported in the literature after administering a similar subcutaneous dose of insulin to sheep. Farraj et al. (1990) reported mean values (± SEM) of C_{max} and AUC of approximately 95 mU/l (± 14) and 8615 mU/l.min (± 1297), respectively and Illum et al. (1994) reported values of approximately 91 (± 18) and 10211 (± 837), respectively. Furthermore, values of C_{min} and AOC obtained from the glucose data in this study (mean (± SD) of approximately 44% (± 14.4) and 10176 % glucose.min (± 2288), respectively) were in agreement with values reported by Illum et al. (1994) (mean values (± SEM) of approximately 37 ± 8.1 and 10040 ± 1062, respectively).

The results show that insulin dosed at 2 IU/kg was not significantly absorbed following the nasal administration of a control solution (Tables 6.2-6.4, Figures 6.1-6.3). Values of bioavailability (Table .6.5, Figure 6.3) and pharmacological availability (Table 6.4, Figure 6.3), relative to subcutaneous, of the nasal control insulin dose were 0.20 % and

1.4%, respectively. The values of relative bioavailability and pharmacological availability are in good agreement indicating that a reasonable estimate of insulin absorption can be obtained from the glucose data. Slight discrepancies between the two values may be expected since basal fluctuations in glucose concentrations in sheep were not taken into account. Also, the elevation of insulin concentrations may not exactly mirror the fall in glucose concentrations which was apparent in one sheep in the group after subcutaneous administration where the insulin AUC was much lower than that of the other two animals in the group although the glucose AOC for the same animal was the second highest in the group (results not shown). The nasal bioavailablity of the control insulin solution was in agreement with values reported by Farraj et al. (1990) and Illum et al. (1994) of 1.8% and 1.2%, respectively.

Intranasal insulin absorption was improved by coadministration of LVCSN, MVCSN or LPC compared to that obtained by administering a control insulin solution (Tables 6.3-6.6, Figures 6.1-6.3). However, it is evident from the plasma glucose concentration versus time curves (Table 6.3, Figure 6.1) that the extent and duration of hypoglycaemia was much more pronounced following the nasal administration of the formulation of insulin with 0.5% MVCSN compared to the other nasal formulations. The greater extent of the hyoglycaemia after administering the formulation containing 0.5% MVCSN was reflected in the lower mean value of C_{min} (~ 43% of basal glucose concentration) obtained for this formulation compared to those obtained after dosing the other formulations (Table 6.5, Figure 6.3). For the other nasal enhancer formulations, values of C_{min} were in the region of 71-84% compared to about 88% for the control insulin solution. The rate of decrease of % glucose concentrations (Table 6.4) after administering the insulin formulations (Table 6.4) was highest after administering the LPC formulation (~ 1 %/min) closely followed by the 0.5% MVCSN formulation (~ 0.9 %/min), whereas for the other enhancer formulations rates were lower (0.37-0.49 %/min). Comparing the chitosan (MVCSN & LVCSN) formulations, the rate of decrease of % glucose concentrations increased as chitosan concentration increased and at concentrations of 0.1% and 0.5%, respectively, values were lower for the LVCSN grade. The plasma glucose profile and values of C_{min} for the 0.5% MVCSN formulation were similar to those following the subcutaneous dose. For the insulin / enhancer formulations, values of relative pharmacological availability of 2.4% (0.1% LVCSN), 3.2% (0.5% LVCSN), 4.0% (0.1% MVCSN), 8.7% (0.5% MVCSN), and 2.6% (0.5% LPC) were obtained compared to a value of 1.4% for the control solution. Thus, from the glucose data the compounds ranked in terms of absorption enhancing efficacy in the order: 0.5% MVCSN >> 0.1% MVCSN > 0.5% LVCSN > 0.5% LPC ~ = 0.1% LVCSN > control solution.

The plasma insulin versus time curves (Table 6.4, Figure 6.2) show that prolonged elevation in insulin concentrations, indicating sustained insulin absorption, occurred after nasally dosing insulin / 0.5% MVCSN or following the subcutaneous dose. The mean value of C_{max} (Table 6.6, Figure 6.3) for the 0.5% MVCSN formulation (~ 191 mU/l) was higher than that obtained for the other formulations (~ 142, 69, 10 and 79 mU/l for

LPC, 0.1% MVCSN, insulin control and subcutaneous formulations, respectively). The highest T_{max} (40 minutes) was observed after administering the insulin / 0.5% MVCSN formulation indicating sustained absorption of insulin from this formulation compared to that from the other formulations. The rate of increase of insulin concentrations was highest for the LPC formulation (0.17 mU/l/min) with values of 0.09 and 0.05 mU/l.min for the formulation containing 0.5% and 0.1% MVCSN, respectively. Values of relative bioavailability of 1.8% (0.1% MVCSN), 8.4% (0.5% MVCSN), and 2.1% (0.5% LPC) were obtained compared to 0.20% for the control solution (no data for 0.2% or 0.5% LVCSN). Thus, from the insulin data the compounds ranked in terms of absorption enhancing efficacy in the order: 0.5% MVCSN >> 0.5% LPC > 0.1% MVCSN.

Statistical analysis of the data with a one-way ANOVA showed that there were significant differences between the dose groups in the values of C_{min}, AOC (hence relative pharmacological availability), C_{max} and AUC (hence relative bioavailability). The Tukey-Kramer multiple comparisons test following ANOVA showed that only values of C_{min} and AOC obtained for the 0.5% MVCSN dose group were significantly different to those of the insulin control group. Values of C_{min} of 0.5% MVCSN group were also significantly lower than those in the 0.1% LVCSN dose groups and values of AOC were significantly higher than those in all the other groups. The Tukey-Kramer multiple comparisons test showed that significantly higher values of C_{max} were obtained for the 0.5% MVCSN and 0.5% LPC dose groups compared to the insulin control group although there was no difference between the 0.1% MVCSN and the control group. Values of C_{max} were also significantly higher after administering the higher dose of MVCSN. Values of AUC of the 0.5% MVCSN dose group were significantly higher than those of the other dose groups although there were no differences between the other groups.

The results show that the formulation containing 0.5% MVCSN, performed better than the other formulations in terms of enhancing nasal insulin absorption in the sheep model. Values of relative pharmacological availability obtained in the sheep (8.7% ± 2.39) after administering a solution of insulin with 0.5% MVCSN were of similar order of magnitude to those achieved in the rat (13.8% ± 1.37) after dosing insulin / 0.5% MVCSN. In contrast, after dosing a formulation of insulin and 0.5% LPC, values of relative pharmacological availability of 2.6% (± 2.16) and 16.2% (± 1.59) were obtained in the sheep and rat models respectively, indicating that the absorption enhancing efficacy of LPC was much lower in the sheep than in the rat. However, after nasal administration of a control insulin solution, values of relative pharmacological availability were of the same order of magnitude in the two species (1.4% ± 1.02 and 2.1% ± 1.89 in sheep and rats, respectively).

Impaired mucociliary clearance in the rat model due to the effects of the anaesthetic, thereby increasing the contact time between the drug formulation and the nasal mucosa, may account for the increased absorption of insulin in this species. Mayor and Illum (1994) showed that nasal insulin absorption was reduced in rats which were

anaesthetised compared to those which were sedated for dose administration or remained conscious. In the conscious sheep model, clearance of drug formulation from the nasal cavity due to mucociliary clearance mechanisms may reduce the contact time between the drug formulation and the nasal mucosa and this could explain the reduction in absorption for this species. Normal movements of the sheep after dose administration may also contribute to the drainage of the drug formulation from the nasal cavity and sneezing or snorting during the post-dose period may result in the physical loss of dose. In contrast to the LPC formulation, the nasal absorption enhancing efficacy of the formulation containing 0.5% MVCSN was not significantly reduced in the sheep. The reasons for this have not been elucidated. However, the bioadhesive and/or viscosity enhancing properites of chitosan may increase the residence time of the drug formulation in the nasal cavity, thus increasing the contact time between the drug and the nasal mucosa.

The degree of enhancement of nasal insulin absorption in sheep by coadministering MVCSN was comparable with values reported by Aspden (1996). From plasma glucose data, Aspden reported a value of C_{min} of about 55% and a T_{min} of 54 minutes (concentration of 0.25% chitosan base) compared to values of about 43% and 85 minutes, respectively in this study. Values of AOC could not be compared because of differences in the duration of the respective studies.

The reasons for the apparently greater absorption enhancing efficacy of MVCSN compared to LVCSN has not been elucidated. However, differences in the performance of the two grades are probably due to differences in the viscosities, hence molecular weights, of the respective grades. The values of intrinsic viscosity of MVCSN and LVCSN are 1010 and 388 ml/g respectively (refer to Section 10.4). Thus, it is probable that the relatively less viscous formulations containing LVCSN is cleared more rapidly from the sheep nasal cavity than formulations containing MVCSN (at the same concentration). The viscosity grade may also influence the bioadhesive properties of chitosan. Furthermore, there may be an optimal molecular weight range of chitosan at which optimal absorption enhancing efficacy is exhibited. In view of the lack of differences in the performance of different grades of chitosan in the rat model, this should be evaluated in the sheep model.

6.4 Conclusions

The study showed that in the sheep model, insulin dosed at 2 IU/kg was not significantly absorbed without coadministration with absorption enhancers. Each of the compounds coadministered with insulin in solution formulations improved nasal insulin absorption although the formulation of insulin with 0.5% MVCSN performed significantly better than the other formulations tested. The relative bioavailability of insulin from the 0.5% MVCSN formulation was about 8% which was approximately a 40-fold improvement on absorption compared to the control solution and a 4-fold to that of the other enhancers.

Table 6.2. Summary of formulations and dose groups in the study to investigate the intranasal or subcutaneous absorption of insulin from various formulations in sheep

Formulation or Group No.	Insulin (IU/kg)	Enhancer (mg/kg)	Dose volume (ml/kg)
1. Insulin control solution	2.0	-	0.01
2. Insulin + 0.1% LVCSN	2.0	0.01	0.01
3. Insulin + 0.5% LVCSN	2.0	0.05	0.01
4. Insulin + 0.1% MVCSN	2.0	0.01	0.01
5. Insulin + 0.5% MVCSN	2.0	0.05	0.01
6. Insulin + 0.5% LPC	2.0	0.05	0.01
7. Subcutaneous reference solution	0.2	-	0.0476

* Dose of LVCSN / MVCSN expressed as dose of chitosan glutamate

Table 6.3. Summary of plasma glucose concentrations following the intranasal administration of 2 IU/kg insulin in combination with LVCSN, MVCSN or LPC or the subcutaneous administration of 0.2 IU.kg insulin in sheep

	Mean plasma glucose concentration (% of basal) (± SD)													
Time before or after dosing (min)	insulin control	SD	0.1% LVCSN	SD	0.5% LVCSN	SD	0.1% MVCSN	SD	0.5% MVCSN	SD	0.5% LPC	SD	Subcut insulin	SD
-15	100.0	0.0	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
-5	100.0	0.0	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100 0	0.00
5	100.0	3.4	103.3	3.43	95.8	2.37	96.0	1.73	95.2	2.92	100.0	1.54	97.6	9.74
10	103.3	3.3	103.2	5.54	97.7	0.77	96.1	4.26	96.0	3.63	97.4	4.44	100.8	6.94
15	104.6	4.0	102.2	6.52	98.6	1.39	94 9	1.85	97.8	0.76	98.3	1.73	95.3	8.67
20	104.6	4.0	99.4	6.62	93.9	2.04	92.3	4.20	94.3	2.18	88.6	10.33	93.0	11.39
30	105.3	2.6	97.4	8.27	90.1	1.41	88.3	0.83	84.7	5.52	78.0	14.23	76.4	14.59
40	102.7	2.7	93.6	6.82	82.7	6.33	80.6	3.28	75.2	4.87	75.9	20.50	72.0	18.14
50	101.2	4.4	87.9	7.02	77.1	6.19	76.8	4.48	64.9	5.13	76.9	16.00	58.9	16.61
60	99.2	4.7	85.9	8.70	74.3	8.44	73.8	7.14	57.2	5.11	79.0	15.19	57.7	18.11
75	98.7	3.8	86.9	7.84	71.5	10.04	71.8	8.63	47.9	14.66	82.2	12.56	49.9	16.14
90	96.7	4.0	86.9	10.89	78.1	10.37	76.6	9.54	44 6	20.11	83.2	8.30	47.9	20.13
120	94.1	8.1	86.9	8.14	84.6	7.57	76.9	4.38	47.2	19.98	91.8	10.47	49.0	15.46
150	91.3	7.3	88.9	6.57	85.5	4.68	81.0	1.43	52.4	16.59	92.7	5.70	48.8	9.59
180	90.6	9.7	88.0	5.15	89.3	3.34	85.0	4 90	59.2	13.99	94.0	8.77	47.8	10.26
240	89.3	6.3	89.9	3.81	94.0	3.97	90 0	7.54	77.2	6 64	96.5	9.15	62.8	3.56

Table 6.4. Summary of plasma insulin concentrations following the intranasal administration of 2 IU/kg insulin in combination with MVCSN or LPC or the subcutaneous administration of 0.2 IU.kg insulin in sheep

	Mean plasma insulin concentration (mU/l)									
Time before or after dosing (min)	Insulin control		Insulin/0.1% MVCSN		Insulin/0.5% MVCSN		Insulin/0.5% LPC		subcut. insulin	
		SD		SD		SD		SD		SD
-15	6.0	1.3	6.4	4.23	6.5	1.62	12.4	-	7.0	8.89
-5	5.4	1.8	10.0	9.46	8.2	3.45	13.3	1.31	6.7	5.51
5	5.9	1.1	14.6	5.58	24.2	22.08	104.1	51.33	57.7	45.08
10	9.0	3.7	21.3	1.36	89.1	116.77	142.4	73.94	40.0	20.78
15	9.4	5.1	42.9	8.65	110.7	34.82	74.3	21.82	48.7	16.04
20	6.8	2.0	59.6	37.40	54.4	17.72	53.3	2.57	54.7	7.02
30	5.4	0.5	55.2	35.95	59.7	47.47	30.7	9.06	71.7	16.17
40	6.5	1.1	47.3	29.35	73.2	54.20	17.8	3.54	72.0	30.51
50	5.3	1.5	17.0	12.10	87.8	80 04	13.8	5.09	55.3	32.39
60	5.5	1.5	9.3	6.34	100.8	85 69	8.3	1.10	60.3	29.70
75	6.2	1.8	5.0	2.23	82.9	75.71	7.2	1.14	55.3	31.90
90	5.7	1.8	3.7	0.81	52.6	45.78	7.2	3.08	45.0	17.00
120	5.5	0.9	3.6	2.32	23.6	19.28	5.4	1.99	38.0	12.17
150	6.7	2.0	2.9	1.40	11.7	6 90	5.9	2.40	35.3	11.02
180	7.6	3.2	2.3	0 99	9.3	4.56	6.2	2.26	21.7	5.69
240	5.3	2.3	3.2	2.06	7.8	3.96	6.2	3.10	19.7	11.59

Table 6.5. Summary of pharmacokinetic paramenters determined from plasma glucose data following the intranasal administration of 2 IU/kg insulin in combination with LVCSN, MVCSN or LPC or the subcutaneous administration of 0.2 IU.kg insulin in sheep

		Mean (± SD)				
Formulation or Group number	No. sheep in group	Cmin (% glucose)	Tmin (min)	AOC (% glucose. min)	Rate of decrease of % glucose (%/min)	pharmacological availability * (%)
1. Insulin control	4	88.0 (± 6.49)	105 (± 75.5)	1379 (± 1032.6)	-	1.4 (± 1.02)
2. Insulin / 0.1% LVCSN	3	84.0 (± 8.28)	133 (± 93 8)	2486 (± 1427.9)	0.37 (± 0.032)	2.4 (± 1.40)
3. Insulin / 0.5% LVCSN	3	70.6 (± 10.10)	70 (± 8.7)	3295 (± 1166.5)	0.49 (± 0.171)	3.2 (± 1.14)
4. Insulin / 0.1% MVCSN	3	70.9 (± 8.50)	83 (± 27.0)	4065 (± 152.6)	0.46 (± 0.225)	4 0 (± 0.15)
5. Insulin / 0.5% MVCSN	3	42.9 (± 17.05)	85 (± 22.9)	8815 (± 2433.7)	0.87 (± 0.055)	8.7 (± 2.39)
6. Insulin / 0.5% LPC	3	74.9 (± 19.57)	48 (± 10.4)	2644 (± 2200.8)	0.95 (± 0.384)	2.6 (± 2.16)
7. Sucutaneous insulin	3	44.3 (± 14.40)	130 (± 56.8)	10176 (± 2794 8)	-	100.0

* Relative to subcutaneous

Statistical Analysis (nasal groups only)

One-way ANOVA

Sig. dif. in values of Cmin ($P<0.01$), Rate of decrease of % glucose ($P<0.05$) and AOC ($P<0.001$).
No sig. diff. in values of Tmin ($P>0.05$).

Tukey-Kramer Multiple Comparison Test following ANOVA

Cmin: Sig. dif. between groups 1 vs 5, 2 vs 5 ($P<0.01$).
Rate of decrease of % glucose: Sig. dif. between group 2vs6 ($P<0.05$).
AOC: Sig. dif. between groups 1 vs 5 ($P<0.001$), 2 vs 5, 3 vs 5, 6 vs 5 ($P<0.01$) and 4 vs 5 ($P<0.05$).
No sig. dif. ($P>0.05$) between all other formulation groups.

Table 6.6. Summary of pharmacokinetic paramenters determined from plasma insulin data following the intranasal administration of 2 IU/kg insulin in combination with MVCSN or LPC or the subcutaneous administration of 0.2 IU.kg insulin in sheep

Formulation or Group number	No. sheep in group	Mean (± SD) Cmax (mU/l)	Tmax (min)	AUC (mU/l.min)	Rate of increase of insulin ([logmU/l]/min)	Bioavailability * (%)
1. Insulin control	4	10.4 (± 3.91)	20 (± 13.5)	169 (± 130.4)	-	0.2 (± 0.14)
4. Insulin / 0.1% MVCSN	3	68.8 (± 31.64)	22 (± 7.6)	1682 (± 1189.3)	0.05 (± 0.034)	1.8 (± 1.29)
5. Insulin / 0.5% MVCSN	3	191.1 (± 28 49)	40 (± 26.5)	7707 (± 4632.1)	0.09 (± 0.050)	8.4 (± 5.03)
6. Insulin / 0.5% LPC	3	142.4 (± 73.94)	10 (± 0.0)	1942 (± 610.0)	0.17 (± 0.034)	2.1 (± 0.66)
7. Subcutaneous insulin	3	79.3 (± 26 08)	25 (± 18.0)	9205 (± 2287.5)	-	100.0

* Relative to subcutaneous

Statistical Analysis (Performed on nasal groups 1, 4, 5, & 6 only)

One-way ANOVA
Sig. dif. in values of Cmax (P<0.001), rate of increase of insulin (P<0.05) and AUC (P<0.01).
No sig. dif. in values of Tmax (P>0.05).

Tukey-Kramer Multiple Comparison Test following ANOVA
Cmax: Sig. dif. between groups 1 vs 5 (P<0.001), 1 vs 6 (P<0.01) and 4 vs 5 (P<0.05).
Rate of increase of insulin: Sig. dif. between group 4vs6 (P<0.05).
AUC: Sig. dif. between groups 1 vs 5 (P<0.01) and 4 vs 5, 6 vs 5 (P<0.05).
No sig. dif. (P>0.05) in values of Cmax, Rate of increase of insulin conc. and AUC between other groups.

Fig 6.1 Plasma glucose concentration versus time profiles following the intranasal administration of 2 IU/kg insulin in combination with LVCSN, MVCSN or LPC or the subcutaneous administration of 0.2 IU.kg insulin in sheep

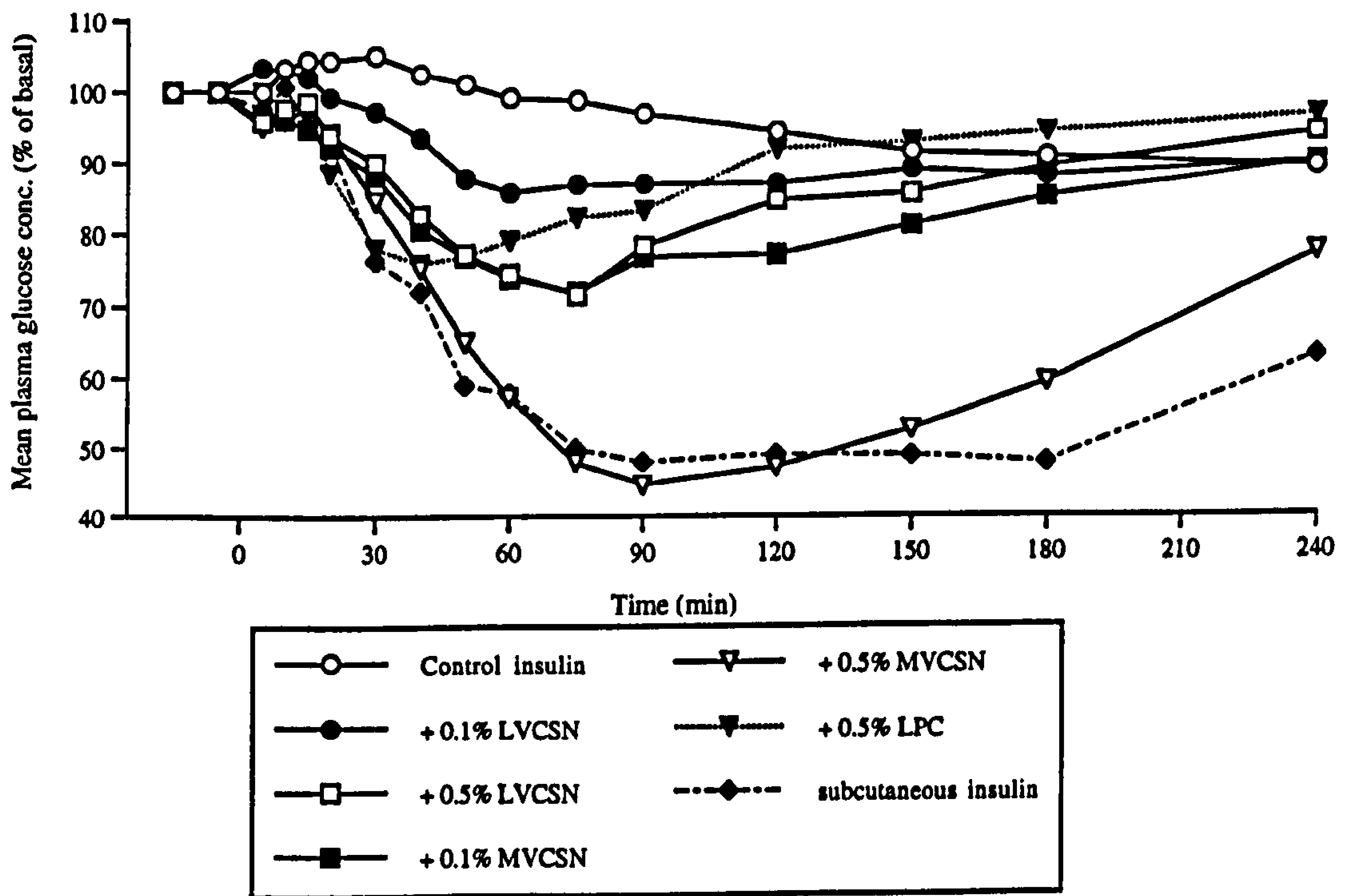


Fig 6.2 Plasma insulin concentration versus time profiles following the intranasal administration of 2 IU/kg insulin in combination with MVCSN or LPC or the subcutaneous administration of 0.2 IU.kg insulin in sheep

Legend as in Figure 6.1

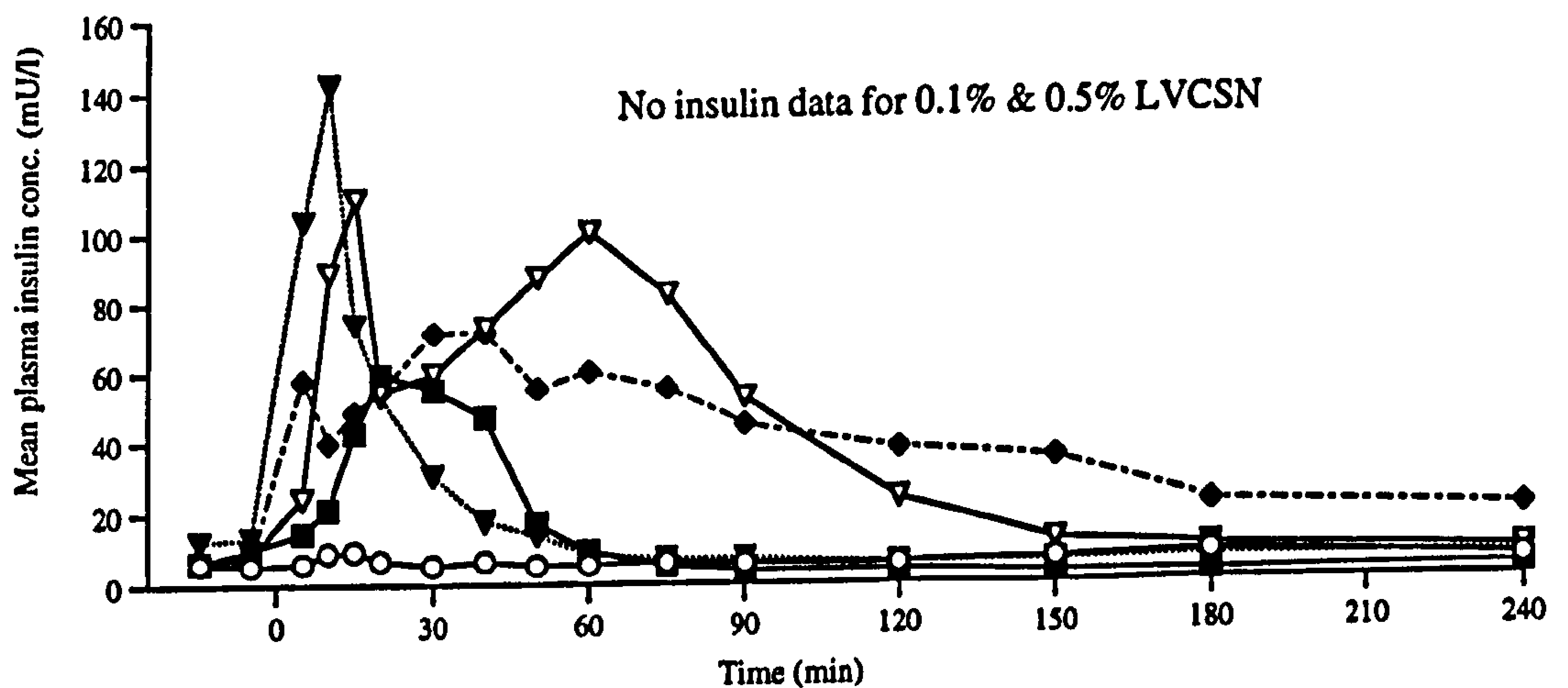
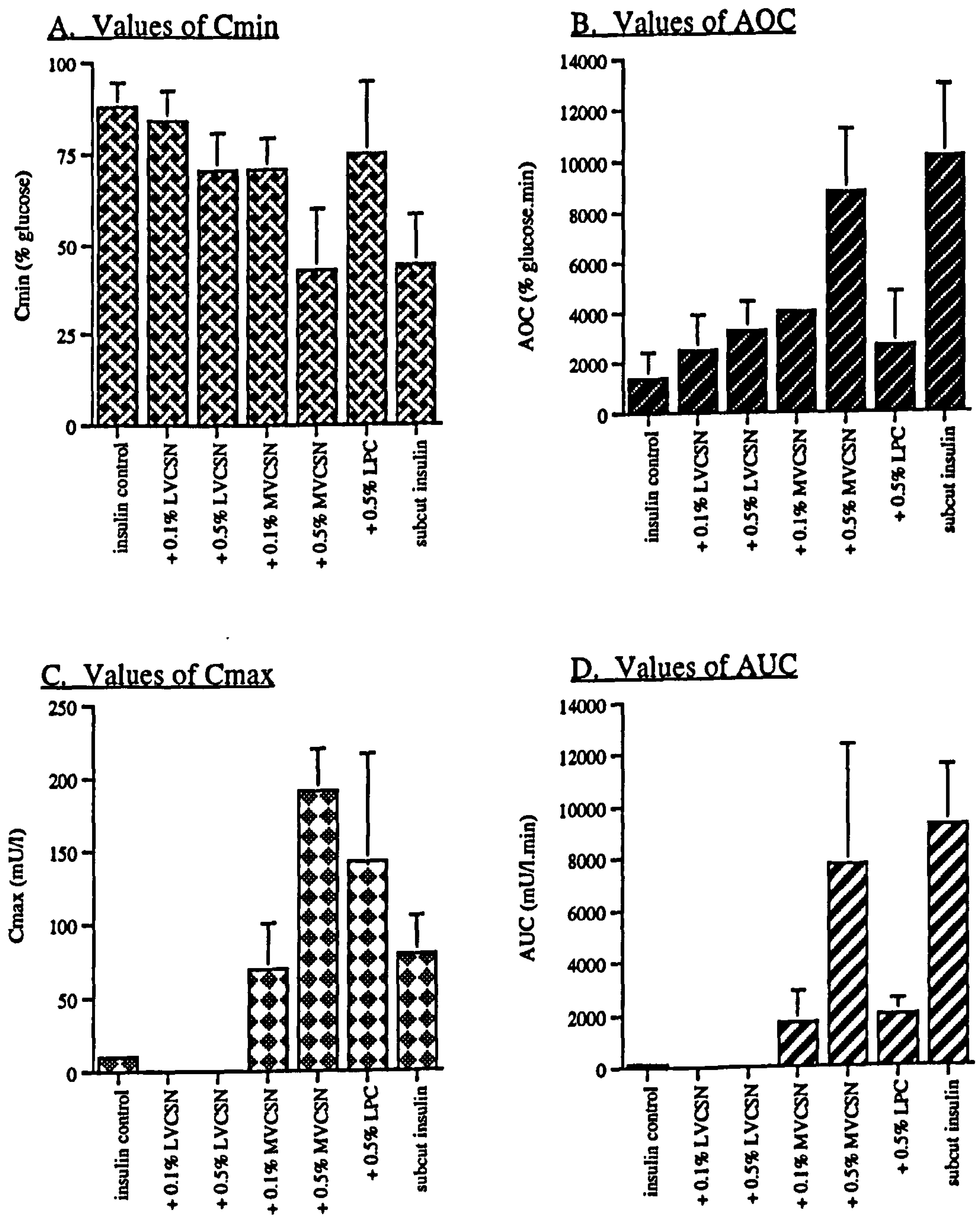


Figure 6.3. Selected pharmacokinetic parameters following the intranasal administration of 2 IU/kg insulin in combination with LVCSN, MVCSN or LPC or the subcutaneous administration of 0.2 IU.kg insulin in sheep



CHAPTER 7

EVALUATION AND FURTHER OPTIMISATION OF NASAL INSULIN FORMULATIONS CONTAINING CHITOSAN IN SHEEP

7.1 Introduction

In Chapter 6 it was demonstrated that a formulation containing MVCSN was highly effective in enhancing the intranasal absorption of insulin in the sheep model. In further optimising chitosan solution formulations for the nasal delivery of insulin there are several factors relating to the formulation, such as the concentrations and doses of insulin and chitosan, the dose volume administered, the type of vehicle used and the characteristics of the grade of chitosan used, which should be considered. In this chapter, a number of these factors are investigated further details of which will be given in the appropriate section. The various studies are reported in the following sections:

7.2 Effect of chitosan concentration on the intranasal absorption of insulin in sheep
7.3 Effect of the dose of chitosan on the intranasal absorption of insulin in sheep
7.4 Effect of the dose of insulin on the intranasal absorption of insulin in sheep
7.5 Effect of dose volume on the intranasal absorption of insulin in sheep
7.6 Effect of different vehicles on the absorption enhancing efficacy of chitosan in sheep
7.7 Effect of different grades of chitosan on the intranasal absorption of insulin in sheep
7.8 Reproducibility of the absorption enhancing efficacy of chitosan in sheep

Many of the sheep studies were performed in parallel to the nasal absorption studies in rats. Despite the decision to use isotonic formulations in rat studies, in an early sheep study investigating the effect of chitosan concentration, hypotonic formulations were adinistered. Thus, for consistency throughout the sheep studies, it was decided that hypotonic fomulations would be administered.

In the absorption studies, various insulin formulations were administered nasally to sheep. Blood samples were collected pre- and post administration of the insulin formulations and plasma separated. Plasma glucose and insulin concentrations were measured and insulin absorption was assessed, indirectly, from the decrease in blood glucose concentrations and directly from the elevation in plasma insulin concentrations following insulin dose administration. The efficacy of insulin absorption from the formulations used in each study were compared. For reference, the subcutaneous absorption data reported in Chapter 6 was used.

Methods and materials used were as given in Chapter 2 / Appendix 1. Additional details are given in the relevant sections.

7.2 Effect of chitosan concentration on intranasal insulin absorption in sheep

7.2.1 Aims and Objectives

In a previous study in rats (refer to Section 4.3), the absorption enhancing efficacy of chitosan was shown to increase with increasing concentration of chitosan in the formulation until an optimal range of concentrations was reached. In this study the effect of different concentrations of chitosan on the intranasal absorption of insulin was investigated in sheep. Five different concentrations of MVCSN, ranging from 0.1-1.0% were tested.

7.2.2 Materials and Methods

7.2.2.1 Materials

Insulin (specific activity 24.22 IU/mg) was used as given in Section 3.2. Additional materials are given in Section 3.3.

7.2.2.2 Preparation of insulin formulations

Five solution formulations were prepared for nasal administration to sheep as listed below. Each nasal solution contained 200 IU/ml insulin (sufficient to administer a dose of 2 IU/kg insulin in a volume of 0.01 ml/kg). All formulations were prepared in 14.65 mM phosphate buffer of pH 7.4. and were adjusted to pH 4 during preparation by the addition of HCl. Each formulation was freshly prepared on the day of the study.

Table 7.1. Outline composition of formulations

Formulation No.	Outline composition
1	200 IU/ml insulin with 0.10% (1.0 mg/ml) MVCSN, pH 4
2	200 IU/ml insulin with 0.20% (2.0 mg/ml) MVCSN, pH 4
3	200 IU/ml insulin with 0.35% (3.5 mg/ml) MVCSN, pH 4
4	200 IU/ml insulin with 0.50% (5.0 mg/ml) MVCSN, pH 4
5	200 IU/ml insulin with 1.00% (1.0 mg/ml) MVCSN, pH 4

* Concentration of MVCSN expressed as the concentration of chitosan glutamate

7.2.2.3 Absorption study in the sheep model

Sheep were dosed nasally with the appropriate insulin formulation. A summary of the dose groups is given in Table 7.2. Nasal doses of 2 IU/kg insulin were administered at a volume of 0.01 ml/kg. Each nasal dose was divided between both nostrils.

7.3 Results & Discussion

The results show that insulin dosed at 2 IU/kg with chitosan was absorbed nasally from each of formulations administered to sheep (Tables 7.3-7.6, Figures 7.1-7.2). However, increasing the formulation concentration of chitosan improved insulin absorption.

The pharmacokinetic parameters determined from the glucose data (Table 7.5, Figure 7.2 A-C) showed that decreases in plasma glucose concentrations and the rate of decrease in glucose concentrations reached a plateau at a chitosan concentration between 0.5% and 1.0% (values of approximately 40% and 0.9 % glucose/min, respectively between 0.5-1.0% MVCSN). However, the effect on the overall extent and duration of the hypoglycaemia, reflected in the values of AOC and pharmacological availability, was marginally better at 1.0% (values of 4319 % glucose.min and 4.2%, respectively at 1.0%). These findings were supported by the pharmacokinetic parameters determined from the insulin data (Table 7.6, Figure 7.2 D-F) which showed that increasing the formulation concentration of chitosan improved insulin absorption. Taking into account the inter-animal variations in the data, the absorption enhancing efficacy of chitosan in terms of values of C_{max} and AUC (hence bioavailability) appeared to plateau at a concentration between 0.35-1.0% (At 0.35, 0.5 and 1.0% MVCSN values of C_{max} ranged between 76-99 mU/l and values of AUC between 2012-2370 mU/l.min).

Values of pharmacological availability (3.6-4.2%) and bioavailability (2.2-2.6%), relative to a subcutaneous dose of insulin, showed that there was very little difference in insulin absorption from formulations containing between 0.35-1.0% chitosan (Tables 7.6 & 7.5, respectively). However, these values were much lower than those reported in Chapter 6 following the intranasal administration of an insulin formulation containing 0.5% MVCSN (8.7% and 8.4%, respectively). Possible explanations for this are given below.

There were no statistically significant differences between the formulation groups (Tables 7.5 & 7.6) in terms of values of C_{min} and AOC obtained from the glucose data and values of C_{max}, T_{max}, rate of increase of insulin concentrations or AUC, hence bioavailability, obtained from the insulin data. There were, however, significant differences in values of T_{min} and rates of decrease of glucose concentrations. Values of T_{min} of Formulation Group 1 (0.1% MVCSN) were higher than those of the other formulation groups. However, in view of glucose concentrations in this group of animals generally not decreasing to the same extent as those in the other formulation groups and since values of T_{max} were not significantly different from those obtained in the other groups, the difference in values of T_{min} were not considered important and may in-part be due to a natural fluctuation in basal glucose concentrations. The rate of decrease of glucose concentrations after dosing the formulation containing 0.5% chitosan although significantly higher than those obtained with the 0.1% MVCSN formulation were not significantly different from values obtained for the other formulations.

The results of this study show that the intranasal absorption of insulin was improved by increasing the concentration of chitosan in the formulation from 0.1% to 0.35% although further increase in chitosan concentration did not appear to further increase insulin absorption. These findings support those from the previous study in rats (refer to Chapter 4) which showed that maximal insulin absorption was obtained at a chitosan concentration between 0.5-0.75%. The trend of increase in absorption with increasing chitosan concentration until reaching an optimal concentration suggests that the

mechanism (s) of action of chitosan on the nasal membrane, thereby promoting insulin absorption, reaches a maximum. As discussed in Chapter 4, increasing the concentration of chitosan may reduce mucociliary clearance or drainage of the formulation from the nasal cavity due to increased viscosity and/or bioadhesion. The viscosity of chitosan solutions increases with concentration (refer to Section 10.5). For MVCSN solutions prepared in 1% acetic acid, values of apparent viscosity measured at 5 rpm of MVCSN solutions in acetic acid at concentrations of 0.2, 0.5 and 1.0 % (reported in Section 10.5) were 4.7, 15.4 and 55.8 CPS, respectively. Interaction of chitosan with the nasal membrane and the effect on tight junctions may also increase by increase in chitosan concentration.

The results obtained after nasally administering a dose of 2 IU/kg insulin from a formulation containing MVCSN generally agree with those reported by Aspden (1996). Aspden evaluated medium viscosity chitosan glutamate at concentrations of 0.25% and 0.5% w/v, expressed as the concentration of chitosan base, finding only marginal differences between the absorption enhancing efficacy of the two formulations.

In this study the bioavailability, relative to subcutaneous, of insulin from the 0.5% MVCSN formulation was only 2% compared to about 8% in the study reported in Chapter 3. Furthermore, values of C_{max}, pharmacological availability and reductions in glucose concentrations were lower in this study than values reported previously. The reasons for these discrepancies are not known but may be attributed to several factors. Inter-animal variations could be due to differences in the cross-breed of sheep used in the two studies. This may possibly influence absorption due to slight differences in the anatomy and physiology of the nasal cavity and differences in the homeostasis of blood glucose concentrations. Inter-animal variations in the anatomy and physiology of the nasal cavity will influence the exact site of deposition of the dosing solution in the nasal cavity which is likely to influence the subsequent spread and clearance of the dose from the nasal cavity. Differences do not appear to be related to the weight of the animals used since the weights of the sheep were similar in both studies. Differences in the ambient conditions of temperature and humidity on different study days may also influence absorption and ideally experiments should be performed under standardised environmental conditions (Gizurarson, 1990). Due to a heavy wool coat for much of the year and low efficiency of the sweat glands, the sheep uses the surfaces of the respiratory system including the nasal cavity to dissipate heat through water vaporisation (May, 1964). The rate of fluid production in the nasal cavity and the respiratory rate of sheep will help to regulate heat loss. Excess fluid production will also dilute the drug formulation which may subsequently affect the spread and clearance of the formulation from the nasal cavity. Despite the lower values of bioavailability obtained in this study, the improvement in nasal absorption compared to a control solution was about 10-fold which for polypeptide drugs represents a substantial increase in absorption.

From the results of this study it was decided to maintain the formulation concentration of chitosans at 0.5% for further investigations. Concentrations above 0.5% may cause

problems with nasal administration from spray devices which would be required for commercial application due to the increased viscosity of the solution. Lower concentrations of chitosan would also be favoured considering toxicological issues. In this study, the formulation containing 0.35% chitosan performed as well as that containing 0.5% chitosan. However, since a formulation concentration of 0.35% MVCSN was probably close to the start of the optimal range, it was decided to allow a margin of safety when selecting an optimal concentration. Thus, a concentration of 0.5% MVCSN was selcted for investigation in future studies.

__Table 7.2. Summary of formulations and dose groups in the study to investigate the effect of MVCSN concentration on the intranasal absorption of insulin in sheep__

Formulation or Group No.	Insulin (IU/kg)	MVCSN (mg/kg)	Dose volume (ml/kg)
1. Insulin + 0.10% MVCSN	2.0	0.01	0.01
2. Insulin + 0.20% MVCSN	2.0	0.02	0.01
3. Insulin + 0.35% MVCSN	2.0	0.035	0.01
4. Insulin + 0.50% MVCSN	2.0	0.05	0.01
5. Insulin + 1.00% MVCSN	2.0	0.1	0.01

* Dose of MVCSN expressed as dose of chitosan glutamate

__Table 7.3. Summary of plasma glucose concentrations following the intranasal administration of 2 IU/kg insulin in combination with different concentrations of MVCSN in sheep__

	Mean (± SD) plasma glucose concentration									
Time before or after dosing (min)	0.1% MVCSN	SD	0.2% MVCSN	SD	0.35% MVCSN	SD	0.5% MVCSN	SD	1.0% MVCSN	SD
-15	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
-5	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
5	97.4	2.17	93.6	1.03	97 8	2.00	97.9	3.86	97.1	2.03
10	98.2	0.69	94.9	1.65	95.8	2.87	97.8	3.24	99.2	2.55
15	95.6	3.16	92.2	0.79	94 4	2.44	93.4	6.52	94.3	2.19
20	94.7	2.07	90.3	1.16	89.2	5.64	87.8	6.85	89.9	5.14
30	91.2	3.45	84.0	2.15	80.0	14.20	77.1	11.01	79.8	8.81
40	90.3	2.91	82.9	4.17	73.6	15.68	66.7	12.66	69.2	8.80
50	87.7	7.21	79.9	6 67	70.9	19.29	61.2	12.80	63.7	7.61
60	86.8	5.60	83.1	7.19	68.7	20.11	60.5	14.64	61.5	8.78
75	83.3	8.09	84.3	6.07	69.6	20.77	65.1	14.27	61.5	8.81
90	84.2	8.56	86.7	4.08	73.2	15.62	72.8	11.94	66.5	7.27
120	84.2	13.09	87.3	3.14	82.3	7.17	84.2	6.81	77.8	6.57
150	85.9	10.16	86.8	3.66	86.6	1.31	93.1	3.78	88.0	5.34
180	85.0	9.70	85.4	2.85	90.8	4.11	93.0	0.70	90.9	7.48
240	88.5	3.35	87.5	4.39	91.5	6.03	96.6	4.74	94.5	6.08

Table 7.4. Summary of plasma insulin concentrations following the intranasal administration of 2 IU/kg insulin in combination with different concentrations of MVCSN in sheep

	Mean (± SD) plasma insulin concentration									
Time before or after dosing (min)	0.1% MVCSN	SD	0.2% MVCSN	SD	0.35% MVCSN	SD	0.5% MVCSN	SD	1.0% MVCSN	SD
-15	7.1	2.32	12.0	1.22	11.7	5.49	6.9	2.13	8.2	3.36
-5	7.4	1.99	8.7	0.86	10.9	6.25	6.0	0.94	8.0	3.33
5	9.4	1.24	29.2	13.30	30.4	11.71	41.2	18.55	50.3	35.19
10	36.2	11.09	47.3	18.48	81.0	68.07	74.3	11.11	54.5	29.18
15	28.5	19.62	43.0	29.75	57.2	12.90	70.6	13.95	86.6	21.17
20	23.0	17.29	29.5	10.85	52.9	19.13	45.1	3.12	69.0	23.79
30	14.1	6.40	27.4	32.81	66.9	43.81	37.2	15.08	57.1	21.49
40	8.7	1.60	10.0	4.54	43.3	28.81	27.4	17.00	33.0	8.49
50	6.2	2.34	6.6	0.92	26.0	19.93	18.2	7.07	15.5	7.86
60	6.3	0.28	6.3	0.27	21.6	20.71	15.7	13.10	23.4	16.94
75	6.6	1.12	5.8	0.80	16.8	16.14	13.6	15.09	10.4	4.20
90	5.7	1.71	5.4	0.89	8.8	6.28	5.6	4.03	7.8	1.84
120	4.0	0.68	5.2	0.62	7.0	3.86	4.3	2.36	5.8	2.32
150	3.7	1.30	4.1	1.11	7.6	3.37	4.4	1.95	5.6	1.27
180	3.6	0.74	4.5	0.11	5.9	2.16	5.0	3.03	5.7	2.66
240	4.3	0.85	5.8	2.27	5.6	1.43	4.1	1.01	5.0	1.86

Table 7.5. Summary of pharmacokinetic paramenters determined from plasma glucose data following the intranasal administration of 2 IU/kg insulin in combination with different concentrations of MVCSN in sheep

		Mean (± SD)					
Formulation or Group number	No. sheep in group	Cmin (% glucose)	Decrease in gluc. conc. (%)	Tmin (min)	AOC (% glucose. min)	Rate of decrease of % glucose (%/min)	pharmacological availability * (%)
1. Insulin with 0.1% MVCSN	3	80.6 (± 9.92)	119.4 (± 9.92)	123 (± 56.3)	2980 (± 1661.3)	0.23 (± 0.0.121)	2.9 (± 1.63)
2. Insulin with 0.2% MVCSN	4	79.9 (± 6.67)	20.1 (± 6.67)	50 (± 4.1)	3113 (± 759.3)	0.42 (± 0.148)	3.1 (± 0.75)
3. Insulin with 0.35% MVCSN	4	68.1 (± 19.73)	31.9 (± 19.73)	65 (± 13.7)	3819 (± 1522.8)	0.64 (± 0.538)	3.8 (± 1.50)
4. Insulin with 0.5% MVCSN	4	59.8 (± 14.43)	40.2 (± 14.43)	59 (± 7.8)	3699 (± 1145.3)	0.94 (± 0.208)	3.6 (± 1.13)
5. Insulin with 1.0% MVCSN	4	60.1 (± 8.76)	39.9 (± 8.76)	68 (± 12.8)	4319 (± 1075.7)	0.87 (± 0.177)	4.2 (± 1.06)

* Relative to subcutaneous

Statistical Analysis

One-way ANOVA

Sig. dif. in values of Tmin and rate of decrease of % glucose ($P<0.05$).
No sig. dif. in values of Cmin & AOC ($P>0.05$).

Tukey-Kramer Multiple Comparison Test following ANOVA

Tmin: Sig. dif. between groups 1 vs 2 ($P<0.01$) and 1vs3, 1vs4 ($P<0.05$)
Rate of decrease of % glucose: Sig. dif. between groups 1 vs 4 ($P<0.05$)
No sig. dif. ($P>0.05$) between all other formulation groups.

Table 7.6. Summary of pharmacokinetic paramenters determined from plasma insulin data following the intranasal administration of 2 IU/kg insulin in combination with different concentrations of MVCSN in sheep

		Mean (± SD)				
Formulation or Group number	No. of sheep in group	Cmax (mU/l)	Tmax (min)	AUC (mU/l.min)	Rate of increase of insulin ([log mU/l]/min)	Bioavailability * (%)
1. Insulin + 0.1% MVCSN	3	39.3 (± 14.30)	12 (± 2.9)	351 (± 314.3)	0.08 (± 0.051)	0.5 (± 0.28)
2. Insulin + 0.2% MVCSN	4	53.4 (± 26.22)	13 (± 2.9)	782 (± 666.3)	0.09 (± 0.029)	0.9 (± 0.72)
3. Insulin + 0.35% MVCSN	4	99.2 (± 60.93)	16 (± 9.5)	2260 (± 1493.7)	0.08 (± 0.048)	2.5 (± 1.62)
4. Insulin + 0.5% MVCSN	4	76.2 (± 11.16)	11 (± 2.5)	2012 (± 773.6)	0.12 (± 0.051)	2.2 (± 0.84)
5. Insulin + 1.0% MVCSN	4	94.5 (± 13.77)	14 (± 2.5)	2370 (± 962.5)	0.15 (± 0.098)	2.6 (± 1.05)

* Relative to subcutaneous

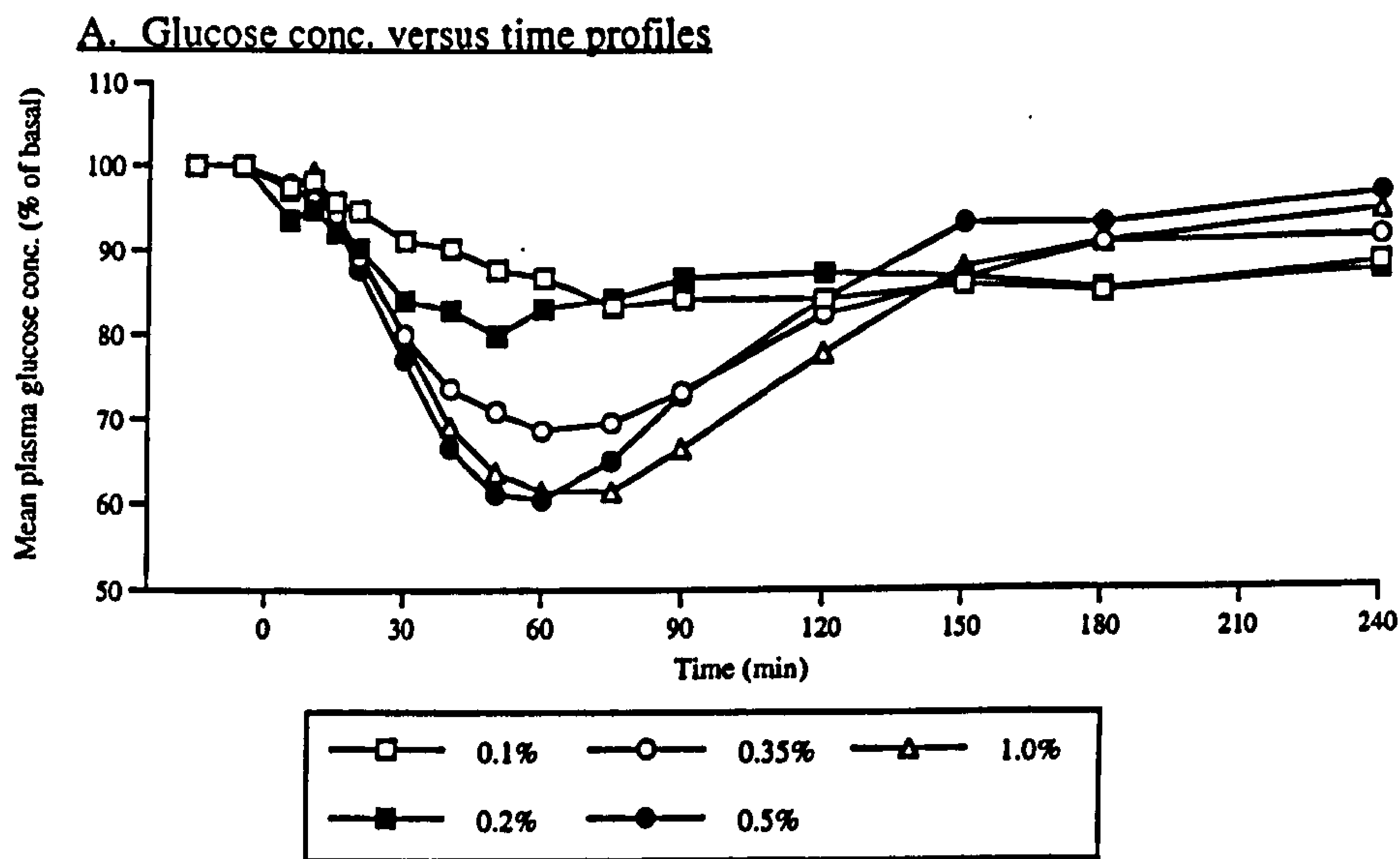
Statistical Analysis

One-way ANOVA

No sig. dif. in values of Cmax, Tmax, AUC and rate of increase of insulin (P>0.05).

Fig 7.1. Plasma glucose and insulin concentration versus time profiles following the intranasal administration of 2 IU/kg insulin in combination with different concentrations of MVCSN in sheep

A. Glucose conc. versus time profiles



B. Insulin conc. versus time profiles

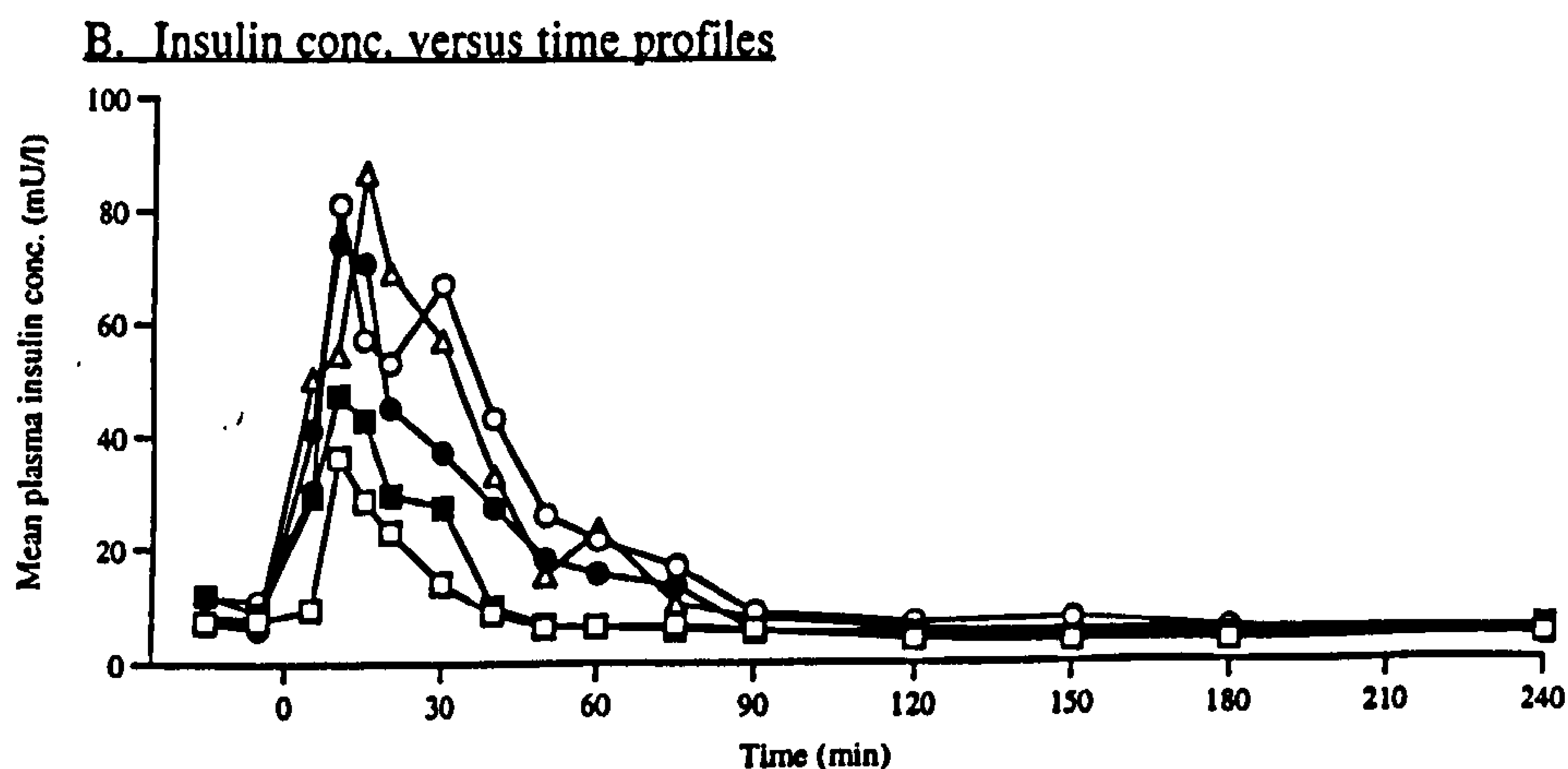
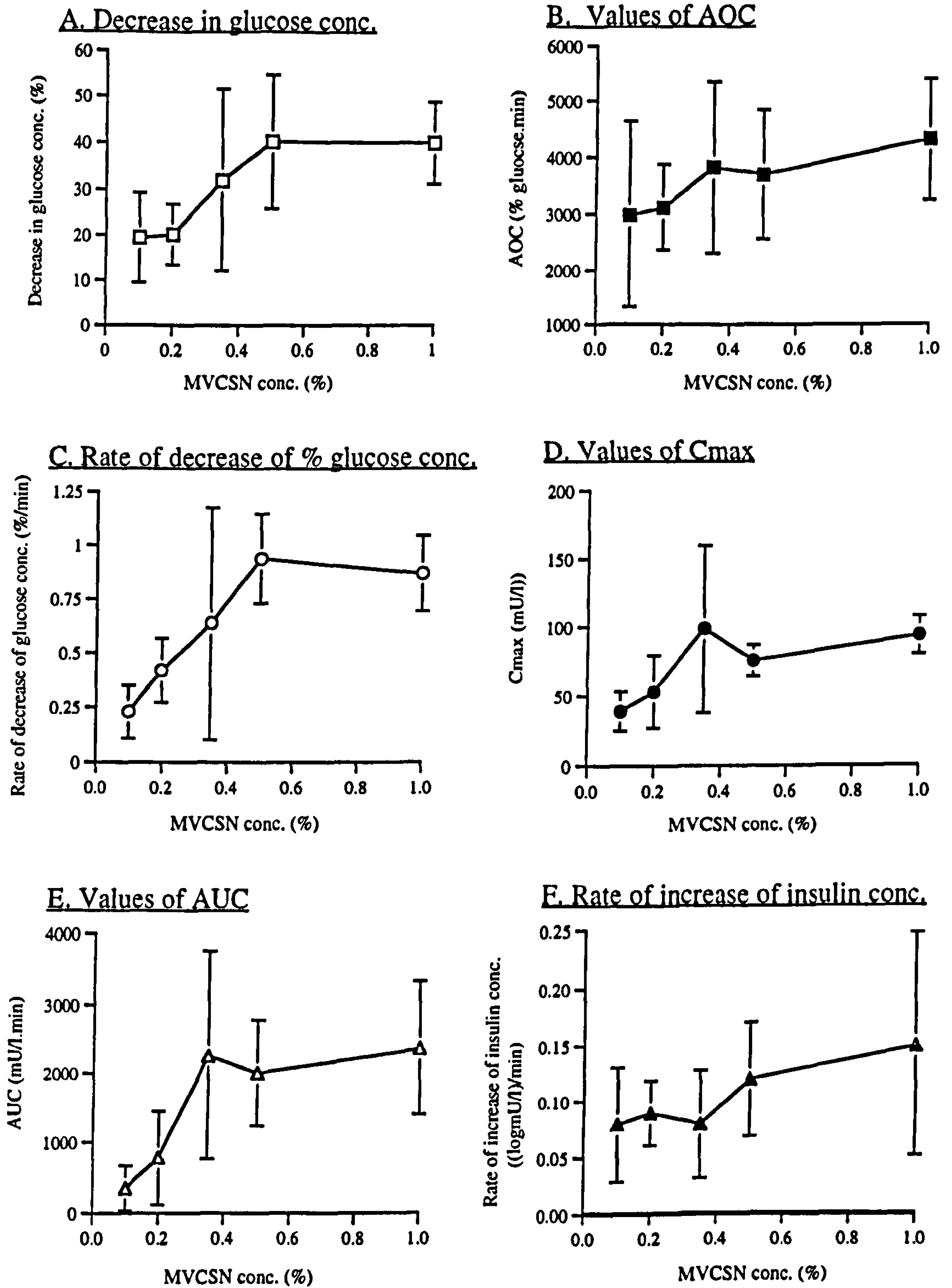


Figure 7.2. Values of pharmacokinetic parameters determined from glucose or insulin data following the intranasal administration of 2 IU/kg insulin in combination with different concentrations of MVCSN in sheep



7.3 Effect of chitosan dose on the intranasal absorption of insulin in sheep

7.3.1 Aims and Objectives

The previous study showed that increasing the concentration of MVCSN in the formulation improved nasal insulin absorption until an optimal concentration was reached. Increasing the concentration of chitosan in the formulation will inevitably increase the viscosity of the formulation which may in-part account for improved efficacy (refer to Section 10.5). Increasing chitosan concentration will also increase the dose of chitosan administered nasally to sheep. Thus, the aim of this study was to investigate the effect of the dose of chitosan on its nasal absorption enhancing efficacy. The dose of chitosan administered to sheep was adjusted by varying the dose volume administered. The use of a fixed concentration of chitosan in the formulations (0.5% MVCSN) ensured that any changes in the absorption enhancing efficacy of the formulations was not attributed to changes in the viscosity of the solutions. The insulin concentration of the formulations was appropriately adjusted to maintain a fixed dose of insulin at 2 IU/kg. The nasal dose of MVCSN was varied between 0.0125-0.2 mg/kg by varying dose volume between 0.0025-0.04 ml/kg.

7.3.2 Materials and Methods

7.3.2.1 Materials

Materials used were as given in Section 7.2.

7.3.2.2 Preparation of insulin formulations

Five solution formulations were prepared for nasal administration to sheep as given in Table 7.8. Each formulation contained a sufficient concentration of insulin to administer a dose of 2 IU/kg at the appropriate dose volume (refer to Table 7.7). All formulations were prepared in 14.65 mM phosphate buffer of pH 7.4 and were adjusted to pH 4 during preparation by the addition of HCl. Each formulation was freshly prepared on the day of the study.

Table 7.7. Outline composition of formulations

Formulation No.	Outline composition
1	50 IU/ml insulin with 0.5% (5.0 mg/ml) MVCSN, pH 4
2	100 IU/ml insulin with 0.5% (5.0 mg/ml) MVCSN, pH 4
3	200 IU/ml insulin with 0.5% (5.0 mg/ml) MVCSN, pH 4
4	400 IU/ml insulin with 0.5% (5.0 mg/ml) MVCSN, pH 4
5	800 IU/ml insulin with 0.5% (5.0 mg/ml) MVCSN, pH 4

* Concentration of MVCSN is expressed as the concentration of chitosan glutamate

7.3.2.3 Absorption study in the sheep model

Sheep were dosed nasally with the appropriate insulin formulation. A summary of the dose groups is given in Table 7.8. Nasal doses of 2 IU/kg insulin were administered.

The dose volume was varied between 0.0025-0.04 ml/kg. Each nasal dose was divided between both nostrils. In view of the results obtained following glucose analysis, plasma insulin analysis was not performed.

7.3.3 Results & Discussion

The results showed that at a fixed dose of insulin (2 IU/kg), variations in the dose of MVCSN and the dose volume did not significantly influence the intranasal absorption of insulin in sheep (Tables 7.9-7.10, Figures 7.3-7.4). Furthermore, altering the concentration of insulin in the formulation over a wide range (50-800 IU/ml) had no apparent effect on the performance of the formulation. The plasma glucose versus time profiles of each group of sheep followed a similar trend (Figure 7.3). The return towards basal concentrations of plasma glucose appeared to be more rapid in sheep dosed with 0.2 mg/kg MVCSN (0.04 ml/kg). However, the inter-animal variations for this group of animals were high (Table 7.9). A dose of 2 IU/kg insulin with 0.5% MVCSN administered at 0.01 ml/kg, did not perform as well as the other formulations in terms of the lowering of plasma glucose concentrations over the experimental period (Table 7.9, Figure 7.3). The reasons for this are not known but in view of the results from the other dose groups in which much higher and lower doses of chitosan and dose volumes were administered without apparently affecting glucose concentrations the results for this dose group were considered anomalous.

The pharmacokinetic parameters determined from the glucose data (Table 7.10, Figure 7.4) show that taking into account inter-animal variability there was little difference between the dose groups. Values of C_{min} ranged between about 63-77 % glucose with values of T_{min} of 70-105 minutes. The rates of decrease of glucose concentrations ranged from 0.5-0.9 % glucose/min, values of AOC from 2624-4532 % glucose.min and values of pharmacological availability from 2.6-4.5%. Statistical analysis of the data with a one-way ANOVA (Table 7.10) showed that there were no significant differences between the dose groups in values of C_{min}, T_{min}, AOC (hence pharmacological availability) and the rate of reduction of glucose concentrations. Values of pharmacological availability obtained for the different dose groups ranged between 2.6-4.5 % (Table 7.10).

The intranasal absorption of insulin was not apparently affected by changes in the dose of MVCSN coadministered or by changes in the dose volume administered. In view of these results and those from the previous study which assessed different concentrations / doses of chitosan, at a fixed dose of insulin it appears that it is the concentration of chitosan in the formulation rather than the dose of chitosan coadministered which is the important factor influencing nasal absorption. This suggests that the effect of chitosan on the nasal membrane reaches a maximum. Increasing the dose volume administered should increase the surface area of the nasal cavity covered by the insulin formulation, thus, increase the absorptive surface area. This would be expected to increase insulin absorption. However, since the degree of absorption was not affected by varying the dose volume then absorption may be limited by the dose of insulin administered in the

formulation. Thus, it was decided that the effect of the nasal dose of insulin should be evaluated.

Table 7.8. Summary of formulations and dose groups in the study to investigate the effect of chitosan concentration on the intranasal absorption of insulin in sheep

Formulation or Group No.	Insulin (IU/kg)	MVCSN (mg/kg)	Dose volume (ml/kg)
1. 50 IU/ml Insulin + 0.5% MVCSN	2.0	0.2	0.04
2. 100 IU/ml Insulin + 0.5% MVCSN	2.0	0.1	0.02
3. 200 IU/ml Insulin + 0.5% MVCSN	2.0	0.05	0.01
4. 400 IU/ml Insulin + 0.5% MVCSN	2.0	0.025	0.005
5. 800 IU/ml Insulin + 0.5% MVCSN	2.0	0.0125	0.0025

* The dose of MVCSN was expressed as the dose of chitosan glutamate

Table 7.9. Summary of plasma glucose concentrations following the intranasal administration of 2 IU/kg insulin with different doses of MVCSN and at various dose volumes in sheep

	Mean (± SD) plasma glucose concentration (% of basal)									
Time before or after dosing (min)	0.2 mg/kg MVCSN		0.1 mg/kg MVCSN		0.05 mg/kg MVCSN		0.025 mg/kg MVCSN		0.0125 mg/kg MVCSN	
		SD		SD		SD		SD		SD
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
-15	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
-5	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
5	97.2	2.86	98.2	2.89	99.4	2.27	100.5	3.86	96.1	5.52
10	96.5	2.21	100.0	3.26	99.5	3.69	101.3	1.91	98.6	6.22
15	94.5	4.37	98.7	3.52	99.6	5.30	100.1	3.18	96.9	5.98
20	91.7	5.93	95.1	6.43	97.1	6.02	95.6	2.46	94.4	7.86
30	81.7	12.41	89.1	11.45	90.5	9.22	85.3	5.76	87.6	7.01
40	72.3	15.01	81.9	16.97	85.5	9.05	76.2	9.54	80.3	9.00
50	69.0	18.89	73.4	20.40	82.3	8.60	68.8	10.81	73.6	10.61
60	65.9	20.33	69.2	20.41	78.4	4.20	63.3	14.16	68.9	12.74
75	72.4	17.68	70.5	15.65	77.1	4.98	62.7	14.58	68.5	15.89
90	80.0	8.45	71.2	12.31	80.6	6.33	64.6	15.38	69.8	19.38
120	87.4	5.59	79.6	7.57	86.8	7.18	75.1	13.70	73.0	21.35
150	90.2	4.96	86.2	5.82	92.3	9.94	82.5	12.21	81.9	15.88
180	91.8	6.85	90.5	5.99	94.8	9.86	87.8	6.46	87.5	7.52
240	91.3	7.83	90.4	5.22	96.2	10.19	93.1	2.59	91.3	6.25

Table 7.10. Summary of pharmacokinetic paramenters determined from plasma glucose data following the intranasal administration of 2 IU/kg insulin with different doses of MVCSN and at various dose volumes in sheep

Formulation or Group number	Mean ± SD (n=4)				
	Cmin (% glucose)	Tmin (min)	AOC (% glucose. min)	Rate of decrease of glucose (%/min)	Pharmacological availability * (%)
1	64.9 (± 18.88)	105 (± 90.0)	3351 (± 1085.3)	0.90 (± 0.570)	3.3 (± 1.07)
2	66.3 (± 17.58)	86 (± 33.3)	3774 (± 1576.1)	0.73 (± 0.470)	3.7 (± 1.55)
3	76.5 (± 3.83)	73 (± 13.7)	2624 (± 1377.7)	0.50 (± 0.136)	2.6 (± 1.35)
4	62.7 (± 14.57)	70 (± 61.3)	4532 (± 1887.8)	0.85 (± 0.228)	4.5 (± 1.86)
5	65.4 (± 17.27)	75 (± 21.2)	4349 (± 2525.3)	0.60 (± 0.194)	4.3 (± 2.48)

* Relative to subcutaneous

Statistical Analysis

One-way ANOVA

No sig. dif. in values of Cmin, Tmin, AOC and rate of decrease of glucose concentrations & pharmacological availability (P>0.05).

Fig 7.3. Plasma glucose concentration versus time profiles following the intranasal administration of 2 IU/kg insulin with different doses of MVCSN and at various dose volumes in sheep

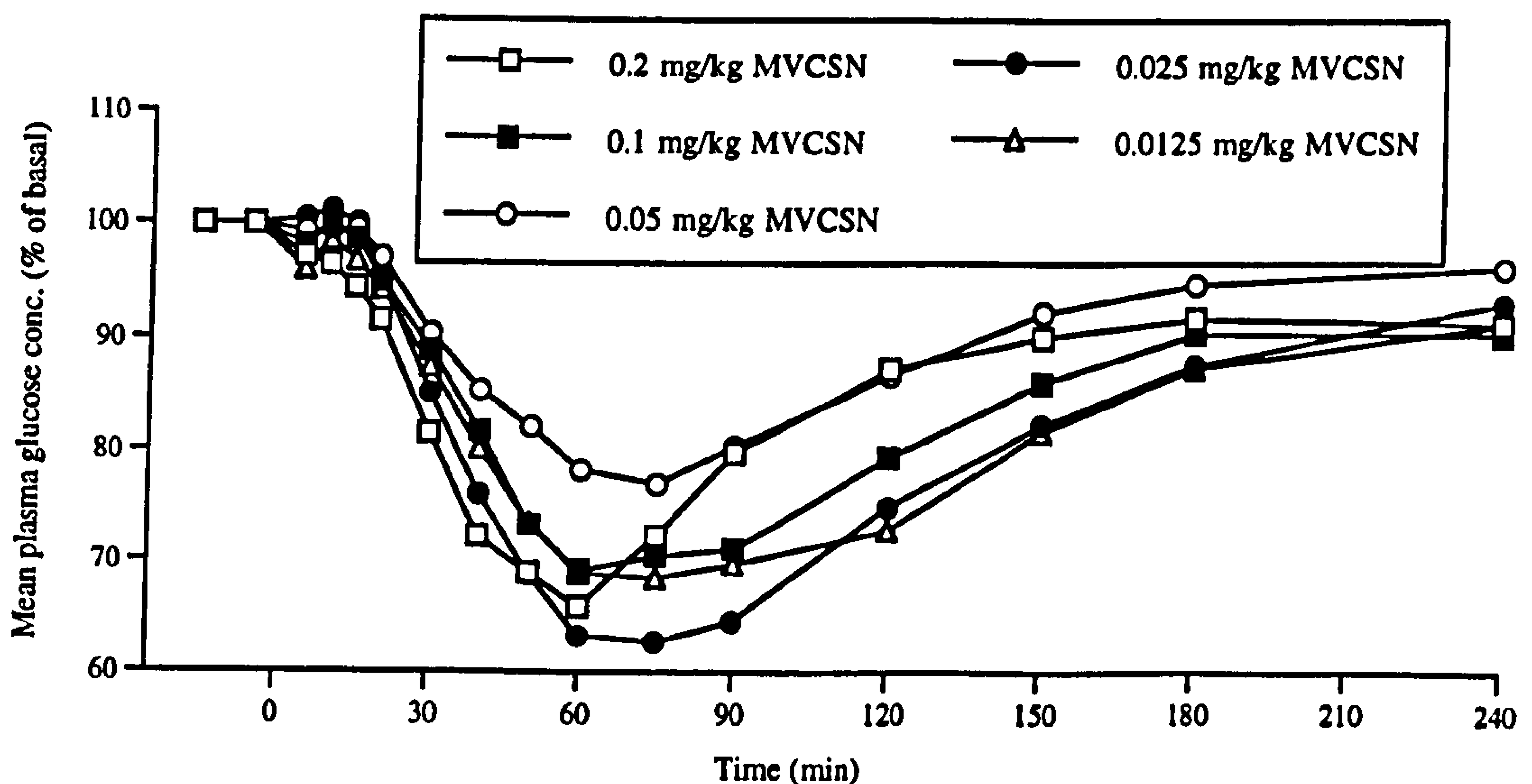
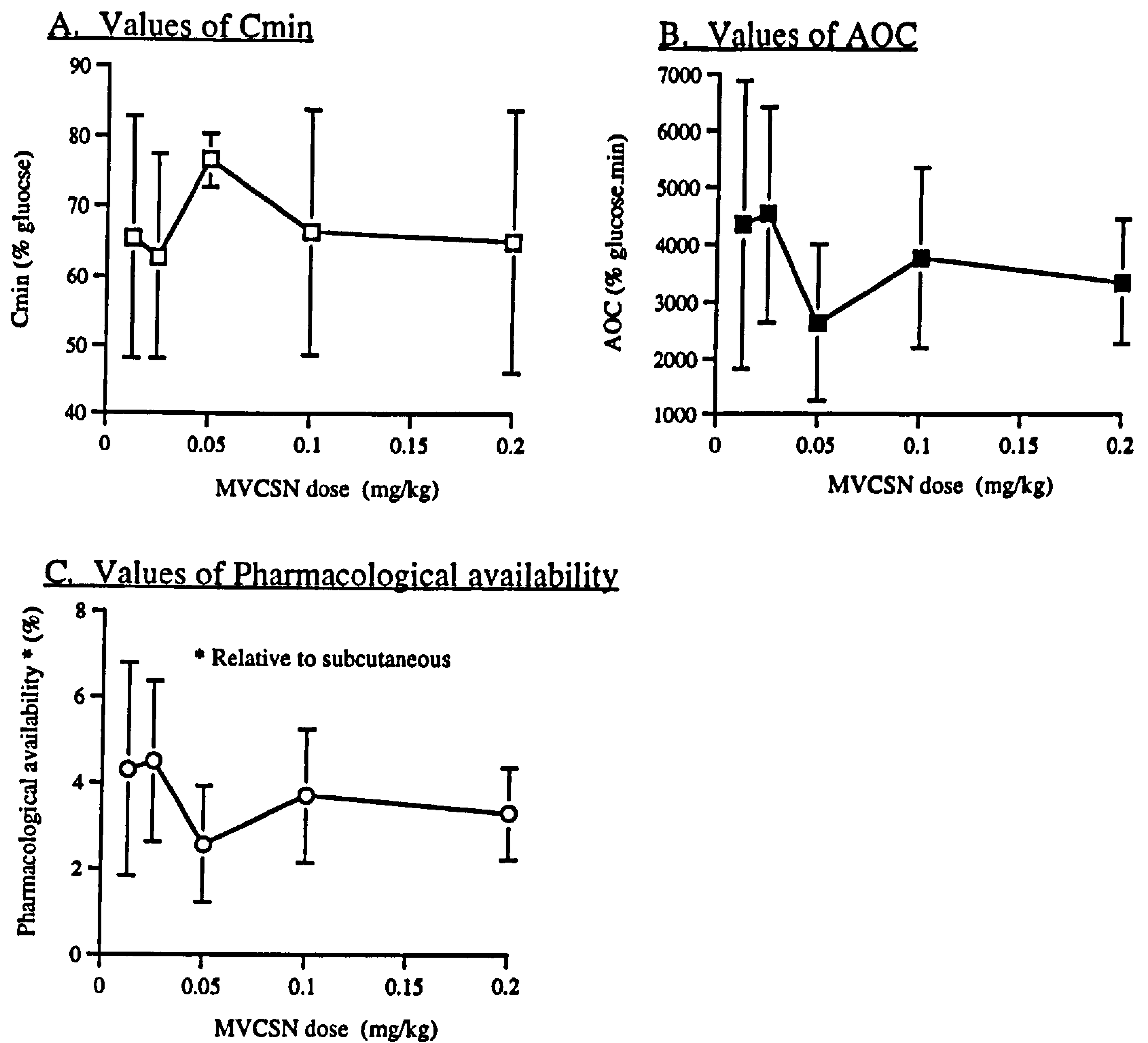


Figure 7.4. Values of pharmacokinetic parameters determined from glucose data following the intranasal administration of 2 IU/kg insulin with different doses of MVCSN and at various dose volumes in sheep



7.4 Effect of insulin dose concentration on the nasal absorption enhancing efficacy of chitosan in sheep

7.4.1 Aims and Objectives

The previous study suggested that the absorption of insulin may be limited by the dose concentration of insulin administered rather than by the absorptive capacity of the nasal cavity. Thus, in this study the effect of varying the dose concentration of insulin on nasal absorption from an insulin / MVCSN formulation was assessed. The concentration of insulin in the formulation was varied between 50-400 IU/ml whereas the concentration of chitosan was fixed at 0.5%. Thus, by administering fixed volumes (0.01 ml/kg) of each solution formulation, doses of insulin between 0.5-4.0 IU/kg were administered.

7.4.2 Materials and Methods

7.4.2.1 Materials

Materials used were as given in Section 7.2.

7.4.2.2 Preparation of insulin formulations

Four solution formulations were prepared for nasal administration to sheep as given in Table 7.11. Each formulation contained an appropriate concentration of insulin to administer the insulin doses specified in Table 7.12 (dose volume 0.01 ml/kg). All formulations were prepared in 14.65 mM phosphate buffer of pH 7.4 and were adjusted to pH 4 during preparation by the addition of 1 M HCl. Each formulation was freshly prepared on the day of the study.

Table 7.11. Outline composition of formulations

Formulation No.	Outline composition
1	50 IU/ml insulin with 0.5% (5.0 mg/ml) MVCSN, pH 4
2	100 IU/ml insulin with 0.5% (5.0 mg/ml) MVCSN, pH 4
3	200 IU/ml insulin with 0.5% (5.0 mg/ml) MVCSN, pH 4
4	400 IU/ml insulin with 0.5% (5.0 mg/ml) MVCSN, pH 4

* Concentration of MCSN expressed as concentration of chitosan glutamate

7.4.2.3 Absorption study in the sheep model

Sheep were dosed nasally with the appropriate insulin formulation. A summary of the dose groups is given in Table 7.12. Nasal doses of 0.5-4.0 IU/kg insulin were administered at a dose volume of 0.01 ml/kg. Each nasal dose was divided between both nostrils.

7.4.3 Results & Discussion

The results showed that increasing the nasal dose of insulin increased both the reduction of plasma glucose concentrations and the elevation of plasma insulin concentrations (Tables 7.13-7.14, Figure 7.5). Values of C_{min} and AOC obtained from the glucose data and C_{max} and AUC obtained from the insulin data displayed a curvilinear relationship with increase in insulin concentration (Figure 7.6). Values of C_{min} were approximately 88,70, 58 and 42 % glucose for doses of 0.5, 1.0, 2.0 and 4.0 IU/kg insulin, respectively. Corresponding values of AOC were 1087, 3814, 5211 and 6915 % glucose.min, respectively. Values of C_{max} after dosing 0.5, 1.0, 2.0 and 4.0 IU/kg insulin were approximately 184, 166, 395 and 951 mU/l, respectively with values of AUC of 6296, 7228, 9926 and 33415 mU/l.min, respectively.

Increase in the dose of insulin from 0.5-2.0 IU/kg had little affect on rates of reduction of glucose concentrations (Table 7.15) or rates of increase of insulin concentrations (Table 7.16) although rates were increased following the administration of 4.0 IU/kg insulin. Considering interanimal variations, values of pharmacological availability were

approximately of the same order of magnitude for each dose group (3.4-7.5 %) (Table 7.15). The slightly lower value (3.4%) after administering a higher nasal dose of insulin (4 IU/kg) could be due to saturation of the mechanisms which lower blood glucose concentrations during glucose homeostasis. In sheep glucose is not the main substrate of tissue metabolism and insulin secreting cells account for only about 12% of the pancreatic islet cells (Hecker, 1983) compared to about 75% of islet cells in man (Espinal, 1989). Thus, glucose homeostasis in the sheep is probably not as important as that in monogastric animals. Values of bioavailability for the dose groups ranged between approximately 11-27% (Table 7.16). Considering that in previous studies the values of pharmacological availability have provided a reasonable estimate of values of bioavailability, the values of bioavailability obtained in this study would appear to be anomalously high. However, no source of error in the analysis or in the processing of the data has been found although there were initial problems with the insulin radioimmunoassay. Thus, in this study, comparison of the performance of the formulations using values of bioavailability should be made with caution.

There were statistically significant differences in values of C_{min}, AOC and rates of reduction of glucose concentrations between the formulation groups although there were no significant differences between values of T_{min} and pharmacological availability (Tables 7.15). Also, there were significant differences in values of C_{max}, AUC and relative bioavailability but not in values of T_{max} and rates of increase in insulin concentrations (Table 7.16). Differences in values of C_{min}, AOC, C_{max} and AUC are not unexpected considering the different doses of insulin administered in the study.

Increase in the dose of insulin coadministered with MVCSN at a fixed dose volume will increase the concentration gradient of the drug across the nasal mucosa. This will increase the passsive absorption of insulin across the nasal membrane. The results indicated that the absorptive capacity of the nasal membrane was not saturated by an increase in the dose of insulin administered. The dose of insulin and/or its release appeared to be the factor limiting the overall absorption of insulin from the formulation.

Table 7.12. Summary of formulations and dose groups in the study to investigate the effect of the dose of insulin on the intranasal absorption of insulin in sheep

Formulation or Group No.	Insulin (IU/kg)	MVCSN (mg/kg)	Dose volume (ml/kg)
1. 50 IU/ml Insulin + 0.5%MVCSN	0.5	0.05	0.01
2. 100 IU/ml Insulin + 0.5% MVCSN	1.0	0.05	0.01
3. 200 IU/ml Insulin + 0.5% MVCSN	2.0	0.05	0.01
4. 400 IU/ml Insulin + 0.5% MVCSN	4.0	0.05	0.01

* Dose of MVCSN expressed as dose of chitosan glutamate

Table 7.13. Summary of plasma glucose concentrations following the intranasal administration of different doses of insulin with fixed doses of MVCSN and fixed dose volumes in sheep

	Mean (± SD) glucose concentration (% of basal)							
Time before or after dosing (min)	0.5 IU/kg insulin	SD	1.0 IU/kg insulin	SD	2.0 IU/kg insulin	SD	4.0 IU/kg insulin	SD
-15	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
-5	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
5	99.3	1.82	96.2	2.00	100.0	1.97	97.0	1.63
10	102.2	2.59	97.0	3.56	100.0	1.15	97.1	3.82
15	101.0	6.90	95.5	5.07	98.0	1.74	94.4	4.28
20	99.0	6.94	90.1	7.83	93.8	2.96	89.2	4.91
30	95.5	6.88	83.9	10.25	86.9	2.05	74.5	6.19
40	91.3	6.85	79.0	10.30	80.8	1.83	63.9	6.46
50	92.0	7.35	74.8	8.81	74.6	2.41	55.2	5.34
60	91.8	7.39	74.7	8.50	69.8	4.80	46.6	5.14
75	91.6	6.08	77.3	10.43	65.7	12.96	41.9	5.17
90	93.6	5.53	76.5	13.77	64.7	19.24	42.5	4.17
120	96.7	3.25	79.9	14.10	69.3	25.99	49.4	6.62
150	95.3	2.49	82.9	8.23	74.0	24.73	69.4	10.14
180	95.2	2.71	86.5	2.98	79.5	20.45	87.8	4.33
240	98.0	4.23	90.7	2.96	88.8	5.91	99.0	4.74

Table 7.14. Summary of plasma insulin concentrations following the intranasal administration of different doses of insulin with fixed doses of MVCSN and fixed dose volumes in sheep

	Mean (± SD) insulin concentration (% of basal)							
Time before or after dosing (min)	0.5 IU/kg insulin	SD	1.0 IU/kg insulin	SD	2.0 IU/kg insulin	SD	4.0 IU/kg insulin	SD
-15	13.0	2.51	11.0	3.06	9.5	2.74	10.2	2.04
-5	14.1	5.75	10.0	3.28	9.9	0.89	10.0	2.29
5	111.6	78.61	67.2	32.57	62.6	46.45	158.9	40.15
10	74.5	68.44	112.2	62.88	101.8	40.59	269.5	187.43
15	71.8	75.17	100.9	65.61	241.6	174.41	649.8	307.55
20	54.2	38.28	96.8	81.17	192.9	137.96	755.0	165.96
30	92.8	60.96	87.6	31.27	256.2	195.69	848.5	324.88
40	99.6	66.29	93.8	79.62	93.1	93.70	415.7	201.50
50	52.4	31.80	95.1	62.41	98.0	71.90	328.9	201.29
60	43.3	21.27	62.8	88.01	49.5	27.86	204.9	97.60
75	33.7	4.86	47.5	37.70	43.6	9.07	98.2	40.30
90	65.7	9.78	30.0	11.97	28.5	16.39	63.1	35.84
120	25.6	13.28	17.1	8.28	16.9	9.18	29.0	21.22
150	22.0	7.86	20.2	7.10	22.8	6.84	25.1	9.22
180	20.1	5.66	24.6	8.84	17.4	5.71	28.4	13.03
240	17.2	3.27	13.0	2.17	12.8	2.97	15.8	4.02

Table 7.15. Summary of pharmacokinetic paramenters determined from plasma glucose data following the intranasal administration of different doses of insulin with fixed doses of MVCSN and fixed dose volumes in sheep

		Mean ± SD (n=4)				
Formulation or Group number	Insulin (IU/kg)	Cmin (% glucose)	Tmin (min)	AOC (% glucose. min)	Rate of decrease of glucose (%/min)	Pharmacological availability * (%)
1	0.5	88.4 (± 5.02)	88 (± 63.9)	1087 (± 689.5)	0.43 (± 0.079)	4.3 (± 2.71)
2	1.0	69 8 (± 10.96)	67 (± 22.7)	3814 (± 1381.4)	0.63 (± 0.208)	7.5 (± 2.72)
3	2.0	57.8 (± 18.37)	85 (± 27.4)	5211 (± 3282.2)	0.62 (± 0.084)	5.1 (± 3.23)
4	4.0	41.5 (± 4.72)	81 (± 7.2)	6915 (± 1028.8)	0.90 (± 0.102)	3.4 (± 0.51)

* Relative to subcutaneous

Statistical Analysis

One-way ANOVA

Sig. dif. in values of Cmin (P<0.001), AOC and rate of decrease of glucose concentration (P<0.01).
No sig. dif. in values of Tmin & pharmacological availability (P>0.05).

Tukey-Kramer Multiple Comparison test following one-way ANOVA

Cmin: Sig. dif. between groups 1vs4 (P<0.001) & 1vs3, 2vs4 (P<0.05).
AOC: Sig. dif. between groups 1vs4 (P<0.01) & 1vs3 (P<0.05).
Rate of decrease of glucose conc.: Sig. dif. between groups 1vs4 (P<0.01) and 3vs4 (P<0.05).
No sig. dif. (P>0.05) between other formulation groups.

Table 7.16. Summary of pharmacokinetic paramenters determined from plasma insulin data following the intranasal administration of different doses of insulin with fixed doses of MVCSN and fixed dose volumes in sheep

		Mean ± SD (n=4)				
Formulation or Group number	Insulin (IU/kg)	Cmax (mU/l)	Tmax (min)	AUC (mU/l. min)	Rate of increase of insulin ([log mU/l]/min)	Bioavailability * (%)
1	0.5	184.0 (± 17.65)	15 (± 10.8)	6296 (± 867.4)	0.15 (± 0.104)	27.4 (± 3.77)
2	1.0	166 (± 41.9)	24 (± 18.0)	7228 (± 4178.8)	0.14 (± 0.067)	15.7 (± 9.08)
3	2.0	394.5 (± 107.73)	23 (± 8.7)	9926 (± 4309 4)	0.12 (± 0 069)	10.8 (± 4 68)
4	4.0	951.1 (± 254.84)	24 (± 7.5)	33415 (± 11252.8)	0.24 (± 0.028)	18.2 (± 6.11)

* Relative to subcutaneous

Statistical Analysis

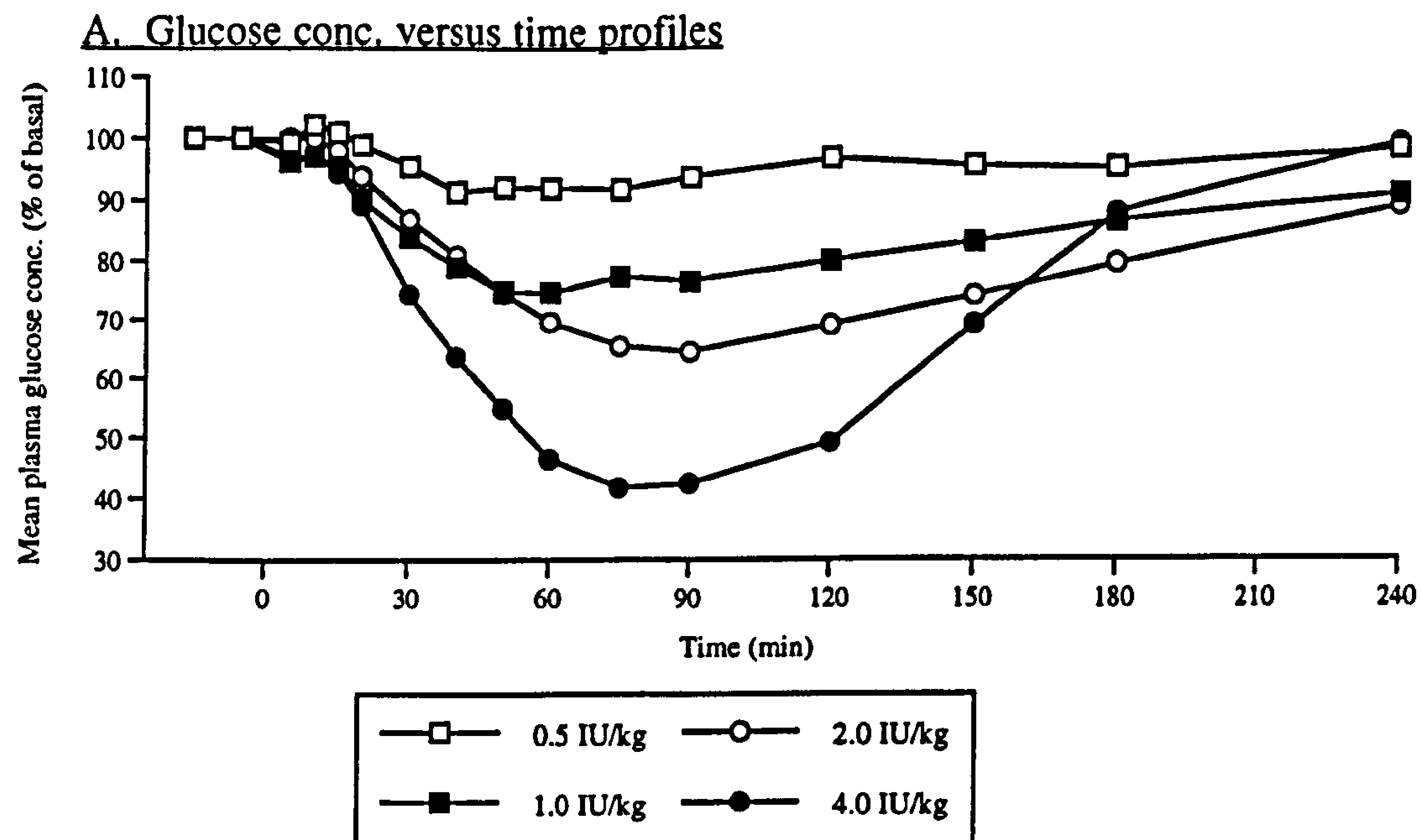
One-way ANOVA

Sig. dif. in values of Cmax, AOC (P<0.001) and Bioavailability (P<0.05).
No sig. dif. in values of Tmax and rate of increase of insulin (P>0.05).

Tukey-Kramer Multiple Comparison test following one-way ANOVA

Cmax: Sig. dif. between groups 1vs4, 2vs4 & 3vs4 (P<0.001)
AUC: Sig. dif. between groups 1vs4, 2vs4 & 3vs4 (P<0.001)
Bioavailability: Sig. dif. between group 1vs3 (P<0.05).
No sig. dif. (P>0.05) between all other formulation groups

Figure 7.5. Plasma glucose and insulin concentration versus time profiles following the intranasal administration of different doses of insulin with fixed doses of MVCSN and fixed dose volumes in sheep



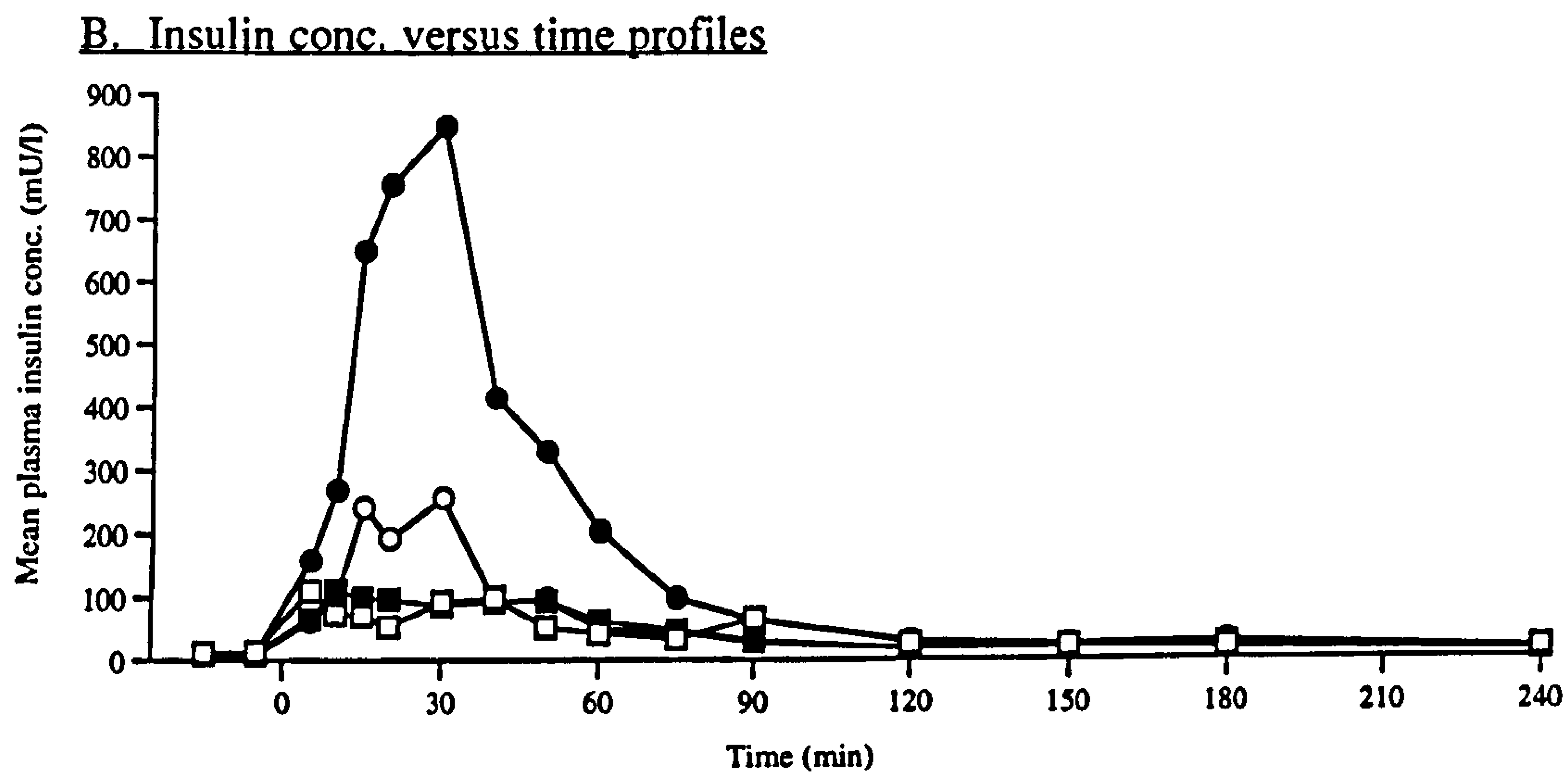
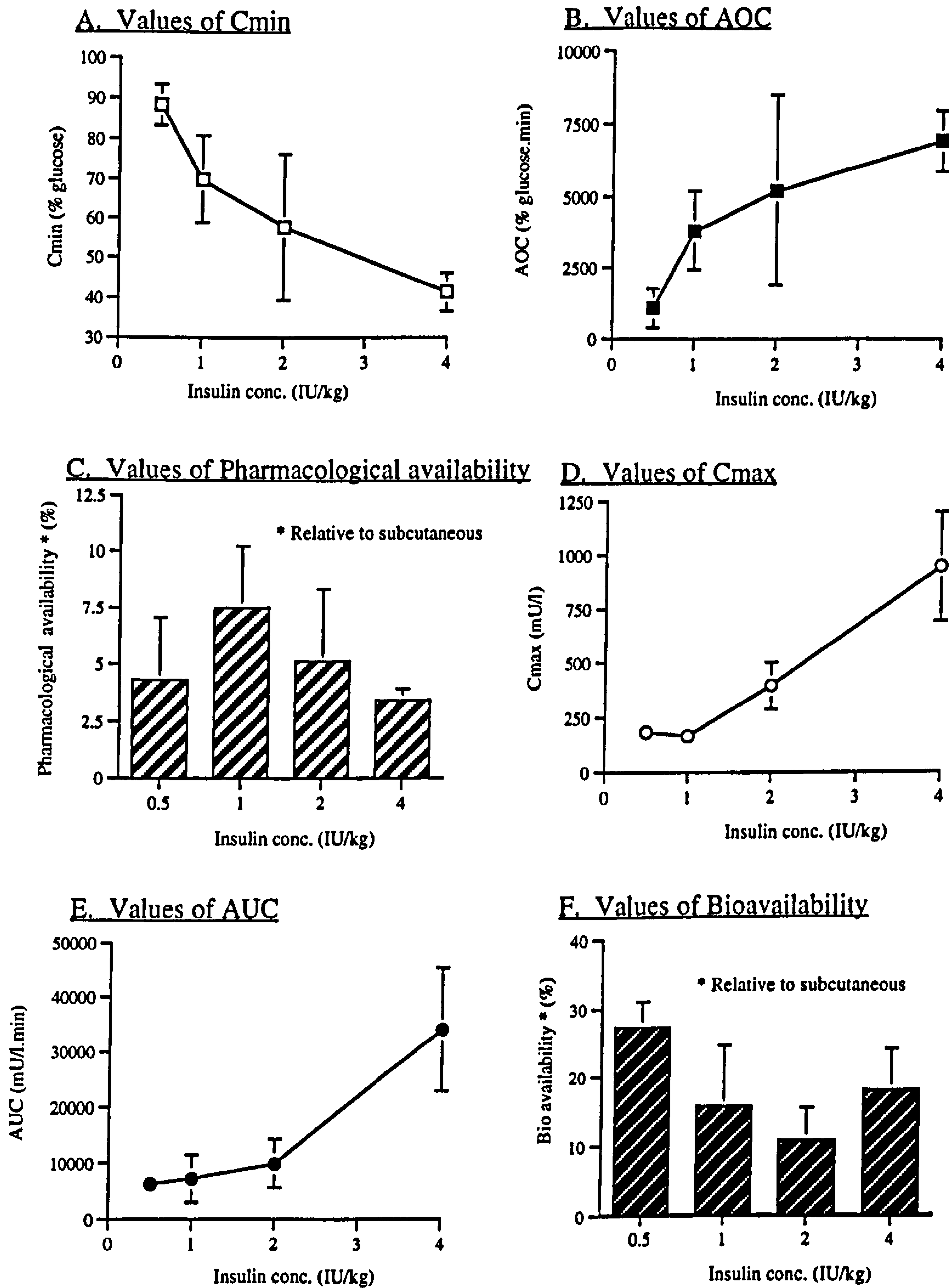


Figure 7.6. Values of pharmacokinetic parameters determined from glucose and insulin data following the intranasal administration of different doses of insulin with fixed doses of MVCSN and fixed dose volumes in sheep



7.5 Effect of administration of different dose volumes of the same formulation on the intranasal absorption of insulin in sheep

7.5.1 Aims and Objectives

In the last of a series of studies to investigate the relationship between formulation concentrations and doses of insulin / chitosan and dose volumes on the efficacy of intranasal insulin absorption, the effect of nasally administering different volumes of a formulation containing fixed concentrations of insulin and MVCSN was investigated. This study also mimics a situation which may occur if an insulin / chitosan formulation is used therapeutically to treat diabetes. Poor patient compliance may inevitably lead to under or overdose of the formulation and hence the likely affects of this should be anticipated.

7.5.2 Materials and Methods

7.5.2.1 Materials

Materials used were as given in Section 7.2.

7.5.2.2 Preparation of insulin formulation

A solution formulation was prepared for nasal administration to sheep containing 200 IU/ml insulin with 0.5% (5.0 mg/ml) MVCSN, expressed as the concentration of chitosan glutamate. The solution was prepared in 14.65 mM phosphate buffer of pH 7.4 and was adjusted to pH 4 during preparation by the addition of HCl. The formulation was freshly prepared on the day of the study.

7.5.2.3 Absorption study in the sheep model

The 200 IU/ml insulin with 0.5% (5.0 mg/ml) MVCSN solution formulation was administered at different dose volumes. By varying the dose volume administered (between 0.0025-0.03 ml/kg) , the insulin and chitosan doses were altered (0.5-6.0 IU/kg and 0.0125-0.015 mg/kg, respectively). A summary of the dose groups is given in Table 7.17. The dose volume was varied between 0.0025-0.03 ml/kg. Each nasal dose was divided between both nostrils.

7.5.3 Results & Discussion

The results showed that the absorption of insulin, in-terms of deceases in plasma glucose concentrations and elevations of plasma insulin concentrations, increased with an increase in the volume administered of the 200 IU/ml insulin / 0.5% MVCSN formulation (Tables 7.18-7.19, Figure 7.8). Thus, insulin absorption increased with increase in the doses of insulin and MVCSN administered. The relationship between dose volume and selected pharmacokinetic parameters is given in Tables 7.20-7.21 and Figure 7.9. Figure 7.9 shows a curvilinear relationship between values of C_{min} and AOC and the approximately linear relationship between values of C_{max} and AUC.

Values of pharmacological availability were shown to decrease slightly at higher dose volumes which may reflect saturation of the mechanisms involved in lowering blood glucose concentrations. This is supported by values of C_{min} and AOC which generally appear to be reaching a plateau towards the higher dose volume. Values of bioavailability show that, taking into account the high interanimal variability at low dose volume, values were similar for each dose group. Rates of reduction of plasma glucose concentrations and rates of increase of plasma insulin concentrations were shown to increase with increase in dose volume followed by a plateau between 0.02-0.03 ml/kg (Tables 7.20-7.21).

As previously reported, the concentration of chitosan in the formulation rather than chitosan dose influenced the absorption of insulin. Thus, in this study the increase in insulin absorption with increase in dose volume is probably due to the increase in the dose of insulin rather than the increase in chitosan dose. Furthermore, increase in dose volume is likely to increase the spread of the formulation in the nasal cavity and hence the absorptive surface area.

Table 7.17. Summary of formulations and dose groups in the study to investigate the effect of administering different dose volumes of the same formulation on the intranasal absorption of insulin in sheep

Formulation or Group No.	Insulin (IU/kg)	MVCSN (mg/kg)	Dose volume (ml/kg)
1	0.5	0.0125	0.0025
2	1.0	0.025	0.005
3	2.0	0.05	0.01
4	4.0	0.01	0.02
5	6.0	0.015	0.03

* Dose of MVCSN expressed as dose of chitosan glutamate

Table 7.18. Summary of plasma glucose concentrations following the intranasal administration of different dose volumes of the same formulation in sheep

	Mean (± SD) glucose concentration (% of basal)									
Time before or after dosing (min)	0.0025 ml/kg	SD	0.005 ml/kg	SD	0.01 ml/kg	SD	0.02 ml/kg	SD	0.03 ml/kg	SD
-15	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
-5	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
5	95.3	5.27	98.1	2.64	98.3	2.63	98.7	3.37	98.0	1.73
10	96.7	4.84	98.1	2.64	100.4	4.35	98.6	4.07	98.5	2.06
15	98.2	3.20	99.5	2.99	98.9	4.20	95.9	2.95	94.1	7.17
20	95.3	5.13	98.7	2.56	93.9	3.39	93.0	2.00	88.8	10.23
30	93.9	7.76	91.8	6.61	82.5	4.49	77.0	7.81	73.4	13.87
40	89.5	13.16	85.8	5.78	73.2	7.74	69.3	8.32	64.0	16.65
50	91.0	11.79	83.0	5.93	70.3	9.85	63.8	7.01	61.9	17.82
60	91.7	12.49	78.4	7.08	68.8	12.05	62.5	2.03	58.5	13.51
75	93.2	4.01	77.8	9.91	70.3	14.79	63.9	1.62	60.0	13.96
90	96.0	5.91	83.1	10.08	75.3	16.06	73.1	9.10	69.9	18.63
120	96.1	4.34	89.3	9.20	86.8	8.05	84.2	10.57	76.1	15.81
150	98.3	2.16	91.4	6.81	93.3	8.06	91.1	6.94	88.6	7.39
180	98.9	2.19	95.4	4.00	97.6	8.82	98.0	9.57	97.5	3.10
240	99.6	2.49	99.5	3.77	103.3	7.43	106.3	8.45	106.4	3.71

Table 7.19. Summary of plasma insulin concentrations following the intranasal administration of different dose volumes of the same formulation in sheep

	Mean (± SD) insulin concentration (mU/l)									
Time before or after dosing (min)	0.0025 ml/kg		0.005 ml/kg		0.01 ml/kg		0.02 ml/kg		0.03 ml/kg	
		SD		SD		SD		SD		SD
-15	18.2	6.54	13.8	3.93	12.6	4.29	10.2	2.34	15.6	5.98
-5	20.6	5.18	15.4	2.63	11.8	2.31	16.8	4.76	22.0	6.34
5	36.0	9.77	22.3	3.58	36.8	16.75	55.5	29.84	88.2	15.95
10	56.6	32.98	46.2	9.60	62.8	19.51	141.8	102.91	202.8	97.45
15	51.0	21.70	56.2	21.83	70.0	16.54	134.1	76.68	167.6	87.69
20	59.1	47.45	50.3	12.64	81.0	26.53	129.6	64.06	183.7	109.14
30	37.4	29.28	32.8	8.67	62.3	41.35	126.9	62.16	164.0	114.80
40	39.1	16.59	31.8	8.60	79.8	81.01	98.9	51.51	157.8	119.58
50	20.1	2.96	26.8	10.29	43.9	20.28	62.8	25.82	100.3	63.87
60	33.4	16.67	26.0	11.93	33.1	8.20	54.0	13.31	76.3	53.10
75	18.0	1.74	17.1	5.13	23.7	7.52	24.3	7.61	41.2	23.23
90	24.0	7.56	14.2	2.63	16.5	1.91	18.5	5.39	32.3	11.69
120	21.9	1.36	16.8	3.67	19.3	12.38	21.2	0.80	23.4	3.58
150	16.1	6.31	11.4	3.03	13.7	1.99	15.8	3.64	17.0	4.20
180	14.9	4.47	15.4	4.73	11.2	1.76	16.8	3.33	18.7	3.59
240	16.0	8.98	15.4	2.11	14.8	2.18	12.0	2.48	17.1	5.42

Table 7.20. Summary of pharmacokinetic paramenters determined from plasma glucose data following the intranasal administration of different dose volumes of the same formulation in sheep

		Mean (± SD)				
Formulation or Group number	Dose vol. (ml/kg)	Cmin (% glucose)	Tmin (min)	AOC (% glucose. min)	Rate of decrease of gluocse (%/min)	Pharmacological availability *
1	0.0025	88.1 (± 11.83)	58 (± 20.2)	887 (± 933.0)	0.32 (± 0.389)	3.5 (± 3.67)
2	0.005	75.6 (± 8.32)	69 (± 11.3)	2197 (± 1141.4)	0.48 (± 0.208)	4.3 (± 2.24)
3	0.01	65.4 (± 10.32)	59 (± 12.5)	2964 (± 1435.6)	0.84 (± 0.245)	2.9 (± 1.41)
4	0.02	60.4 (± 4.07)	62 (± 10.3)	3453 (± 1028.0)	1.01 (± 0.548)	1.7 (± 0.51)
5	0.03	55.6 (± 14.05)	58 (± 11.9)	4012 (± 1957.5)	1.11 (± 0.295)	1.3 (± 0.64)

* Relative to subcutaneous

Statistical Analysis

One-way ANOVA

Sig. dif. in values of Cmin ($P<0.01$) and AOC ($P<0.05$).

No sig. dif. in values of Tmin, rate of decrease of glucose conc. & pharmacological availability ($P>0.05$).

Tukey-Kramer Multiple Comparison test following ANOVA

Cmin: Sig. dif. between groups 1vs5 ($P<0.01$) & 1vs3, 1vs4 ($P<0.05$).

AOC: Sig. dif. between group 1vs5 ($P<0.05$).

No sig. dif. ($P>0.05$) between other formulation groups.

Table 7.21. Summary of pharmacokinetic paramenters determined from plasma insulin data following the intranasal administration of different dose volumes of the same formulation in sheep

Formulation or Group number	Dose vol. (ml/kg)	Mean ± SD (n=4) Cmax (% glucose)	Tmax (min)	AUC (mU/l. min)	Rate of increase of insulin ([log mU/l]/min)	Bioavailability * (%)
1	0.0025	69.0 (± 41.12)	24 (± 25.0)	1539 (± 996.9)	0.04 (± 0.015)	6.7 (±4.33)
2	0.005	60.7 (± 18.17)	15 (± 4.1)	1126 (± 399.9)	0.05 (± 0.014)	2.5 (± 0.87)
3	0.01	108.6 (± 64.61)	25 (± 12.9)	3753 (± 2369.0)	0.08 (± 0.034)	4.1 (± 2.57)
4	0.02	178.0 (± 93.50)	25 (± 12.9)	6079 (± 2887.9)	0.11 (± 0.060)	3.3 (± 1.57)
5	0.03	240.0 (± 110.56)	19 (± 14.4)	8652 (± 5261.0)	0.10 (± 0.018)	3.1 (± 1.90)

* Relative to subcutaneous

Statistical Analysis

One-way ANOVA

Sig. dif. in values of Cmax and AUC ($P<0.05$).

No sig. dif. in values of Tmax, rate of increase of insulin conc. & Bioavailability ($P>0.05$).

Tukey-Kramer Multiple Comparison test following one-way ANOVA

Cmax: Sig. dif. between groups 1vs5 & 2vs5 ($P<0.05$).

AUC: Sig. dif. between group 1vs5 & 2vs5 ($P<0.05$).

No sig. dif. ($P>0.05$) between other formulation groups.

Fig 7.7. Plasma glucose and insulin concentration versus time profiles following the intranasal administration of different dose volumes of the same formulation in sheep

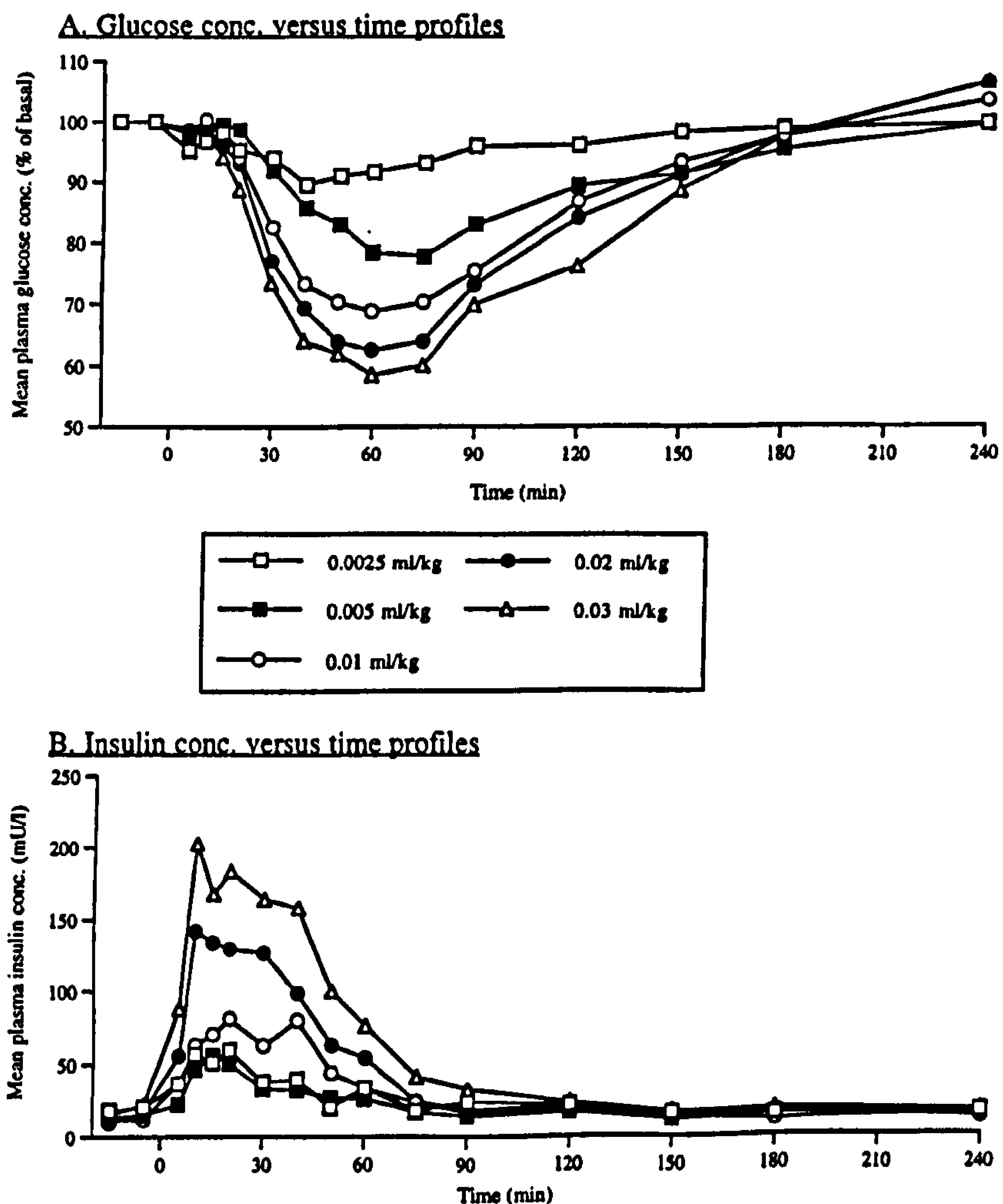
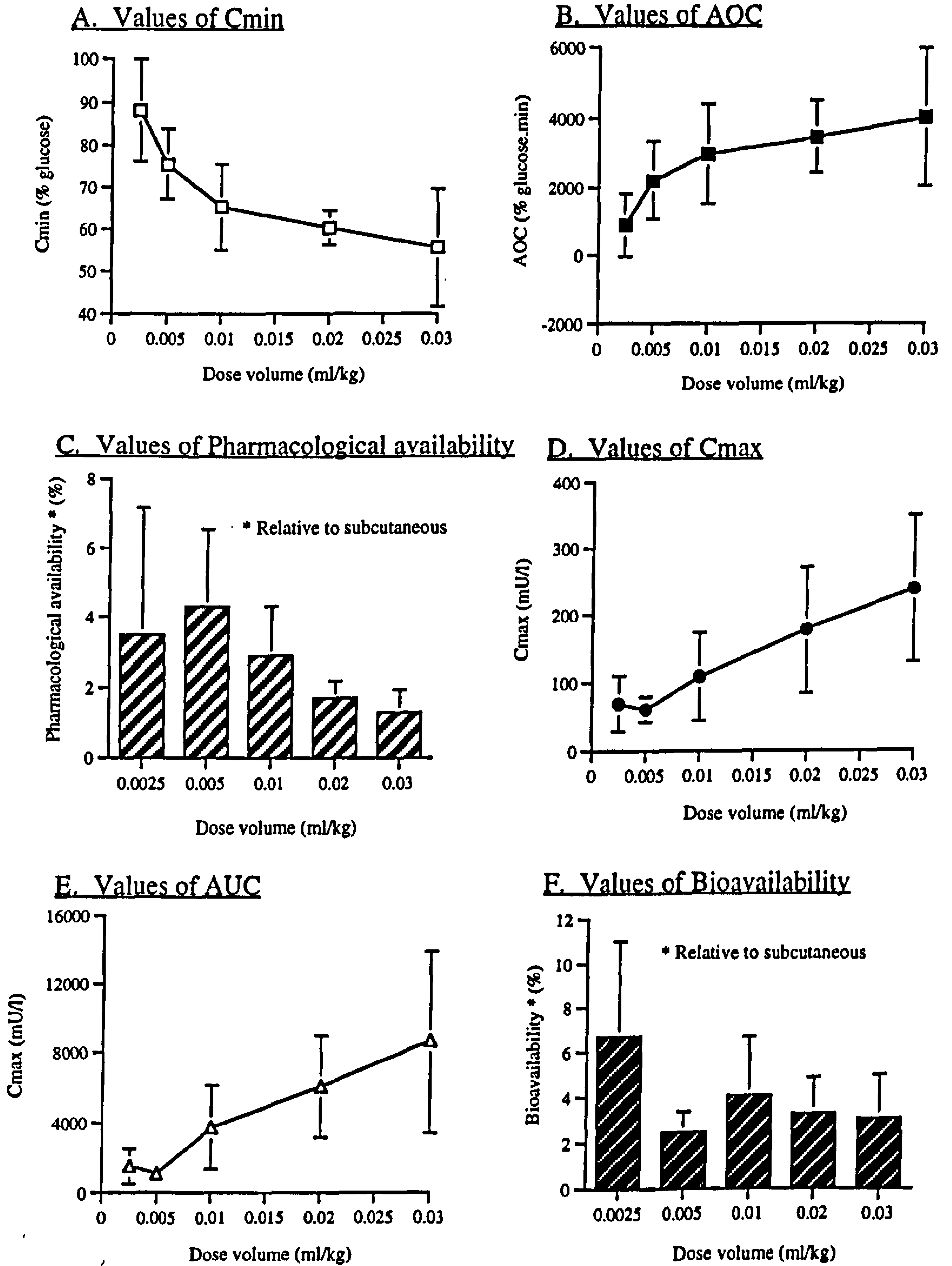


Figure 7.8. Values of selected pharmacokinetic parameters following the intranasal administration of different dose volumes of the same formulation in sheep



7.6 Effect of different vehicles on the absorption enhancing efficacy of chitosan

7.6.1 Aims and Objectives

Previous studies in the rat showed that a hypertonic formulation of insulin / chitosan was shown to increase the nasal absorption of insulin relative to that obtained in hypotonic and isotonic media. There were no apparent differences in the performance of the formulation in hypotonic or isotonic media. In the previous studies performed in sheep, formulations were prepared in a hypotonic phosphate buffer. Thus, the aim of this study was to investigate the effect of hypotonic or isotonic vehicles on the absorption enhancing efficacy of MVCSN. A hypotonic solution prepared in 14.65 mM phosphate buffer was compared to a isotonic solution prepared in 0.85% sodium chloride (NaCl). As a control, an insulin solution without MVCSN was prepared in 14.65 mM phosphate buffer. All three formulations were prepared at pH 4.0.

7.6.2 Materials and Methods

7.6.2.1 Materials

The materials used were as given in Section 7.2.

7.6.2.2 Preparation of insulin formulation

Three solution formulation, each containing 200 IU/ml insulin, were prepared for nasal administration to sheep. A summary of the formulations prepared is given in Table 7.21. Formulations were prepared in either 14.65 mM phosphate buffer of pH 7.4 or 0.9% NaCl and each formulation was adjusted to pH 4 during preparation by the addition of 1 M HCl. The formulations were freshly prepared on the day of the study.

Table 7.22. Outline composition of the formulations

Formulation No.	Outline composition	Osmolality (Osmol/kg)
1	200 IU/ml insulin in phosphate buffer, pH 4 (control solution)	0.091
2	200 IU/ml insulin with 0.5% (5.0 mg/ml) MVCSN in phosphate buffer, pH 4	0.110
3	200 IU/ml insulin with 0.5% (5.0 mg/ml) MVCSN in 0.85% NaCl, pH 4	0.310

* Concentration of MVCSN expressed as concentration of chitosan glutamate

7.6.2.3 Absorption study in the sheep model

Sheep were dosed with 2 IU/ml insulin from the appropriate insulin formulation at 0.01 ml/kg. A summary of the dose groups is given in Table 7.22. Each nasal dose was divided between both nostrils. In view of the glucose results, plasma insulin concentrations were not measured.

7.6.3 Results & Discussion

As expected from previous results, the nasal absorption of insulin was poor without coadministration with MVCSN (Tables 7.22-7.23, Figure 7.10). The pharmacological availability, relative to subcutaneous, for the control solution at pH 4.0 was about 0.7% compared to about 2.9% for the formulations containing MVCSN. There was no significant difference in the absorption enhancing efficacy of MVCSN in hypotonic or isotonic vehicles. The plasma glucose versus time profiles (Figure 7.10) and values of the pharmacokinetic parameters (Table 7.23) were similar for the two formulations. Thus, the two vehicles used did not appear to influence nasal insulin absorption.

The values of relative pharmacological availability obtained for the control solution of insulin at pH 4 (0.7% ± 0.87) were slightly lower than those reported in Chapter 6 for the control solution at pH 7.4 (1.4% ± 1.02). There were also differences between values of C_{min} and T_{min} obtained for the control formulation. However, these differences were not significant. It must be taken into account that these studies were performed in different sheep and on different occasions where there may have been differences in experimental conditions. Also, subcutaneous dose administration was only performed on one occasion. Furthermore, measurement of a physiological parameter such as plasma glucose although giving a good indication of absorption is not as precise as measurement of plasma insulin concentrations. Intranasal insulin absorption has been shown to be pH dependent (Hirai et al., 1978). In a study in anaesthetised dogs, a high nasal dose of insulin (50 IU/kg) was shown to be poorly absorbed at a pH of about 6 which is close to the isoelectric point of insulin. Insulin absorption was improved slightly in the pH range approximately 6 to 7.4 although dramatic increases in absorption were observed in the pH range pH 6 to 3.1 with maximum absorption occurring at the latter pH. The reduced absorption at the isoelectric point of insulin was attributed to the low solubility of insulin at this pH where it forms a suspension and the formation of isomers, particularly hexomers and octomers, compared to the predominance of monomers at acid pH. In the sheep studies reported in this project, insulin absorption was not shown to be pH dependent which may be attributed to differences in the two species and the much lower dose of insulin administered in the sheep studies. Furthermore, in contrast to the conscious sheep model used in this project, Hirai et al. used anaesthetised dogs in which mucociliary clearance mechanisms may be impaired.

Although this study showed that the same degree of insulin absorption was obtained from both hypotonic and isotonic vehicles, for therapeutic application, particularly for chronic use, isotonic formulations are desirable to prevent or limit nasal irritation. Illum et al (results not published), investigated the nasal irritancy of buffers prepared at pH 4 in two human volunteers finding that the nature of the bufffer, its buffering capacity and tonicity influenced the degree of nasal irritation. Generally, isotonic solutions tended to be less irritant than hypotonic solutions. The nature of the excipient used to adjust the tonicity of the formulation was also shown to influence the degree of- nasal irritancy. The pH of the buffer will also influence the degree of irritation to mucous membranes (Walz,1985). Walz reported that the irritancy of isotonic solutions on mucous membranes was minimal

in the pH range approximately 4-11.

Table 7.23. Summary of formulations and dose groups in the study to investigate the effect of two different vehicles on the intranasal absorption of insulin from a formulation containing MVCSN in sheep

Formulation or Group No.	Insulin (IU/kg)	MVCSN (mg/kg)	Dose volume (ml/kg)
1. Control insulin solution, pH 4	2.0	-	0.01
2. Insulin / MVCSN in phosphate buffer, pH 4	2.0	0.05	0.01
3. Insulin / MVCSN in 0.85% NaCl, pH 4	2.0	0.05	0.01

* Dose of MVCSN expressed as the dose of chitosan glutamate

Table 7.24. Summary of plasma glucose concentrations following the intranasal administration of an insulin / MVCSN formulation in two different vehicles in sheep.

	Mean (± SD) glucose concentration (% of basal)					
Time before or after dosing (min)	Insulin control pH 4 phosphate buffer		Insulin + 0.5% MVCSN pH4 in phos. buffer		Insulin + 0.5% MVCSN pH 4 in 0.85% NaCl	
		SD		SD		SD
-15	100.0	0.00	100.0	0.00	100.0	0.00
-5	100.0	0.00	100.0	0.00	100.0	0.00
5	99.2	2.87	100.5	4.45	101.2	3.20
10	99.9	4.79	96.8	10.60	100.4	1.49
15	100.0	6.86	95.8	7.82	97.3	3.41
20	101.7	9.84	91.0	11.57	93.5	4.94
30	99.2	9.83	81.3	13.19	83.4	8.10
40	96.7	8.80	76.5	12.82	76.5	10.06
50	97.5	10.33	71.5	10.85	74.1	11.33
60	98.3	6.88	76.4	7.72	74.9	14.92
75	99.2	6.18	84.4	5.53	78.7	16.93
90	99.9	9.17	86.7	2.92	85.6	12.77
120	98.2	7.38	93.9	0.56	93.4	3.99
150	98.3	7.25	94.6	2.76	98.1	4.06
180	101.7	4.93	99.7	1.99	98.9	3.16
240	101.7	6.90	99.8	3.96	102.0	5.47

Table 7.25. Summary of pharmacokinetic paramenters determined from plasma glucose data following the intranasal administration of an insulin / MVCSN formulation in two different vehicles in sheep.

	Mean ± SD (n=4)				
Formulation or Group number	Cmin (% glucose)	Tmin (min)	AOC (% glucose. min)	Rate of decrease of gluocse (%/min)	Pharmacological availability * (%)
1. Insulin control pH 4 in phosphate buffer	94.0 (± 8.03)	49 (± 35.2)	678 (± 797.5)	-	0.7 (± 0.87)
2. Insulin + 0.5% MVCSN in phosphate buffer	70.7 (± 9.67)	54 (± 8.8)	2638 (± 1574.4)	0.72 (± 0.128)	2.9 (± 1.71)
3. Insulin + 0.5% MVCSN in 0.85% NaCl	71.0 (± 11.58)	56 (± 11.6)	2667 (± 1392.7)	0.73 (± 0.247)	2.9 (± 1.51)

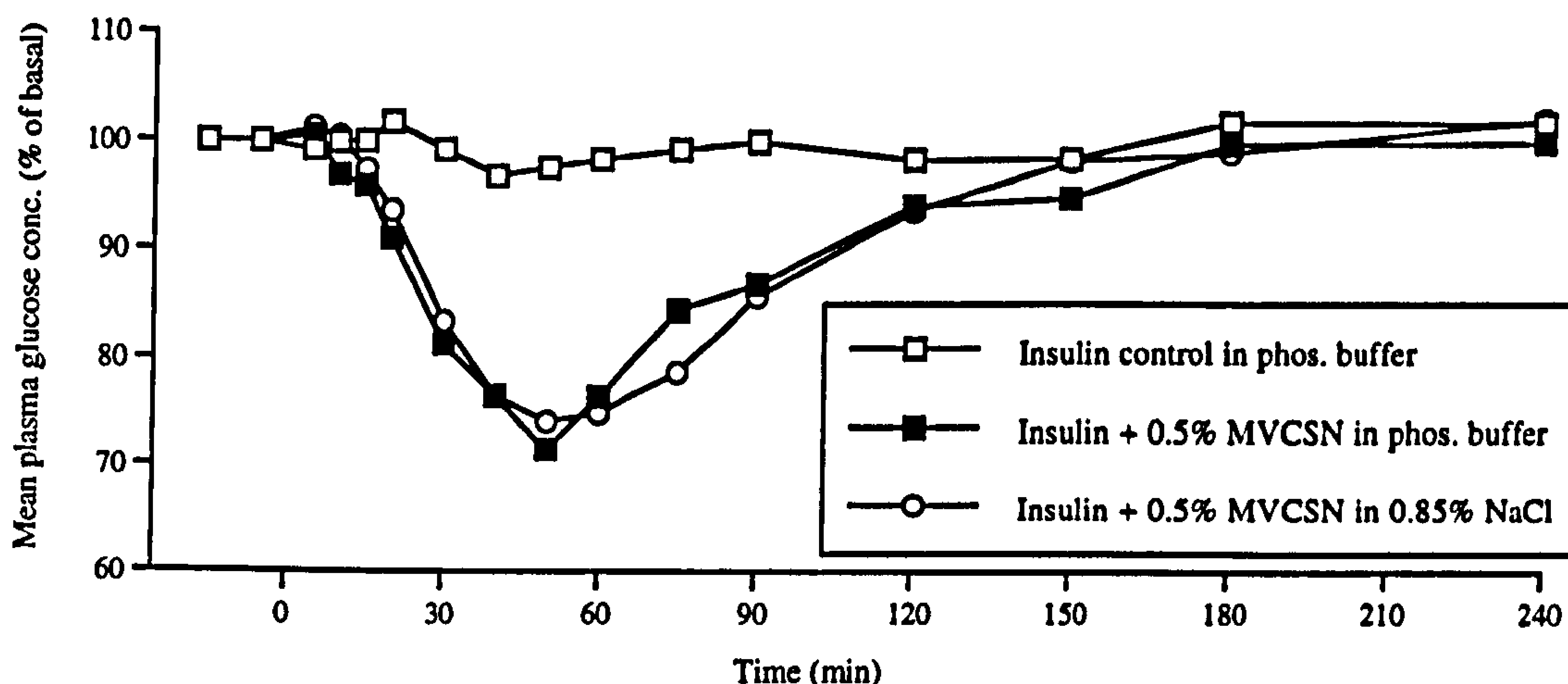
* Relative to subcutaneous

Statistical Analysis

One-way ANOVA
Sig. dif. in values of Cmin (P<0.05). No sig. dif. in values of Tmin and AOC (P>0.05)

Tukey -Kramer Multiple Comparisons Test following ANOVA
Cmin: Sig. dif. between groups 1vs2 and 1vs3 (P<0.05). No sig. dif. between groups 2vs3 (P>0.05).

Fig 7.9. Plasma glucose concentration versus time profiles following the intranasal administration of an insulin / MVCSN formulation in two different vehicles in sheep.



7.7 Effect of grade and molecular weight of chitosan on the intranasal absorption of insulin in sheep

7.7 Aims and Objectives

This project has predominantly investigated the absorption enhancing efficacy of a medium viscosity grade of chitosan glutamate (MVCSN). MVCSN was shown in a previous sheep study to perform better than a low viscosity grade in terms of enhancing nasal insulin absorption. However, there are many grades of chitosan available commercially and the characteristics of the chitosan utilised in the formulation, such as the degree of acetylation, molecular weight (related to the viscosity grade) and salt type, may influence insulin absorption via the nasal route. Thus, the chitosan employed in the nasal delivery system should be optimised. In this study, the effect of chitosan grade and salt type in promoting nasal insulin absorption was investigated. Each grade of chitosan used had a similar degree of deacetylation. The performance of a medium viscosity grade of chitosan hydrochloride (CSN HCL) and a low viscosity grade of chitosan lactate (CSN lacate) were compared to that of MVCSN.

7.7.2 Materials and Methods

7.7.2.1 Materials

The characteristics of the chitosans used are given in Table 2.1

7.7.2.2 Preparation of insulin formulation

Solution formulation was prepared for nasal administration to sheep containing 200 IU/ml insulin and either 0.5% (5.0 mg/ml) of MVCSN, CSN HCL or CSN lactate or 1.0% (10.0 mg/ml) CSN lactate. A summary of the formulations prepared is given in

Table 7.26. All formulations were prepared in 14.65 mM phosphate buffer of pH 7.4 and were adjusted to pH 4 during preparation by the addition of 1 M HCl. The formulations were freshly prepared on the day of the study.

Table 7.26. Outline composition of the formulation

Formulation No.	Outline composition	Viscosity * (CPS)
1	200 IU/ml insulin with 0.5% (5.0 mg/ml) MVCSN, pH 4	9.4
2	200 IU/ml insulin with 0.5% (5.0 mg/ml) CSN HCL, pH 4	17.8
3	200 IU/ml insulin with 0.5% (5.0 mg/ml) CSN lacate, pH 4	2.7
4	200 IU/ml insulin with 1.0% (10.0 mg/ml) CSN lactate, pH 4	5.5

* Concentration of each chitosan expressed as the concentration of chitosan salt

* Viscosity measured at 10 rpm / 25°C using a Brookfield DVIII rheometer with CP40 cone and plate (refer to Section 10.5)

7.7.2.3 Absorption study in the sheep model

Sheep were dosed with 2 IU/ml insulin from the appropriate insulin formulation administered at 0.01 ml/kg. A summary of the dose groups is given in Table 7.22. Each nasal dose was divided between both nostrils.

7.7.3 Results & Discussion

The results showed that formulations containing 0.5% or 1.0% CSN lactate were less effective than those containing 0.5% of either MVCSN or CSN HCL in enhancing the intranasal absorption of insulin in sheep (Tables 7.24-7.27, Figures 7.10-7.11). Increase in the concentration of CSN lactate from 0.5% to 1.0% did not improve insulin absorption.

The plasma glucose versus time profiles were similar following the nasal administration of formulations containing 0.5% MVCSN or CSN HCL (Figure 7.10 A) although with the MVCSN formulation the profile was shifted slightly to the right which was also indicated by the slightly lower rate of decrease of glucose concentrations for this formulation (Table 7.30). Despite the similarities in the plasma glucose profiles, there were differences in the plasma insulin versus time profiles for the MVCSN and CSN HCL formulations with higher peak plasma concentrations attained for the MVCSN formulation (Figure 7.10 B). The plasma insulin profiles were similar for the 0.5% and 1.0% CSN lactate formulations although the latter did not perform as well in terms of lowering plasma glucose concentrations (Figure 7.10).

The pharmacokinetic parameters determined from the glucose data (Table 7.30, Figure 7.11 A-B) showed that values of C_{min} and AOC (hence pharmacological availability) were similar for the MVCSN and CSN HCL formulations and values of C_{min} were lower and values of AOC higher than the values obtained for the CSN lactate formulations. The rate of decrease in glucose concentrations was marginally higher for the CSN HCL formulation compared to the MVCSN formulation although values in both these

formulations were higher than those obtained for the CSN lactate formulations (Table 7.30). Pharmacokinetic parameters determined from the insulin data supports the glucose data in highlighting the differences in the performance of the medium viscosity and low viscosity grades of chitosan (Table 7.31, Figure 7.11 C-D). Values of C_{max} and AUC (hence bioavailability) obtained for the MVCSN formulation were higher for those obtained for CSN HCL although the rates of increase in plasma insulin concentrations were similar for the two formulations. For the MVCSN and CSN HCL formulations these values were higher than those obtained for the CSN lactate formulations.

Despite apparent differences in the pharmacokinetic parameters of the formulations, the only statistically significant differences between the formulation groups was in values of C_{min} and T_{min} (Table 7.30) and C_{max} (Tables 7.31) which reflects the considerable inter-animal variations in the results.

The grade of chitosan used in the formulations was shown to influence the degree of absorption enhancing efficacy with the low viscosity grade (lactate salt) being less effective than the medium viscosity grades (glutamate and hydrochloride salts) at the same concentration of chitosan salt. This may be a reflection of the different viscosities of the formulations since it was shown previously that increasing the concentration of MVCSN, thereby increasing solution viscosity improved the degree of nasal absorption enhancing efficacy. However, for the medium viscosity grades, the salt type also appeared to be of importance since the formulation containing the glutamate salt form was generally more effective than the hydrochloride salt despite the latter formulation being more viscous. The hydrochloride salt would be expected to be more viscous than the glutamate salt at the same concentration of chitosan salt since the hydrochloride salt form is composed of 80-90% chitosan compared to only 55-65% chitosan in the glutamate salt. An extensive study would be required to establish the relationship between salt type and viscosity or molecular weight. Evaluation of the performance of different molecular weight / viscosity fractions prepared from the same chitosan salt would be useful to establish the relationship between viscosity and absorption enhancing efficacy.

Aspden (1996) showed that a medium viscosity glutamate salt of chitosan (42% salt) performed better, in terms of lowering plasma glucose concentrations, than low, medium and high viscosity grades of chitosan hydrochloride (9-16% salt), respectively at concentration of 0.25% and 0.5% chitosan base. Thus, in the above study, differences in absorption enhancing efficacy between the grades tested may have been observed if the salt content of the chitosans had been taken into account.

Table 7.27. Summary of formulations and dose groups in the study to investigate the effect of different grades of chitosan on the intranasal absorption of insulin in sheep

Formulation or Group No.	Insulin (IU/kg)	Chitosan (mg/kg)	Dose volume (ml/kg)
1. Insulin + 0.5% MVCSN	2.0	0.05	0.01
2. Insulin + 0.5% CSN HCL	2.0	0.05	0.01
3. Insulin + 0.5% CSN lactate	2.0	0.05	0.01
4. Insulin + 1.0% CSN lactate	2.0	0.05	0.01

* Dose of chitosan expressed as dose of chitosan salt

Table 7.28. Summary of plasma glucose concentrations following the intranasal administration of insulin with different grades of chitosan in sheep

	Mean (± SD) glucose concentration (% of basal)							
Time before or	0.5% MVCSN		0.5% CSN HCl		0.5% CSN lactate		1.0% CSN lactate	
after dosing (min)		SD		SD		SD		SD
-15	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
-5	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
5	98.0	1.73	98.0	2.71	98.3	2.10	98.2	1.84
10	98.7	2.43	97.1	3.04	97.6	2.84	101.2	2.46
15	97.8	3.41	97.1	3.04	97.6	0.66	100.5	2.92
20	94.0	1.32	90.8	3.14	92.7	1.97	99.0	0.71
30	86.9	3.87	81.2	9.22	87.8	4.19	92.7	3.20
40	75.5	3.19	70.6	9.61	82.2	4.03	89.8	4.72
50	72.9	4.34	68.0	10.67	84.3	1.79	88.5	5.89
60	69.4	3.23	69.5	11.61	84.4	4.08	89.2	7.32
75	72.8	11.46	76.0	9.27	86.4	0.51	89.0	8.51
90	76.9	10.55	78.3	12.28	87.1	1.85	90.3	8.40
120	85.6	4.64	84.4	9.05	88.6	2.95	90.4	6.08
150	87.6	4.64	85.1	7.02	90.6	2.82	92.7	3.10
180	90.8	2.39	89.2	6.08	91.3	1.52	92.7	2.57
240	92.4	4.59	91.2	6.05	95.5	2.51	93.4	1.79

Table 7.29. Summary of plasma insulin concentrations following the intranasal administration of insulin with different grades of chitosan in sheep

	Mean (± SD) insulin concentration (mU/l)							
Time before or	0.5% MVCSN		0.5% CSN HCl		0.5% CSN lactate		1.0% CSN lactate	
after dosing (min)		SD		SD		SD		SD
-15	21.9	6.07	14.9	2.87	24.7	7.16	17.8	3.20
-5	15.6	3.15	12.4	2.02	20.1	1.99	22.6	5.78
5	51.9	33.09	51.7	21.78	42.6	14.86	40.1	25.95
10	94.5	53.93	88.1	53.58	62.1	30.09	57.7	23.02
15	159.9	68.73	92.5	34.74	58.6	17.61	60.0	28.04
20	108.6	23.87	103.8	26.27	46.2	10.03	49.4	18.31
30	76.0	24.28	66.0	29.36	47.9	18.31	37.2	20.11
40	41.7	13.26	27.5	1.85	44.5	17.56	29.2	16.18
50	45.3	22.98	33.0	17.06	34.4	8.84	20.2	4.78
60	34.3	10.93	19.4	2.73	22.4	2.25	21.3	3.59
75	24.6	15.73	14.3	4.33	24.6	7.21	20.2	6.86
90	25.3	6.46	19.7	7.17	28.0	9.07	30.4	13.18
120	18.2	0.36	15.2	4.21	19.0	3.66	21.6	8.24
150	17.0	3.17	13.8	2.64	18.4	3.15	16.6	2.13
180	17.1	5.60	15.6	2.00	20.6	7.28	12.8	2.59
240	25.4	10.34	11.4	0.58	20.3	8.34	17.0	7.48

Table 7.30. Summary of pharmacokinetic paramenters determined from plasma glucose data following the intranasal administration of insulin with different grades of chitosan in sheep

Formulation or Group number	Mean ± SD (n=4)				
	Cmin (% glucose)	Tmin (min)	AOC (% glucose. min)	Rate of decrease of glucose conc. (%/min)	Pharmacokinetic availability *
1. 0.5% MVCSN	67.3 (± 6.88)	63 (± 8.7)	3403 (± 901.4)	0.67 (± 0.093)	3.3 (± 0.89)
2. 0.5% CSN.HCl	66.5 (± 10.60)	55 (± 12.2)	3671 (± 1544.7)	0.86 (± 0.252)	3.6 (± 1.52)
3. 0.5% CSN lactate	81.5 (± 3.24)	44 (± 7.5)	2314 (± 454.9)	0.50 (± 0.152)	2.3 (± 0.45)
4. 1.0% CSN lactate	85.6 (± 6.25)	90 (± 36.3)	1819 (± 771.1)	0.43 (± 0.207)	1.8 (± 0.76)

* Relative to subcutaneous

Statistical Analysis

One-way ANOVA
Sig. dif. in values of Cmin and Tmin (P<0.05).
No sig. dif. in values of AOC and rate of fall of gluocse conc. (P>0.05).

Tukey-Krmaer Multiple Comparisons Test following ANOVA
Cmin: Sig. dif between groups 1vs4 and 2vs4 (P<0.05).
Tmin: Sig. dif between group 3vs4 (P<0.05).
No sig. dif. (P>0.05) between other formulation groups.

Table 7.31. Summary of pharmacokinetic paramenters determined from plasma insulin data following the intranasal administration of different grades of chitosan in sheep

Formulation or Group number	Mean ± SD (n=4)				
	Cmax (mU/l)	Tmax (min)	AUC (mU/l. min)	Rate of increase of insulin conc. ([log mU/l]/min)	Bioavailability * (%)
1. 0.5% MVCSN	167.0 (± 54.85)	16 (± 2.5)	4046 (± 1018.1)	0.10 (± 0.042)	4.4 (± 1.11)
2. 0.5% CSN.HCl	115.7 (± 37.85)	13 (± 6.5)	3032 (± 1492)	0.10 (± 0.062)	3.3 (± 1.62)
3. 0.5% CSN lactate	70.9 (± 23.24)	26 (± 16.0)	2012 (± 1363.7)	0.07 (± 0.040)	2.2 (± 1.48)
4. 1.0% CSN lactate	68.3 (± 17.42)	15 (± 4.08)	1629 (± 1634.2)	0.06 (± 0.049)	1.8 (± 1.78)

* Relative to subcutaneous

Statistical Analysis

One-way ANOVA
Sig. dif. in values of Cmax (P<0.01).
No sig. dif. in values of Tmax, AUC and rate of increase of insulin conc. (P>0.05).

Tukey-Krmaer Multiple Comparisons Test following ANOVA
Cmax: Sig. dif between groups 1vs3 and 1vs4 (P<0.05).
No sig. dif. (P>0.05) between other formulation groups.

Fig 7.10. Plasma glucose and insulin concentration versus time profiles following the intranasal administration of insulin with different grades of chitosan in sheep

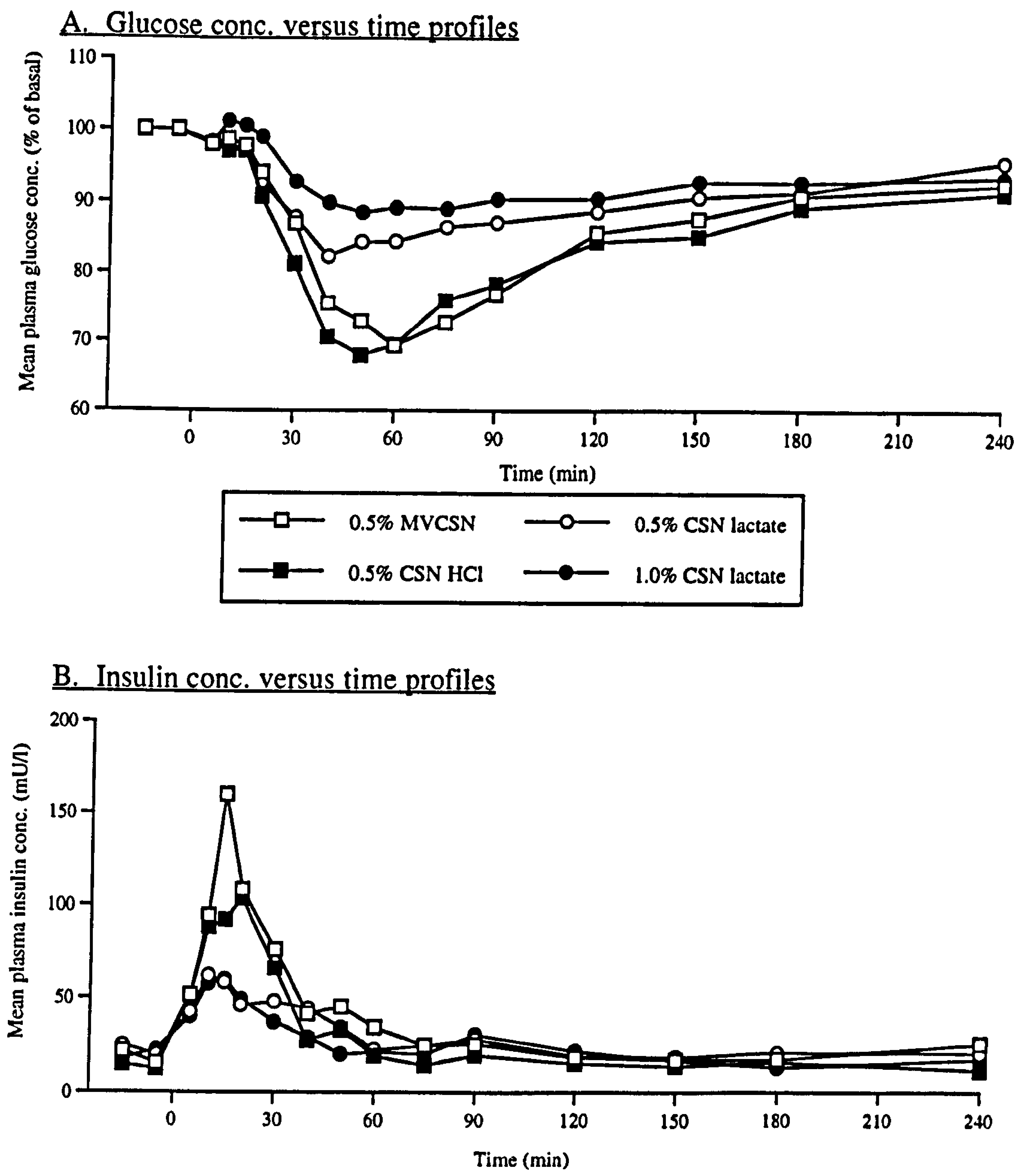
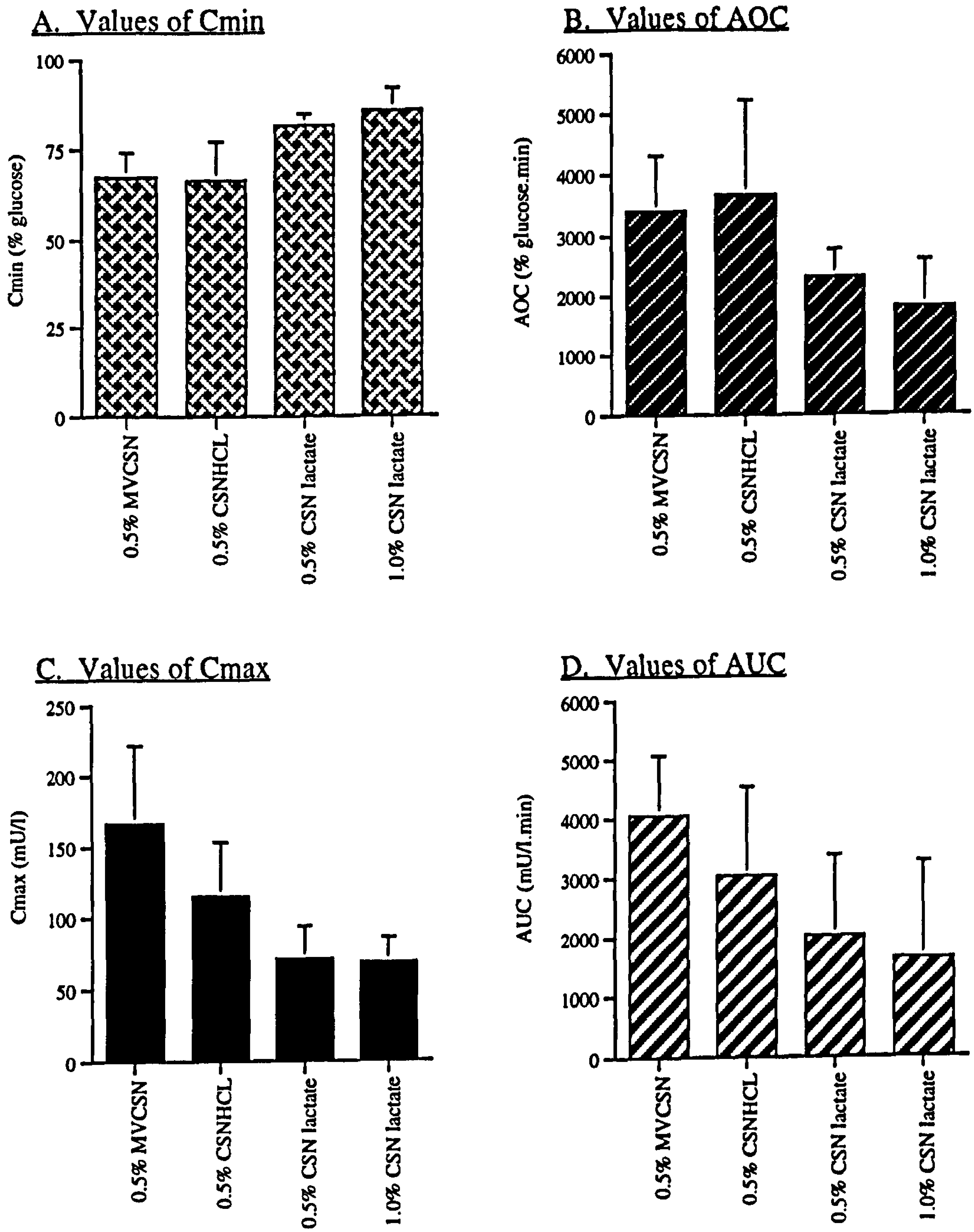


Figure 7.11. Values of selected pharmacokinetic parameters following the intranasal administration of insulin with different grades of chitosan in sheep



7.8 Reproducibility of intranasal insulin absorption following theintranasal administration of an insulin / chitosan formulation on separate occasions in sheep

7.8 Aims and Objectives

In the studies reported previously in this chapter, following the nasal administration of a formulation containing 200 IU/ml insulin with 0.5% MVCSN (pH 4) there was considerable variation in insulin absorption between different groups of animals. This could be attributed to inter-animal variations in nasal absorption as well as differences in experimental conditions on different study days. The aim of this study was to investigate intra- and inter-animal variations in insulin absorption following the nasal administration of the same insulin / MVCSN formulation to the same group of sheep on separate occasions.

7.8.2 Materials and Methods

7.8.2.1 Materials

Insulin of specific activity 21.84 IU/mg and MVCSN was used as given in Section 3.3.

7.8.2.2 Preparation of insulin formulation

A solution formulation containing 200 IU/ml insulin with 0.5% (5.0 mg/ml) MVCSN, was prepared freshly on each study day. Each formulation was prepared in 14.65 mM phosphate buffer of pH 7.4 and adjusted to pH 4 during preparation by the addition of HCl.

7.8.2.3 Absorption study in the sheep model

The study was performed in three sheep on three separate study days. There was a wash-out period of approximately 4 weeks between consecutive study days. Two of the sheep were used on all three study days. However, it was necessary to withdraw the third sheep from the study prior to the third study leg due to illness. A replacement sheep was used on the third study leg. On each study day sheep were dosed with the insulin / MVCSN formulation at 0.01 ml/kg. Each nasal dose was divided between both nostrils. A summary of the dose groups is given in Table 7.32.

It was intended to analyse the glucose and insulin data obtained using a repeated measures analysis of variance which would allow both intra-animal variations, in sheep dosed on different study days, and inter-animal variations, in sheep dosed on each study day, to be evaluated. However, due one of the animal being withdrawn from the third study leg and since the replacement sheep, although a Suffolk and Texel cross-breed was a different strain cross-breed from the sheep previously used and differed in age / weight from the previous animals, repeated measures analysis of variance could not be used. In view of this, insulin analysis was not performed.

7.8.3 Results & Discussion

The glucose data showed that on each study day the plasma glucose concentration versus time profiles followed a similar trend although there were inter-day variations in mean glucose concentrations (Table 7.33, Figure 7.12). The pharmacokinetic parameters further highlight the inter-day variation in the hypoglycaemic response to a 2 IU/kg dose of insulin (Table 7.33, Figure 7.13). However, due to the inter-animal variations on each study day, there were no statistically significant differences between the groups dosed on different study days (Table 7.33). Generally on each study day, inter-animal variations in terms of the extent of the hypoglycaemia (values of C_{min}) tended to be much less than variations in the overall extent and duration of the hypoglycaemia (values of AOC)(Table 7.33). There was also less inter-day variation in values of C_{min} than in values of AOC.

There were intra-animal variations (sheep 1 and 2) in the plasma glucose versus time profiles and pharmacokinetic parameters following the nasal administration of insulin on different study days (Table 7.34-7.35, Figures 7.14-7.15). Generally, intra-animal variability of glucose concentration and pharmacokinetic data tended to be marginally less than inter-animal variability although there was not much difference between the two (Tables 7.32-7.35). A repeated measures analysis of variance would have allowed both intra- and inter-animal variations to be compared by statistical means although as previously mentioned this was not performed.

Reproducibility of the hypoglycaemic response is a prerequisite for the therapeutic use of insulin (Lassmann-Vague, 1991). In the majority of insulin-dependent diabetics, day-to-day metabolic variability in glycaemic control occurs despite consistency in the parenteral dosing regimen (Pickup, 1991). Variability in subcutaneous insulin absorption may occur due to a number of factors. Inaccuracy in dose administration may account for day-to-day variations in a small minority of individuals particularly in elderly patients. The depth of subcutaneous injection may also influence insulin absorption. Perpendicular injection of the insulin dose using a 12 mm needle may inadvertantly lead to intramuscular injection depending on the thickness of the fat layer, hence, oblique injection into a fold of skin is recommended. Absorption from muscle tends to be faster than that following subcutaneous administration which may be due to the faster blood flow in muscle (Banerjee et al., 1991). The anatomical site of subcutaneous or intramuscular injection has a major influence on insulin and peptide absorption (Pickup, 1991, Banerjee et al., 1991). Following subcutaneous injection, the rate of absorption of short-acting insulin preparations tends to be most rapid from the abdomen, particularly the upper abdomen, and slowest from the gluteal region with an intermediate rate in the arm. Thus, for consistency of absorption, the injection site should not be randomly altered. However, at each anatomical site the injection site should be rotated to prevent localised tissue damage or the development of lipohypertrophy which may reduce the rate of absorption of insulin. Within-site variability in insulin absorption may also occur due to the influences of a number of factors such as heat, massage at the site of injection and exercise. The rate of absorption of insulin following subcutaneous injection into a limb has been shown to increase during exercise. Massage at the subcutaneous site of

injection has also been shown to increase the rate of peptide absorption. Degradation or metabolism of insulin at the injection site may also account for variability in insulin absorption. Changes in the physical and chemical stability and biological potency of the insulin preparations are also factors which would influence subcutaneous absorption although these factors would be expected to remain constant within the normal shelf-life of the product

Poor reproducibility in insulin absorption and in the resultant hypoglycaemic response would limit the delivery of insulin via the nasal route (Lassmann-Vague, 1991). In a study in healthy human volunteers, considerable inter-subject variation in serum insulin concentrations (103 ± 49 μU/l at peak concentration) was observed following the intranasal administration of a solution of insulin with the bile salt sodium deoxycholate (Moses et al., 1983). However, there was much less variability in blood glucose concentrations (54 ± 14% of basal concentration) in these subjects. Intra-subject variations in peak serum insulin concentrations and blood glucose response were reported to be less than those between subjects. Paquot et al. (1988) also reported greater inter-subject than intra-subject variation in response to an intranasal formulation of insulin with Laureth-9. The coefficient of inter-subject variation in the maximal decrease in blood glucose concentrations was reported to be 52% compared to 88% for maximal plasma insulin increase. Sinay et al. (1990) investigated the reproducibility in nasal insulin absorption (solution with sodium glychcholate) and its biological effects in patients with insulin-dependent diabetes mellitus (IDDM). From the plasma glucose concentration data the inter-subject coefficient of variation in values of C_{min} and AUC were reported to be 46% compared to intra-subject variability of 17% and 33%, respectively. Likewise, from the plasma insulin concentration data, the inter-subject coefficient of variation in values of C_{max} and AUC were reported to be 48% and 77% compared to intra-subject variability of 31% and 33%, respectively. For therapeutic application inter-subject variations are less important than intra-subject variations since the regimen of insulin therapy should be tailored to the individuals needs (Lassmann-Vague, 1991). When addressing the reproducibility of intranasal insulin absorption and lowering of glucose concentrations, evaluation should be relative to subcutaneous insulin. Frauman et al. (1987) compared the efficacy of intranasal (coadministered with sodium glycocholate) and subcutaneous insulin in non-obese type II diabetics and reported that the inter-subject coefficient of variation in peak serum insulin concentrations was 41% and 44%, respectively. The intra-subject coefficient of variation in peak serum insulin concentrations following the intranasal administration of insulin with meals was reported to be 55% although evaluation for subcutaneous insulin was not reported. The coefficients of variation in the glucose data was not reported. Lassmann-Vague (1991) suggests that in the same individual, the reproducibility of the action of intranasal insulin appears to be acceptable and at least comparable to that administered by subcutaneous injection.

In this study in the sheep model, following the intranasal administration of a formulation of insulin containing 0.5% MVCSN, intra- and inter-animal variability would appear to

be reasonably low. From the glucose data, the coefficients of variation (over the 3 study days) of values of C_{min} and AOC were 11.1-24.6% and 23.3-64.4%, respectively. In sheep 1, the coefficient of variation of values of C_{min} and AOC were 13.3% and 34.3%, respectively. In sheep 2, the coefficient of variation of values of C_{min} and AOC were 11.6% and 18.7%, respectively.

Table 7.32. Summary of plasma glucose concentrations following the intranasal administration of an insulin / MVCSN formulation to a group of 3 sheep on separate occasions (inter-day variability in hypoglycaemic response)

Time before or after dosing (min)	Mean (± SD) plasma glucose conc. (% of basal)					
	Day 1	SD	Day 2	SD	Day 3	SD
-15	100.0	0.00	100.0	0.00	100.0	0.00
-5	100.0	0.00	100.0	0.00	100.0	0.00
5	104.3	0.31	100.0	3.15	103.2	6.00
10	103.4	4.27	101.6	2.71	102.2	6.30
15	102.6	4.62	97.6	4.31	102.2	8.91
20	99.7	4.48	99.5	4.53	100.4	6.06
30	92.9	4.86	86.0	6.35	92.4	9.96
40	87.0	5.53	79.0	4.13	85.1	12.00
50	82.0	12.24	72.3	7.03	79.7	17.40
60	78.1	13.48	71.6	6.82	76.0	16.23
75	80.1	11.44	71.1	8.98	70.5	15.38
90	79.0	13.05	74.6	9.66	72.3	15.96
120	90.8	4.93	79.1	6.20	81.3	9.45
150	92.7	5.24	84.6	4.74	84.9	5.53
180	95.5	5.46	85.6	5.89	88.6	5.07
240	94.5	7.19	87.4	5.23	94.2	5.06

Table 7.33. Summary of pharmacokinetic parameters following the intranasal administration of an insulin / MVCSN formulation to a group of 3 sheep on separate occasions (inter-day variability in hypoglycaemic response)

Formulation or Group number	Mean ± SD (n=4)				
	Cmin (% glucose)	Tmin (min)	AOC (% glucose. min)	Rate of decrease of % glucose (%/min)	pharmacological availability * (%)
1. Study Day 1	78.1 (± 13.48)	68 (± 6.6)	2260 (± 1454.7)	0.66 (± 0.318)	2.2 (± 1.43)
2. Study Day 2	69.2 (± 7.68)	69 (± 18.4)	4078 (± 951.9)	0.73 (± 0.193)	4.0 (± 0.94)
3. Study Day 3	68.7 (± 16.91)	76 (± 18.4)	3513 (± 1820.9)	0.66 (± 0.358)	3.5 (± 1.79)

* Relative to subcutaneous

Statistical Analysis

One-way ANOVA
No sig. dif. in values of Cmin, Tmin, rate of decrease of % glucose and AOC (P<0.05).

Table 7.34. Summary of plasma glucose concentrations following the intranasal administration of an insulin / MVCSN formulation to individual sheep on separate occasions (intra-animal variability in hypoglycaemic response)

Time before or after dosing (min)	Mean (± SD) plasma glucose conc. (% of basal)			
	Sheep 1		Sheep 2	
		SD		SD
-15	100.0	0.00	100.0	0.00
-5	100.0	0.00	100.0	0.00
5	104.5	4.66	99.6	4.33
10	107.0	2.30	100.6	3.69
15	105.2	5.88	98.7	7.80
20	102.4	5.80	98.7	5.24
30	92.1	4.42	87.0	7.49
40	83.7	2.06	79.1	5.39
50	75.9	3.63	72.4	8.85
60	72.4	0.67	69.3	8.07
75	66.4	7.30	73.3	8.21
90	65.7	10.06	75.0	8.53
120	78.1	10.42	82.9	2.89
150	83.1	6.11	86.8	2.76
180	88.6	9.76	87.9	3.59
240	90.2	8.61	89 6	2.99

Table 7.35. Summary of pharmacokinetic parameters following the intranasal administration of an insulin / MVCSN formulation to individual sheep on separate occasions (intra-animal variability in hypoglycaemic response)

	Mean ± SD (n=3)				
	Cmin (% glucose)	Tmin (min)	AOC (% glucose. min)	Rate of decrease of % glucose (%/min)	pharmacological availability * (%)
Sheep 1	64 8 (± 8.60)	81 (± 15.6)	4036 (± 1385.8)	0.75 (± 0.05)	4.0 (± 1.36)
Sheep 2	69.3 (± 8.07)	62 (± 11.5)	3771 (± 704.3)	0.80 (± 0.217)	3.7 (± 0.69)

* Relative to subcutaneous

Statistical Analysis

One-way ANOVA

No sig. dif. in values of Cmin, Tmin, rate of decrease of % glucose and AOC ($P<0.05$).

Figure 7.12. Plasma glucose concentration versus time profiles following the intranasal administration of an insulin / MVCSN formulation to a group of 3 sheep on separate occasions (inter-day variability in hypoglycaemic response)

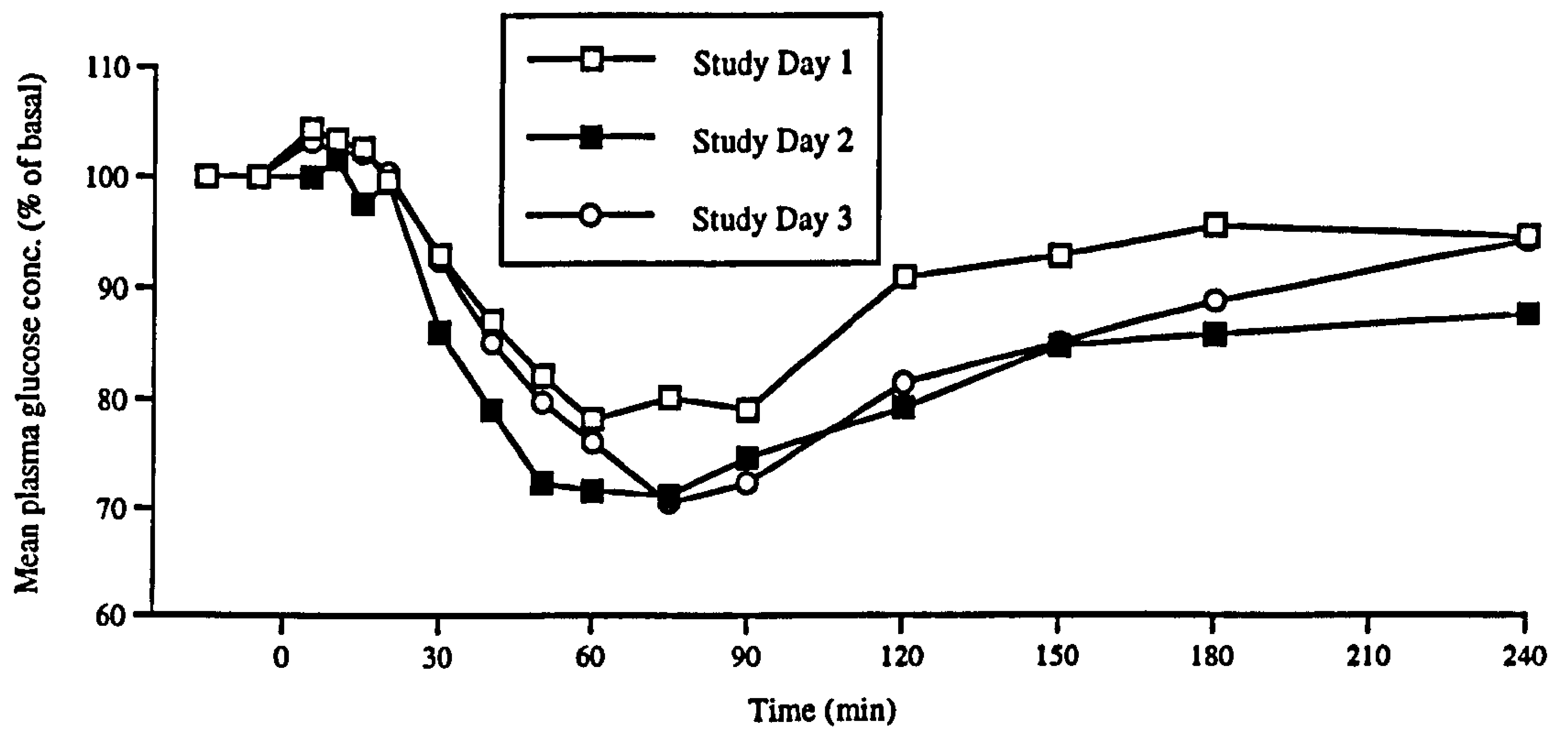


Figure 7.13. Values of selected pharmacokinetic parameters following the intranasal administration of an insulin / MVCSN formulation to a group of 3 sheep on separate occasions (inter-day variability in hypoglycaemic response)

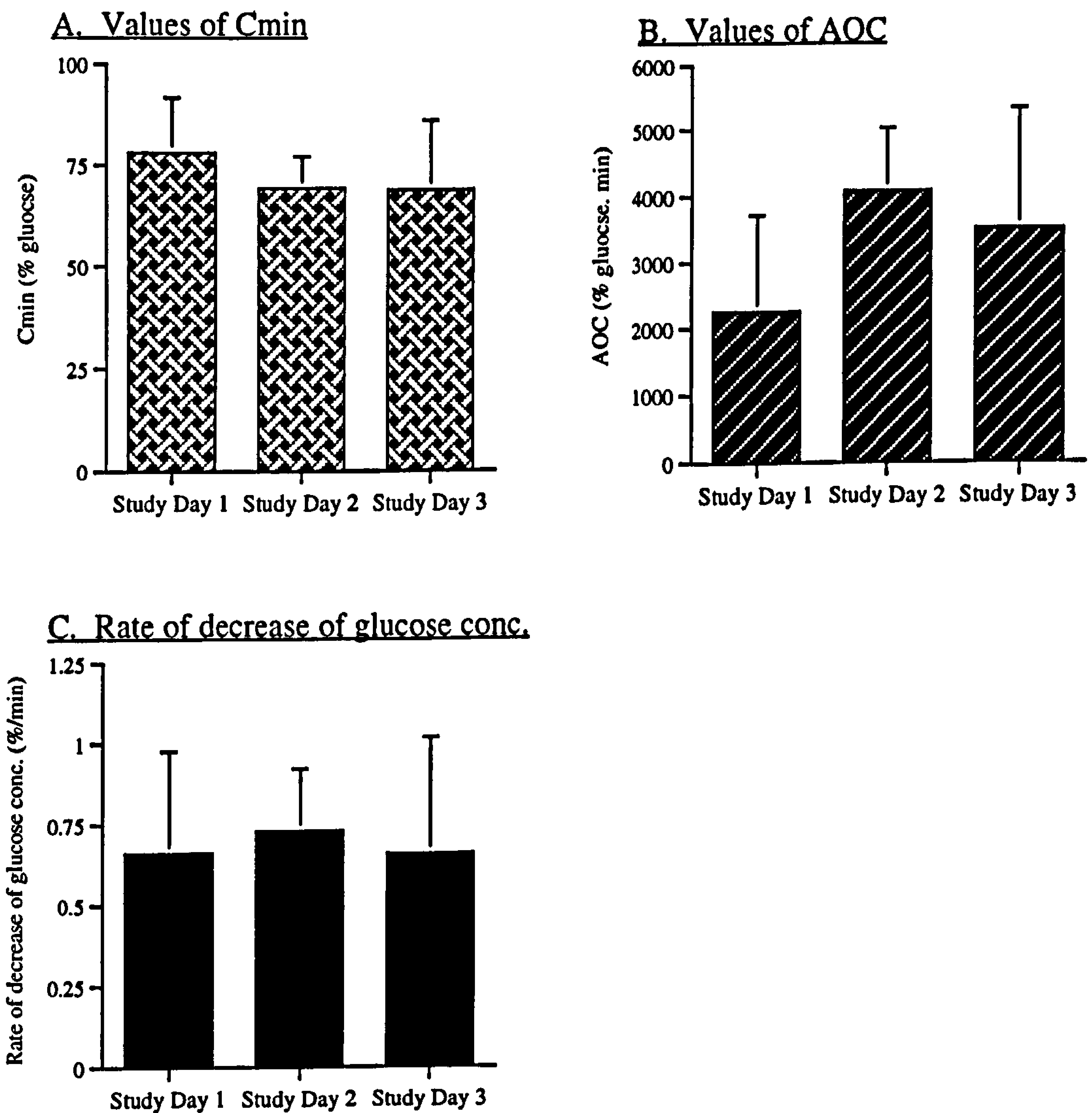


Figure 7.14. Plasma glucose concentration versus time profiles following the intranasal administration of an insulin / MVCSN formulation to individual sheep on separate occasions (intra-animal variability in hypoglycaemic response)

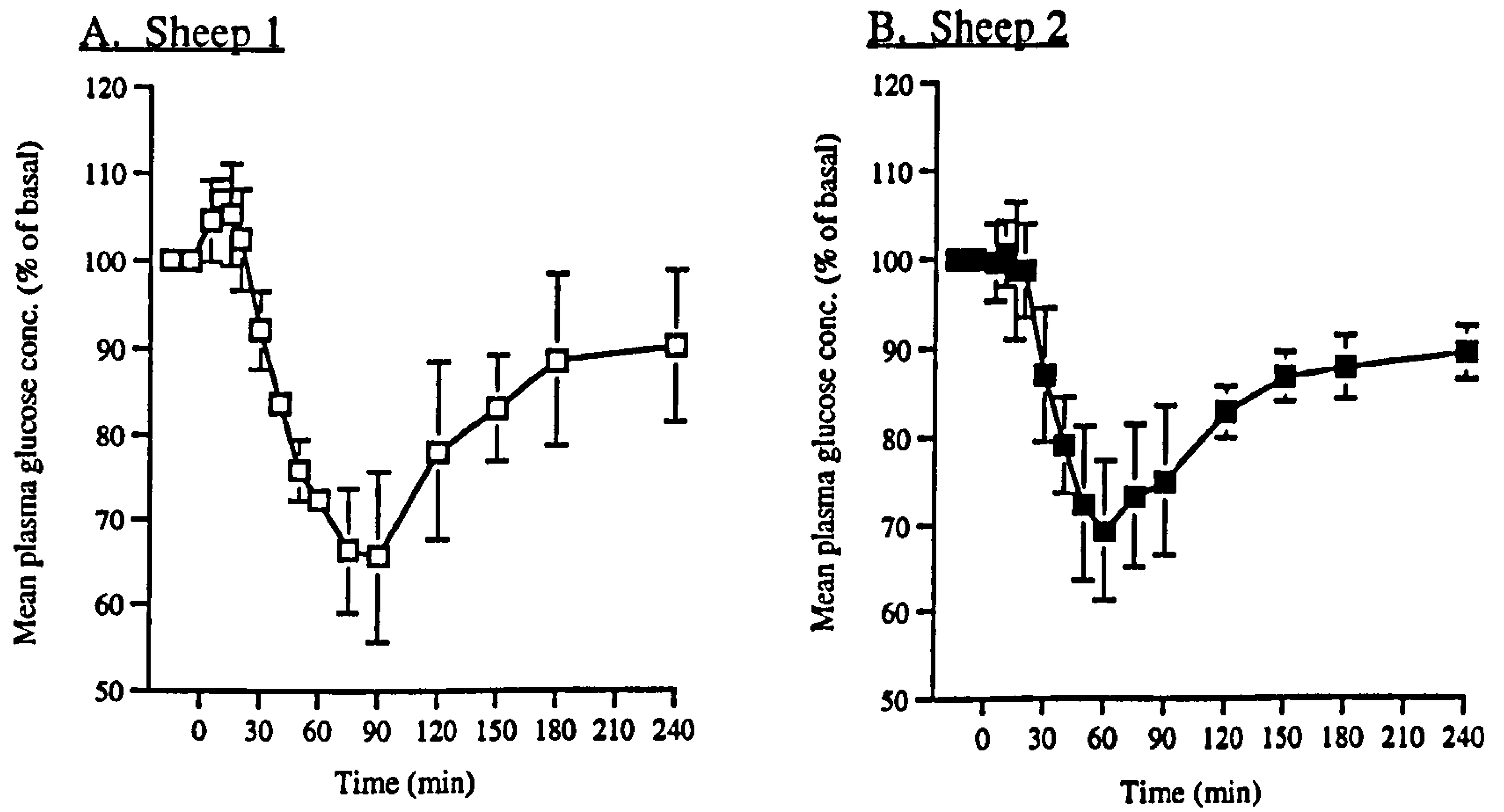
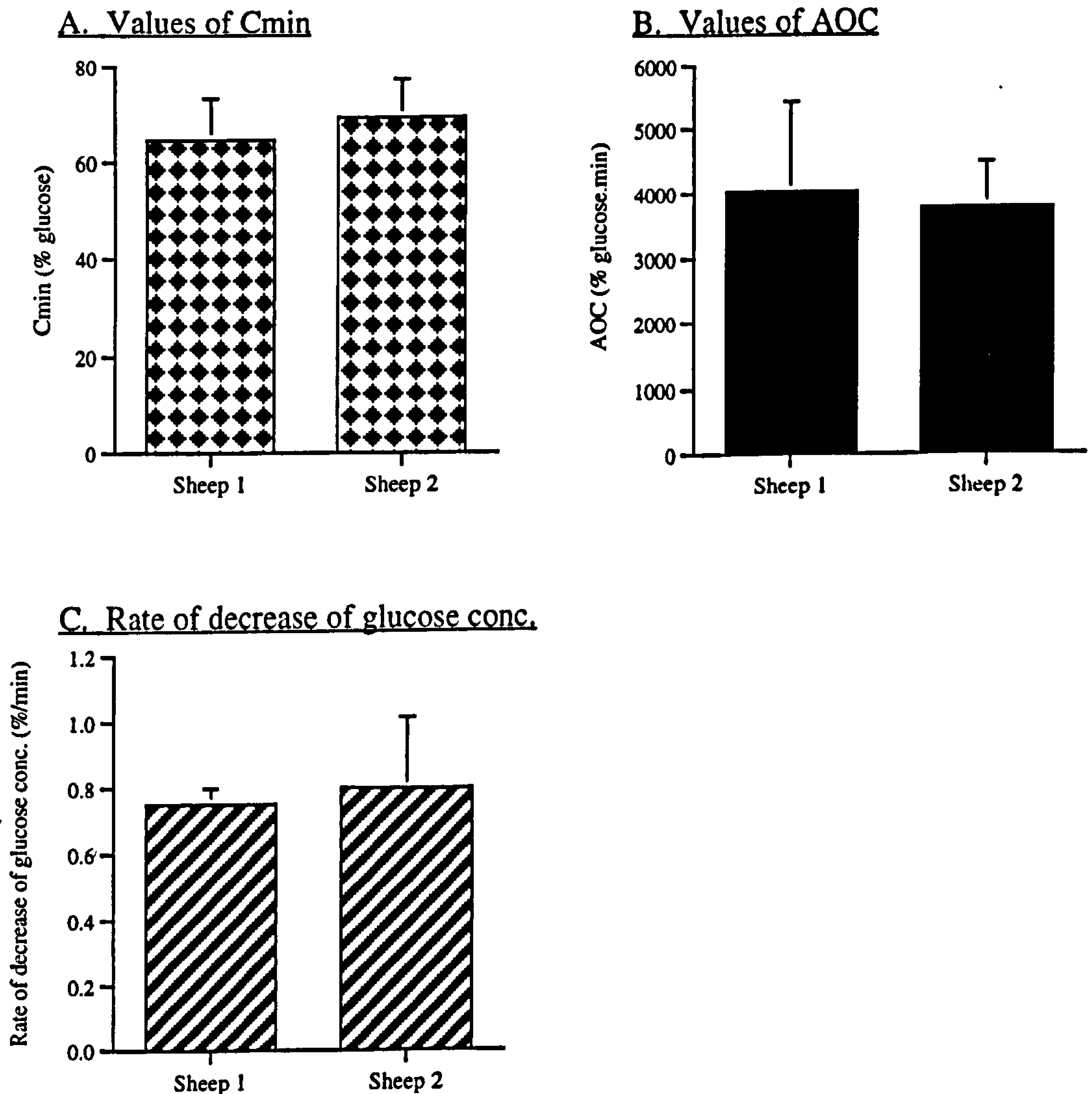


Figure 7.15. Values of selected pharmacokinetic parameters following the intranasal administration of an insulin / MVCSN formulation to individual sheep on separate occasions (intra-animal variability in hypoglycaemic response)



7.9 Conclusions

This chapter presents the results of several nasal absorption studies in sheep aimed at further investigating and optimising chitosan for the nasal delivery of insulin. Consistent with the nasal absorption study in rats reported in chapter 4, increasing the solution concentration of chitosan (MVCSN grade) was shown to improve the intranasal absorption of insulin. Optimal absorption enhancing efficacy was obtained between 0.35 to 1.0 % w/v MVCSN. However, investigation of higher concentrations of chitosan would have been beneficial to establish if there was an upper limit to the optimal range of chitosan concentration above which absorption enhancing efficacy was reduced. Since increase in the solution concentration of MVCSN will not only increase the viscosity of the formulation but also the dose concentration of MVCSN administered, a study was performed to investigate the effect of the dose concentration of MVCSN on intranasal insulin absorption.

At a fixed dose of insulin (2 IU/kg) increase in the dose concentration of MVCSN administered (by increasing the dose volume administered of formulations containing a fixed concentration of MVCSN, thereby altering chitosan dose without altering solution viscosity) did not appear to influence the performance of the formulation in terms of its nasal absorption enhancing efficacy. Thus, it appeared that it was the solution concentration of chitosan rather than dose concentration of chitosan which was important for the nasal absorption enhancement observed. This suggested that the effect of MVCSN in the nasal cavity reached a maximum for the formulations under investigation. The absorption enhancing efficacy of chitosan is proposed to be via a combination of increased residence time of the formulation in the nasal cavity, attributed to the viscosity enhancing and bioadhesive properties of chitosan and the transient effect of chitosan on epithelial tight junctions (Illum et al., 1994). The absorption enhancing efficacy of chitosan due to its affect on tight junctions could be due to the degree of opening of tight junctions or due to the number of tight junctions opened. Increasing the dose concentration of chitosan administered by increasing the dose volume would be expected to increase the spread of chitosan in the nasal cavity, thus, increase the absorptive surface area. This could potentially increase the number of tight junctions opened. In a separate study, nasal insulin absorption was shown to be improved by increasing the dose concentration of insulin administered. Thus, in previous studies, nasal insulin absorption was limited by the dose concentration of insulin. Increasing the dose concentration of insulin increased insulin absorption in terms of the elevation in plasma insulin concentrations and decrease in plasma glucose concentrations although the bioavailability of nasal insulin absorption was not improved. A study was performed in which different volumes of a formulation of 200 IU/ml insulin with 0.5% w/v chitosan were administered. Thus, increasing the dose volume increased both the dose concentrations of insulin and chitosan administered nasally. Increasing the dose volume increased the elevation in plasma insulin concentrations and decreases in plasma glucose concentrations although the bioavailability of nasal insulin absorption was not improved. This study demonstrated that the efficacy of a nasal insulin / chitosan formulation was highly dependent on the volume administered nasally which is an important consideration

if the formulation is to be used therapeutically since poor patient compliance may lead to under- or overdosing.

From the above studies, it appears that it is the formulation concentration of chitosan which is important for its absorption enhancing efficacy since increasing the concentration improved the degree of nasal absorption until an optimal concentration was attained. At the optimal concentration of chitosan, nasal insulin absorption was limited by the dose concentration of insulin.

The efficacy of nasal absorption enhancement of MVCSN appeared to be the same from both hypotonic and isotonic formulations. Thus, differences in the conformation of chitosan in the respective solutions due to differences in ionic concentration (refer to Section 10.5) had no affect on its absorption enhancing efficacy. At chitosan concentrations lower than the optimal concentration, the tonicity of the formulation could be adjusted to regulate the viscosity of the formulation. This should allow the importance of viscosity to be evaluated since solutions containing a lower concentration of chitosan (e.g. 0.1 or 0.2% w/v) could be prepared having a similar viscosity to solutions containing an optimal concentration of chitosan (e.g. 0.5% w/v). The grade of chitosan used was shown to influence the degree of nasal absorption enhancement. Low viscosity chitosan lactate (CSN lactate), at concentrations of 0.5 and 1.0%, was shown to be less effective than medium viscosity chitosan glutamate (MVCSN) at a concentration of 0.5%. This could reflect the lower viscosity of the CSN lactate formulations. The performance of a formulation containing 0.5% MVCSN was shown to be improved compared to that of a formulation containing 0.5% medium viscosity chitosan hydrochloride (CSN HCL) despite the higher viscosity of the latter formulation. Thus, the grade of chitosan appeared to be important for the degree of absorption enhancement attained. It is difficult to compare the efficacy of the different grades of chitosan due to the differences in the chitosan content of the respective formulations. Investigation of the relationship between chitosan molecular weight and the efficacy of nasal absorption enhancement would best be achieved by the use of chitosan fractions of different molecular weights prepared from the same chitosan starting material.

In the final study reported in this chapter, the degree of nasal absorption enhancement, in terms of the lowering of plasma glucose concentrations, was shown to be highly reproducible following the nasal administration of a formulation of insulin with 0.5% MVCSN. Inter- and intra-animal variations appeared to be reasonably low which was encouraging if a nasal delivery system incorporating chitosan is to find therapeutic application.

CHAPTER 8

INTRANASAL ABSORPTION OF SALMON CALCITONIN IN SHEEP

8.1 Aims and objectives

In Chapter 5 it was reported that a formulation containing 0.5% MVCSN enhanced the nasal absorption of salmon calcitonin (S-CT) in the rat model. In this study, the nasal absorption of S-CT from a formulation incorporating 0.5% MVCSN was investigated in the sheep. The performance of the nasal MVCSN formulation was compared to that of a nasal control solution of S-CT. In the study blood samples were collected pre- and post administration of the nasal S-CT formulations. Plasma was separated and plasma calcium concentrations measured. The absorption of S-CT was assessed, indirectly, from the decrease in plasma calcium concentrations following S-CT dose administration.

8.2 Materials and Methods

8.2.1 Materials

Salmon Calcitonin acetate salt (S-CT.4 AcOH.13 H_2O, specific activity 4730 IU/mg, Molecular weight 3431.9), Bachem Feinchemikalien AG, Switzerland.
Chitosan (MVCSN) was used as described in Section 7.2.

8.2.2 Preparation of S-CT formulations

To avoid adsorption losses of S-CT, glassware and plasticware was treated as described in Section 5.2.2. Two solution formulations of S-CT for nasal administration were prepared as given in Table 8.1. Both solutions were prepared in 0.9% NaCl and adjusted to pH 4.0 during preparation by the addition of HCl.

Table 8.1. Outline composition of formulations

Formulation No.	Outline composition
1	2000 IU/ml S-CT control solution, pH 4
2	2000 IU/ml S-CT with 0.5% (5 mg/ml) MVCSN, pH 4

* Concentration of MVCSN expressed as the concentration of chitosan glutamate

8.2.43 Absorption study in the sheep model

Sheep were dosed nasally with the appropriate S-CT formulation. Nasal doses of 20 IU/kg S-CT were administered at a volume of 0.01 ml/kg. MVCSN was administered (Formulation Group 2) at 0.05 mg/kg.

8.3 Results and Discussion

The results showed that nasal absorption of S-CT was poor following the administration of the control solution formulation of S-CT (Tables 8.2-8.3, Figures 8.1-8.2). The intranasal administration of the formulation of S-CT with 0.5% MVCSN appeared to

improve the nasal absorption of S-CT. From the plasma calcium concentration versus time profiles (Figure 8.1) it was observed that the extent and duration of the hypocalcaemia was much greater following the nasal administration of the formulation containing MVCSN. This was reflected in the lower values of Cmin and higher values of AOC for this formulation (Table 8.3, Figure 8.2). Statistical comparison of the formulation groups using a Student's unpaired T-test showed that values of Cmin were significantly lower and values of AOC significantly higher after dosing the S-CT formulation containing MVCSN than after dosing the control S-CT solution.

This study demonstrated that a solution formulation containing 0.5% MVCSN improved the nasal absorption of S-CT in the sheep model, in terms of the lowering of plasma calcium concentrations, compared to a control solution of S-CT. Since a reference (parenteral) dose group was not included in the study then a relative etsimate of S-CT absorption via the nasal route was not obtained. The results support the findings of the nasal absorption study in rats (Chapter 5) by demonstrating that chitosan can potentially be used for the nasal delivery of other peptide drugs. Measurement of plasma S-CT concentrations and the inclusion of a subcutaneous, intramuscular or intravenous reference group in the study would enable values of bioavailability to be calculated. Values of bioavailability could also be compared to values of pharmacodynamic availability. The reference dose should be carefully selected when attempting to estimate nasal absorption from measurement of calcium concentrations to avoid satruation of the hypocalcaemic response (Sinko et al., 1995). Comparison of the performace of the S-CT / MVCSN formulation with a commercially available nasal S-CT formulation would also be useful.

8.4 Conclusions

The study demonstrated that chitosan (MVCSN) enhanced the nasal absorption of S-CT, assessed indirectly from the lowering of plasma calcium concentrations, in the sheep model and thus warrants further investigation.

Table 8.2. Plasma calcium concentration versus time profiles following the intranasal administration of a solution of S-CT, with and without MVCSN, in sheep

	Mean ± SD (n=4) plasma calcium concentration (% of basal)			
Time before or after dosing (min)	S-CT control		S-CT / MVCSN	
		SD		SD
-15	100.0	0.00	100.0	0.00
-5	100.0	0.00	100.0	0.00
15	98.9	1.06	98.5	1.62
30	98.5	1.47	97.6	0.52
45	97.0	0.66	97.3	1.08
60	97.4	1.03	96.6	2.88
90	96.7	1.49	94.3	2.45
120	96.6	0.65	92.6	2.52
150	97.9	2.39	91.6	2.35
180	97.1	2.84	91.4	1.61
240	98.4	1.91	91.2	4.01
300	98.3	2.37	91.2	4.05
360	99.6	3.60	93.2	2.41
420	97.6	1.74	94.5	1.79
480	96.9	1.31	96.0	2.77
600	100.6	2.20	98.4	3.96

Table 8.3. Summary of pharmacokinetic parameters following the intranasal administration of a solution of S-CT, with and without MVCSN, in sheep

Formulation or Group number	Mean ± SD (n=4) Cmin (% calcium)	Tmin (min)	AOC (% calcium.min)
1. S-CT control	95.9 (± 1.53)	206 (± 103.3)	1319 (± 710.7)
2. S-CT / 0.5% MVCSN	89.7 (± 2.01)	229 (± 53.9)	3215 (± 1149.8)

Statistical Analysis

Student's unpaired t-Test

Sig. dif. in values of Cmin (P<0.01). No sig. dif. in values of Tmin and AOC (P>0.05)

Figure 8.1. Plasma calcium concentration versus time profiles following the intranasal administration of a solution of S-CT, with and without MVCSN, in sheep

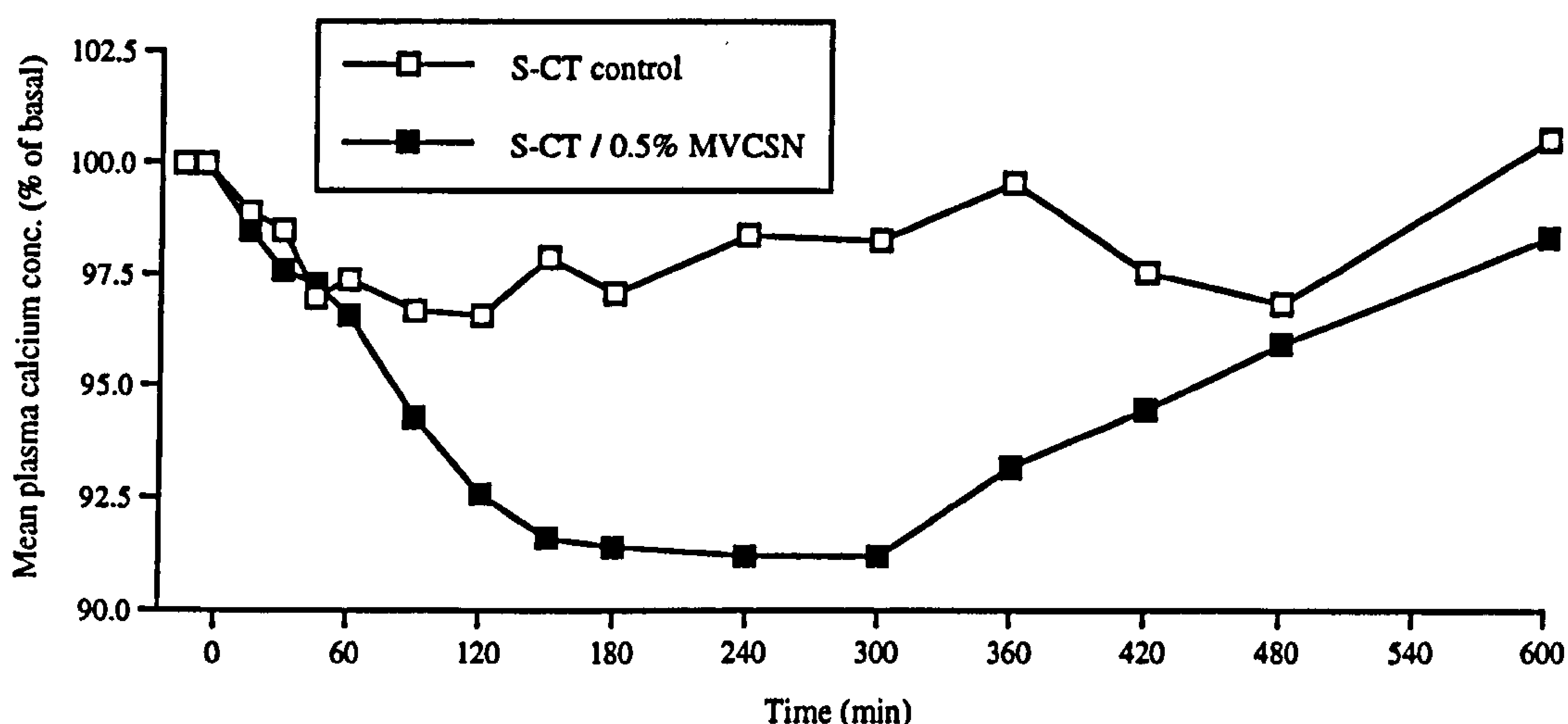
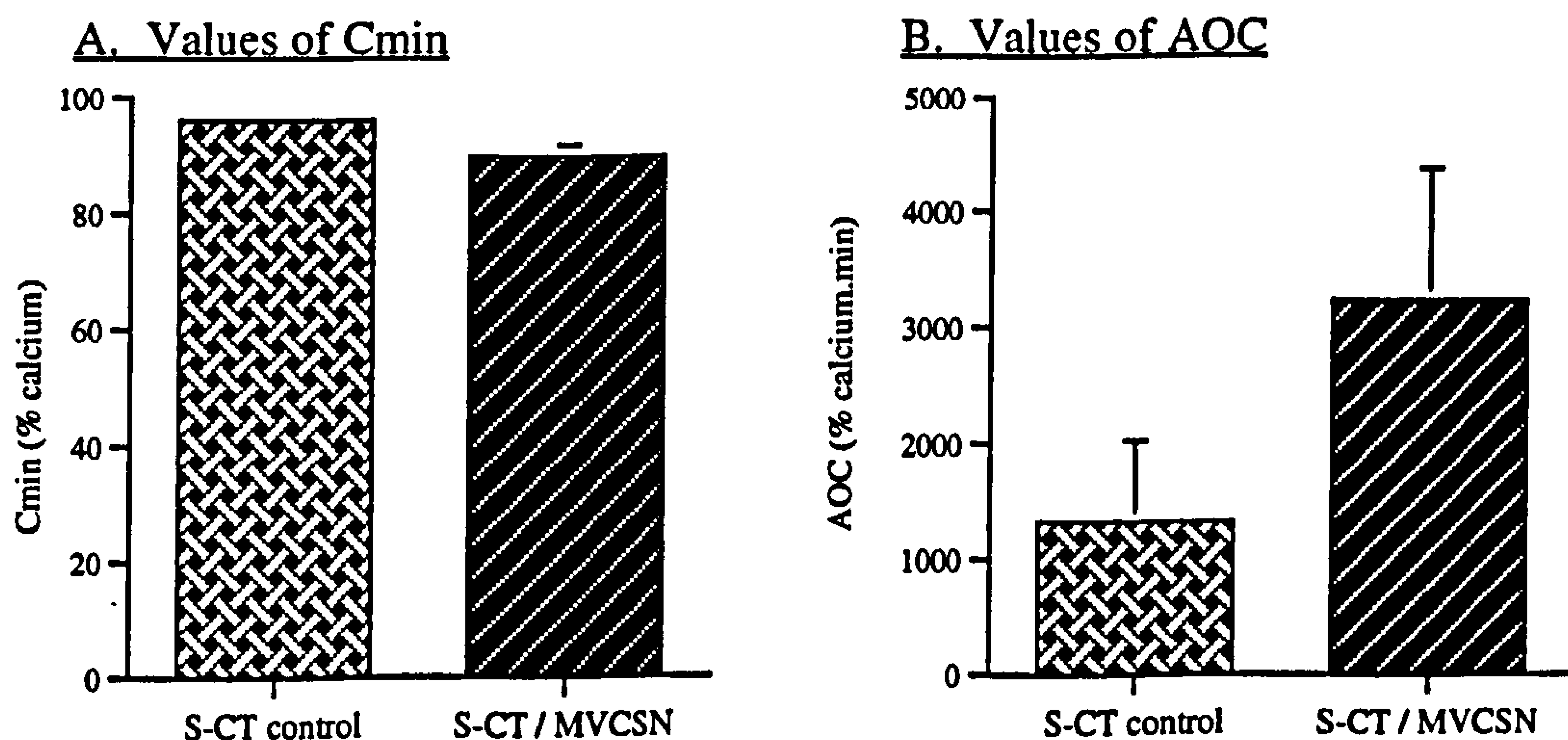


Figure 8.2. Selected pharmacokinetic parameters following the intranasal administration of a solution of S-CT, with and without MVCSN, in sheep



CHAPTER 9

IN VITRO INVESTIGATION OF THE HAEMOLYTIC EFFFECTS OF CHITOSAN AND VARIOUS ABSORPTION ENHANCERS

9.1 General introduction

Erythrocytes (red blood cells) have been frequently used to study the properties of biological membranes and membrane interactions (Cho and Proulx, 1969, Weltzien, 1979). Erythrocytes provide a good natural biological membrane model which can be useful for screening the membrane damaging effects of potential nasal absorption enhancers *in vitro* (Gill et al., 1994a). Damage to the erythrocyte membrane resulting in cell rupture or haemolysis and thus release of haemoglobin can easily be detected spectrophotometrically. Blood from humans or animal models is readily available and the haemolysis assay is relatively easy to set up enabling many compounds to be screened over a short time period. Ultimately, enhancer induced damage to the nasal membrane should be verified by histopathological studies in animal models. However, because histopathology is time consuming and expensive, its application is usually limited to the most promising enhancers selected following screening in haemolysis assays.

The haemolysis assay used in this project was based on that described by Gill et al.. (1994) using rat erythrocytes. In preliminary investigations, the effect of the osmolality and pH of the incubation media used in the assay were investigated prior to investigating the haemolytic effects of chitosan and various absorption enhancers (glycodeoxycholate (GDC), polyoxyethylene-9-lauryl ether (Laureth-9) L-α-lysophosphatidyl choline (LPC)). Following these initial studies, modification of the haemolysis assay was required to allow further investigation of the haemolytic effects of chitosan. The various haemolysis studies are reported in the following Sections:

- 9.2 General materials and methods used in the haemolysis assays
- 9.3 Investigation of the effect of osmolality and pH on the haemolysis assay
- 9.4 Investigation of the haemolytic effects of chitosan and various absorption enhancers
- 9.5 Effect of relative centrifugal field (RCF) and duration of centrifugation on the apparent haemolytic activity of MVCSN
- 9.6 Further investigations of the haemolytic effects of chitosan

9.2 General materials and methods used in the haemolysis assays

9.2.1 Materials

Citrate dextrose phosphate adenine anticoagulant (CDP-A anticoagulant, blood donor pack from Tuta Laboratories Pty Ltd., NSW, Australia) obtained, as a gift, from Department of Haematology, Queen's Medical Centre, Nottingham, UK. The composition of the CDP-A anticoagulant is given in Appendix 1.

Sodium chloride 0.9 % w/v solution (0.9 % NaCl, Steriflex®, 500 ml of 0.9 % sodium chloride intravenous infusion B P) Fresenius Health Care Group, Basingstoke, Hants, UK.
Conical 12 ml polystyrene test tubes (12 ml test tubes), Sarstedt Ltd, Leicester, UK.
MSE Mistral 2000 centrifuge and MSE Microcentaur centrifuge from Fisons Scientific Equipment, Loughborough, Leicestershire, UK.
Water bath (W28) from Grant Instruments Ltd, Cambridge, UK.
Hewlett Packard 845A diode array spectrophotometer (spectrophotometer), Hewlett Packard GmbH, Germany.
Philips quartz cuvettes (matching pair) Unicam Analytical Supplies, Cambridge, UK.
All other materials used are as previously given in previous chapeters.

9.2.2 Methods

Details of the reagents / buffers prepared in these studies are given in Appendix 1.

9.2.2.1 Preparation of stock rat erythrocyte solution

A stock erythrocyte solution was prepared from rat blood collected freshly on the day of the study in isotonic phosphate buffered saline (PBS) at pH 7. Blood was collected from male Wistar rats, of approximate weight 300-500g, under terminal anaesthesia. Rats were anaesthetised and the carotid artery cannulated, for the purpose of blood sample collection, as described in Section 2.2. A tracheotomy was not performed prior to cannulation of the carotid artery. From each rat used in this procedure, 2-3 aliquots of about 4.5 ml of blood were collected into 12 ml test-tubes containing 0.63 ml of CDP-A anticoagulant. Each tube was capped and inverted, gently, several times to ensure complete mixing of the blood and anticoagulant. The rats were killed by an overdose of pentobarbitone sodium

Within approximately 30 minutes of collecting the blood, it was centrifuged at 1500 rpm for 5 minutes in a MSE Mistral 2000 centrifuge at room temperature. The supernatant was removed and discarded. The erythrocytes (pellet) were washed by resuspending them in PBS, centrifuging at 1500 for 5 minutes and discarding the supernatant. This washing procedure was repeated three more times. After the final wash, the supernatant was removed and the erythrocytes pooled in one tube. Erythrocyte stock solutions were prepared by adding 2 ml of pooled erythrocytes to 15.0 ml of PBS in polystyrene Universal bottles. These were mixed by gently inverting several times. The erythrocyte stock solutions were kept at room temperature prior to use.

9.2.2.2 Erythrocyte lysis assay

In the assay it was assumed that total (i.e. 100%) haemolysis occured following incubation of rat erythrocytes with water. Prior to performing the assay, the value of optical density at total haemolysis was tested by incubating erythrocytes with deionized water as described below. The assay required that the optical density at 540 nm was approximately 1.0, otherwise the stock solution was adjusted accordingly by the addition of PBS or pooled erythrocytes and the measurement of optical density at 100%

haemolysis repeated.

The haemolysis assays were performed in triplicate. Since only one assay was done at a time then controls were incorporated in each assay. Each assay consisted of incubations of control media [water to indicate total i.e. 100% haemolysis and isotonic PBS or isotonic NaCl (when testing some of the chitosan solutions) to indicate the basal haemolysis (haemolysis at zero concentration of enhancer solution) or test media [the solutions of enhancers] with erythrocyte stock solution. To 3.5 ml of the control or test media in a 12 ml test tube was added 0.25 ml of stock erythrocyte solution. The tubes were capped and inverted gently three times. The tubes were incubated in a water bath at 37 °C for 5 minutes then centrifuged at 1800 rpm for 2 minutes. The optical density (OD) of the supernatant was measured at 540 nm (peak absorbance of haemoglobin in the test system) using the appropriate water, PBS or NaCl blank. For each assay, the haemolysis (%) was calculated as given below. The mean values of % haemolysis, with SD, were then calculated for each sample.

$$\% \text{ haemolysis} = \frac{\text{optical density (control or test)}}{\text{optical density (Water)}} \times 100$$

9.3 Investigation of the effect of osmolality and pH on the haemolysis assay

9.3.1 Aims and objectives

A preliminary study was performed to investigate the sensitivity of the haemolysis assay to fluctuations in the osmolality and pH of the incubation media. This enabled limits to be set for subsequent assay media in terms of their osmolality and pH. This was an important consideration when evaluating the haemolytic effects of various enhancer solutions since these may considerably alter the osmolality and pH of the diluent particularly at high concentration. The latter is of particular interest for the testing of chitosan solutions since these are prepared at pH 4.

9.3.2 Materials and Methods

The general materials used were as listed in Section 9.2. To investigate the effect of osmolality on erythrocyte haemolysis, solutions of PBS at pH 7.0 were prepared containing 0, 0.05, 0.10, 0.30, 0.50, 0.70, 0.85, 0.90, 0.95, 1.20, 1.50, 1.80, 2.25 and 2.70 % w/v NaCl. The values of osmolality of these solutions ranged from 0.024 to 0.855 Osmol/kg. The osmolality of 'isotonic PBS' (containing 0.9% NaCl) was 0.302 Osmol/kg. Values of osmolality are given in Table 9.1. To investigate the effect of pH on erythrocyte haemolysis, solutions of isotonic PBS were prepared at pH 8.5, 8.0, 7.5, 7.0, 6.5, 6.0, 5.5, 5.0, 4.5, 4.0, 3.5, 3.0, 2.5 and 2.0. The solutions of PBS of varying osmolality and pH were used as the test media. The haemolysis assay was performed as detailed in Section 9.2.

9.3.3 Results and Discussion

In the haemolysis assays performed in this study, values of basal haemolysis (i.e. that caused by incubation with isotonic PBS) were in the region of 22% (Table 9.1. These values were much higher than the values of 5% basal haemolysis reported by Chandler (1992). Gill et al. (1994a) reported that basal values of haemolyssis were less than 20% although specific values were not given. Despite the relatively high values of basal haemolysis obtained, this study was still useful for evaluating the effect of tonicity and pH on the haemolysis of rat erythrocytes.

PBS (pH 7) containing concentrations of NaCl of 0.3 % or lower (osmolality of ≤ 0.118 Osmol/kg) resulted in total (100%) haemolysis of rat erythrocytes (Table 9.1, Figure 9.1). Between concentrations of 0.3 and 0.5 % NaCl (0.118-0.181 Osmol/kg) the haemolytic effect was dramatically reduced. At 0.5 % NaCl the mean haemolysis was approximately 43 % (± 2.7). Increasing the NaCl concentration from 0.5 to 0.7 % NaCl (0.181-0.243 Osmol/kg), reduced the degree of haemolysis further. At 0.243 Osmol/kg values of haemolysis were similar to the values of basal haemolysis (isotonic PBS). Further increase in the concentration of NaCl in the lysate, up to 1.2 % (0.393 Osmol/kg) did not greatly alter the degree of haemolysis from that of the basal value. Increasing osmolality above 0.393 Osmol/kg resulted in only small increases in the degree of haemolysis even at 0.855 Osmol/kg (2.7% NaCl). Thus, in the study, the degree of haemolysis was not affected by changes in lysate osmolality in the range 0.243-0.393 Osmol/kg.

For isotonic PBS between pH 3.5-8.5, values of % haemolysis were similar to basal values (Table 9.1, Figure 9.2). However, further reductions in the pH of the lysate increased erythrocyte haemolysis. Thus in this study, the degree of haemolysis was not affected by changes in lysate pH in the range pH 3.5 to 8.5.

This study showed that rat erythrocytes are stable to a wide range of lysate osmolality and pH values. When evaluating the haemolytic effect of absorption enhancers, the solutions of enhancers should be maintained within these limits of osmolality and pH (0.243-0.393 Osmol/kg and pH 3.5-8.5). Thus, haemolysis, above the basal values which are inherent for the haemolytic assay, can be attributed to the haemolytic effects of the compound under investigation and not due to osmolality or pH effects on erythrocytes. In this study, either osmolality or pH was maintained constant when evaluating the effects of changes in pH or osmolality respectively. However, the sensitivity of erythrocytes to changes in osmolality may be influenced by changes in pH and vice versa which should be kept in mind when performing haemolysis studies. The haemolytic activity of certain compounds may also be influenced by osmolality or pH. Senisterra et al (1988) showed that the haemolytic activity of lysophosphatidylcholine on mutilamellar liposomes (model lipid membrane) increased in hypertonic solutions and decreased in hypotonic solutions. Thus, this should also be considerd when designing experiments.

Table 9.1. Summary of values of % haemolysis (lysis) obtained following the incubation of rat erythrocytes with PBS solutions of different tonicity and pH

Lysate		Mean % haemolysis	SD	Lysate	Mean % haemolysis	SD
Water		100.0	0.00	Water	100.0	0.00
PBS		21.7	4.11	PBS	21.5	3.70
PBS at different values of osmolality (pH 7)				PBS at different values of pH (~0.300 Osmol/kg)		
NaCl conc. (% w/v)	Osmolality (Osmol/kg)	% haemolysis	SD	pH	% haemolysis	SD
0.00	0.024	102.0	2.00	8.5	14.3	1.27
0.05	0.040	103.3	1.28	8.0	14.8	1.20
0.10	0.056	103.4	1.91	7.5	16.0	3.88
0.30	0.118	100.1	1.34	7.0	16.6	2.62
0.50	0.181	43.4	2.73	6.5	14.1	4.51
0.70	0.243	22.9	1.16	6.0	18.3	3.04
0.85	0.293	22.5	3.22	5.5	16.7	1.64
0.90	0.302	19.8	1.78	5.0	18.7	1.24
0.95	0.316	20.4	1.47	4.5	16.0	0.56
1.20	0.393	21.8	5.39	4.0	16.2	2.17
1.50	0.486	28.4	2.17	3.5	22.0	1.81
1.80	0.578	28.9	6.15	3.0	41.8	12.70
2.25	0.717	24.7	2.04	2.5	31.7	9.94
2.70	0.855	24.1	0.54	2.0	74.9	2.42

Figure 9.1. Effect of osmolality on values of % haemolysis

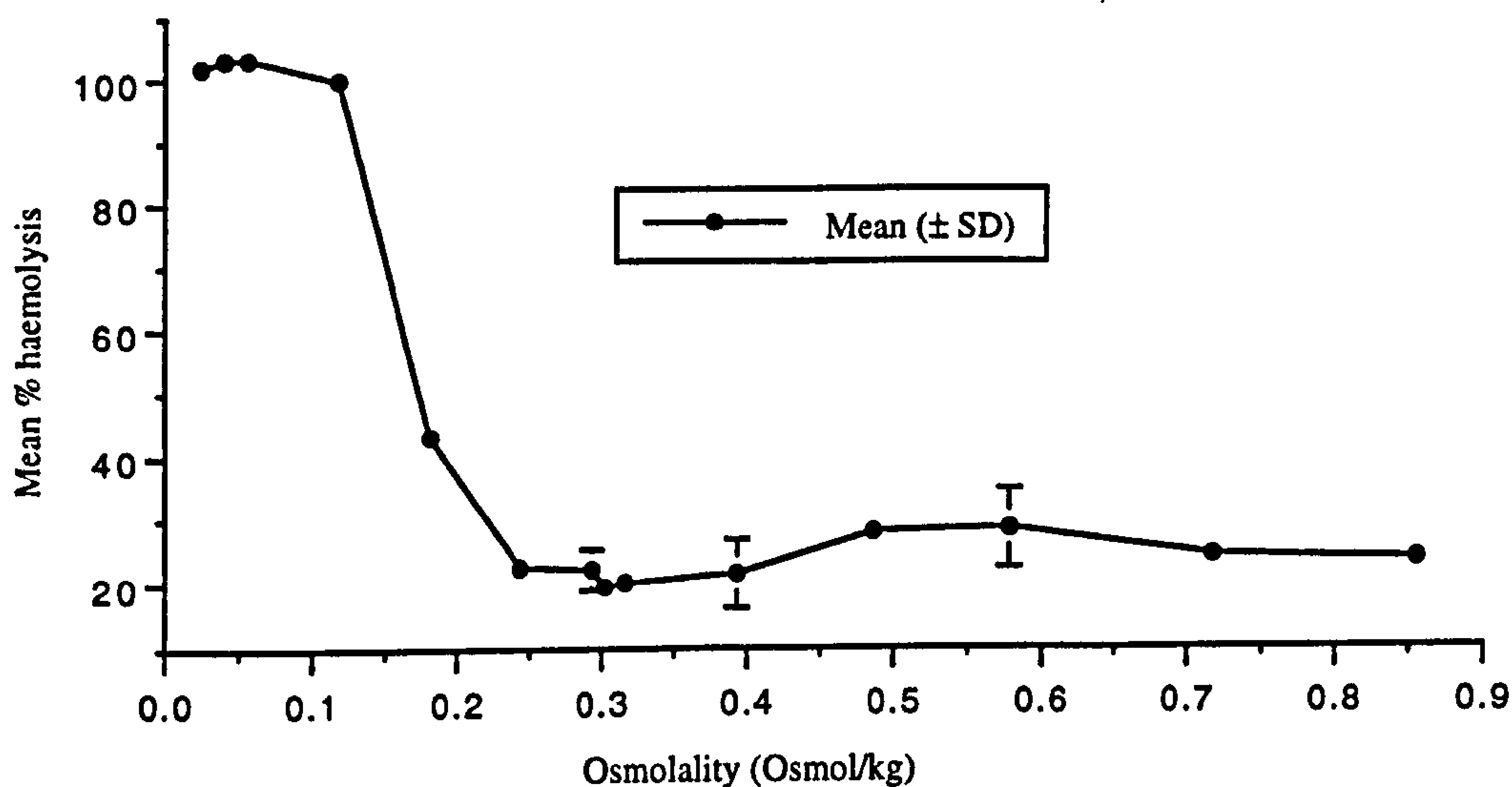
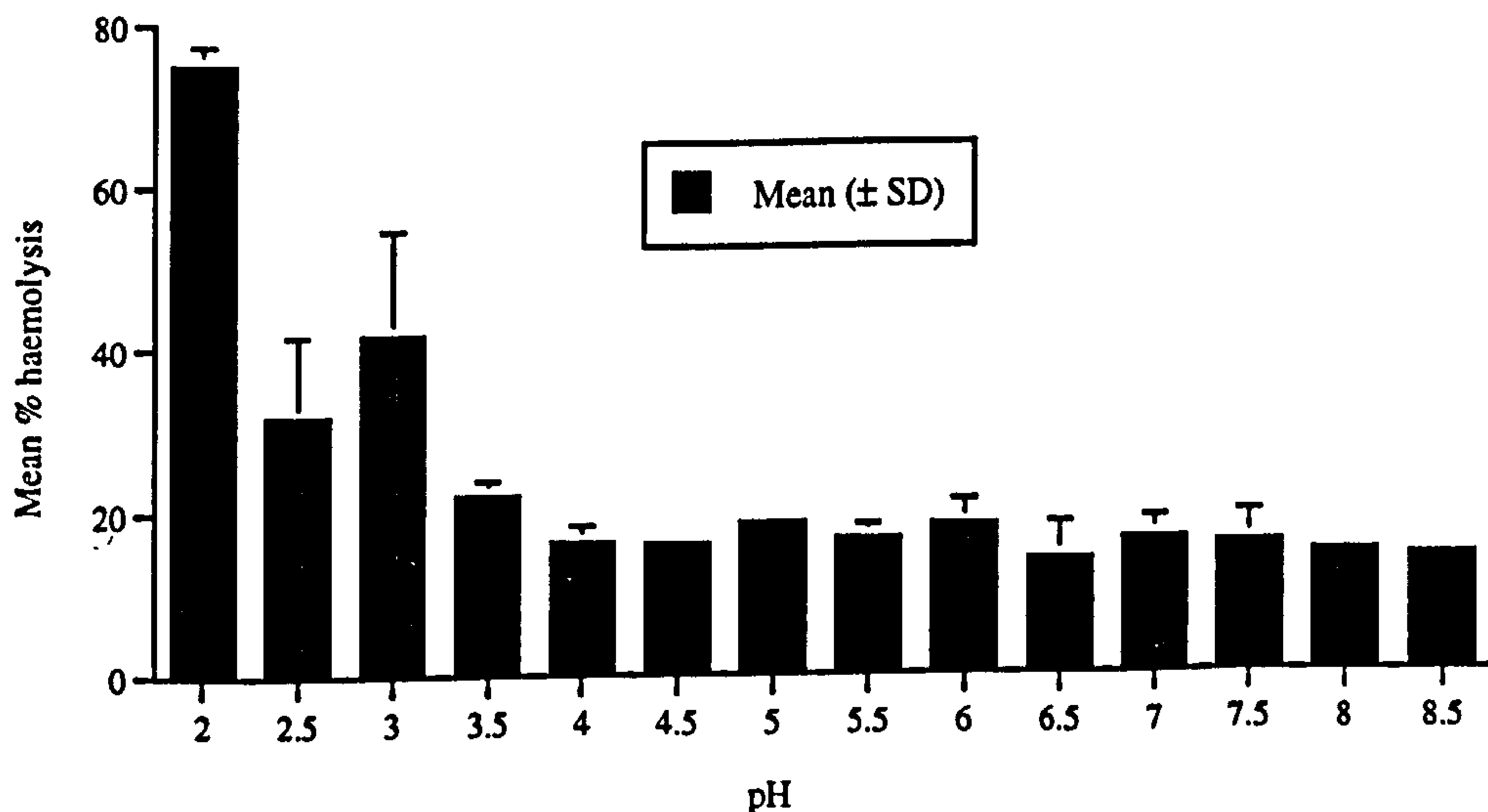


Figure 9.2. Effect of pH on values of % haemolysis



9.4 Investigation of the haemolytic effects of chitosan and various absorption enhancers

9.4.1 Aims and objectives

The nasal absorption enhancing efficacy of chitosan (MVCSN) and several other compounds (GDC, Laureth-9 and LPC) was evaluated in studies in the rat (Chapter 3). However, with the exception of chitosan, these compounds have been shown in various studies reported in the literature to cause damage to biological membranes which may, in-part, account for their absorption enhancing efficacy. The aim of this study, was to compare the haemolytic effects of MVCSN and the absorption enhancers GDC, Laureth-9 and LPC with a view to assessing the safety of chitosan.

9.4.2 Methods

The general materials used are as listed in Section 9.2. Additional materials are given in Section 3.3. Isotonic solutions of MVCSN, GDC, Laureth-9 and LPC were prepared in PBS (pH 7.0). MVCSN solutions in PBS were prepared at pH 4.0 by adjustment with HCl during preparation. MVCSN solutions at pH 4.0 were compared with solutions at pH about 5.3 which were prepared by dissolving / diluting MVCSN directly in PBS. Concentrations of MVCSN were expressed as the concentration of chitosan glutamate. The enhancer concentrations investigated are given in Table 9.2. The erythrocyte lysis assay was performed as described in Section 9.2. The assays were performed with 3.5 ml of control or test media except those which used LPC solutions where the volume of control or test media was reduced to 1.75 ml.

To directly compare the haemolytic activity of the enhancers, the concentration of enhancer at which 50% haemolysis occurred (L_{50}) was estimated. In the calculation of L_{50}, differences between the values of basal haemolysis (incubations with PBS) in each assay were taken in to account. Thus, the haemolysis was attributed solely to the effects of the enhancer. The calculation was performed as follows:

$$\%\ \text{haemolysis} = \frac{\text{optical density (Test)} - \text{optical density (PBS)}}{\text{optical density (Water)}} \times 100$$

Values of mean % haemolysis were plotted against enhancer concentration (not shown) from which values of L_{50} were estimated.

9.4.3 Results and Discussion.

Table 9.2 shows that at enhancer concentrations of 0.0 mg/ml, indicating values of basal haemolysis, there was considerable variation in the values obtained (~ 8-22%). The reasons for these variations are not known but may reflect an inherent sensitivity of rat erythrocytes to slight fluctuations in experimental conditions. The results for MVCSN were misleading, with values of haemolysis for a 0.5% solution at pH~6 calculated in excess of 100% (Table 9.2, Figure 9.3). Following centrifugation of the tubes containing MVCSN (both at pH~6 and pH4), aggregates of apparently intact erythrocytes

were observed in the supernatant and adhered to the walls of the tube especially in the tubes containing the higher concentrations of MVCSN. This was probably due to the relatively high viscosity of MVCSN solutions which prevented the sedimentation of intact erythrocytes. The effect appeared to be more pronounced with MVCSN at pH 5.3 than MVCSN at pH 4 which could have reflected differences in the viscosities of the respective solutions. Subsequently, the apparent viscosity of two 0.5% solutions of MVCSN, one at pH 4 and the second at pH 5.3 were measured using a Brookfield DV-III Programmable Rheometer (refer to Section 10.5 for further details of the method). At 10 rpm, MVCSN at pH 5.3 had a viscosity of 9.78 cps (± 0.060, n=3) compared to 9.52 cps (± 0.046, n=3) at pH 4. The higher viscosity of the solution at pH 5.3 may explain the reasons for the higher optical density readings obtained at this pH. The viscosity of chitosan in solution will usually increase as pH is reduced although at relatively high ionic concentration, such as in 0.9% NaCl, the effects of ionic concentration predominate. The increased viscosity at higher pH is probably due to the combined effects of pH and ionic concentration and the effects of the glutamate moiety of the chitosan salt. Since the centrifugation step employed in the assay appeared to be inadequate for the sedimentation of intact erythrocytes it was probable that the values of haemolysis calculated for chitosan solutions at the higher concentrations were anomalously high. Thus, this was kept in mind when assessing the haemolytic potency of chitosan in this study. Further investigations were performed to evaluate the effect of centrifugation on the values of % haemolysis obtained in the assay the details of which are given in Section 9.5.

The results of this study show that the compounds investigated exhibited varying degrees of haemolytic potency (Tables 9.2-9.3, Figure 9.3). LPC had the greatest haemolytic activity with a value of L_{50} estimated at 4.5×10^{-4} % w/v compared to values of 4.1×10^{-3}, 0.08, 0.18 and 0.25 % w/v for Laureth-9, GDC, MVCSN pH~5.3 and MVCSN pH 4, respectively. The values obtained for LPC and Laureth-9 are in agreement with the values reported by Chandler (1995) of 5.2×10^{-4} % w/v and 4.1×10^{-3} % w/v, respectively. Thus, the compounds investigated in this study ranked in order of haemolytic potency as follows: LPC >> Laureth-9 >> GDC > MVCSN pH 5.3 > MVCSN pH 4

The ranking of the compounds LPC, Laureth-9 and GDC was in agreement with results published by Gill et al. (1994a) (values of L_{50} were not reported). MVCSN was shown to have the lowest haemolytic activity despite suggestions of anomalously high values of haemolysis.

Values of L_{50} used to rank the compounds in order of haemolytic potency will depend on the cell density used in the incubation media and thus depend on the experimental conditions (Weltzien et al., 1977). For lysophospholipids values of L_{50} are thought to reflect their binding affinities to erythrocytes. A more accurate measure of the actual membrane disruption may be obtained by determination of the cell-bound amounts of the

lysate at 50% haemolysis (A_{50}) which is independent of experimental conditions. However, determination of values of A_{50} requires more complex assay procedures involving the use of radiolabelled compounds. In this study, since the haemolytic activity of the compounds was assessed using the same erythrocyte stock solution, thus the cell density of the incubation media was assumed to be identical, then values of L_{50} were considered appropriate for comparing the haemolytic activity of the various compounds. The fluctuations in basal values of haemolysis were taken into account during the calculation of values of L_{50}. Haemolysis may be due to the disruption of the normal molecular organisation of the cell membrane, due to the removal of membrane lipids into mixed micelles with the lysate molecule (Helenius & Simons, 1975) or due to the binding and incorporation of the lysate into the lipid matrix of the membrane (Weltzien , 1979), which lead to permeability changes and leakage of haemoglobin.

The order of haemolytic potency did not correlate with the order of nasal absorption enhancing potency demonstrated in Chapter 3 where the compounds were ranked in the order: LPC > MVCSN pH 4 >> GDC > Laureth-9.
LPC was the most effective absorption enhancer but also had the greatest haemolytic activity. In contrast, the absorption enhancing efficacy of MVCSN pH 4 was only marginally less effective than that of LPC but its haemolytic activity was the lowest of the compounds tested. Laureth-9 was shown to be only moderately effective as an absorption enhancer despite being a potent haemolytic agent. GDC also showed moderate absorption enhancing efficacy although in contrast to Laureth-9 its haemolytic activity was relatively low. With the exception of MVCSN, the compounds investigated in this study, particularly LPC and Laureth-9, were shown to cause maximal haemolysis at concentrations which were much lower than the concentrations at which they effectively enhanced nasal insulin absorption in the rat.

The haemolytic potency of LPC and Laureth-9 correlates with the severe damage that these compounds have been shown to cause to the rat nasal mucosa in vivo (Chandler et al., 1991a & 1991b). In this study the bile salt GDC was shown to exhibit similar haemolytic activity (L_{50} = 0.08 % w/v) to that of the bile salt derivative STDHF (L_{50} = 0.045 % w/v) reported by Chandler et al (1995). Chandler et al. (1991b) showed that STDHF caused less damage to the rat nasal mucosa than Laureth-9 at a similar concentration. The low haemolytic activity of MVCSN supports the histological studies of Illum et al. (1994) which showed that exposure of the rat nasal mucosa to 0.5% chitosan for a period of 60 minutes did not result in any significant changes in nasal morphology. The results from the haemolysis studies results are encouraging since they suggest that MVCSN is relatively non-damaging to biological membranes. Furthermore, since the haemolytic activity of MVCSN recorded in this study was suspected to be anomalously high it was decided that this would be further investigated in haemolysis studies.

Table 9.2. Summary of values of % haemolysis (lysis) following the incubation of rat erythrocytes with solutions of MVCSN or various other absorption enhancers

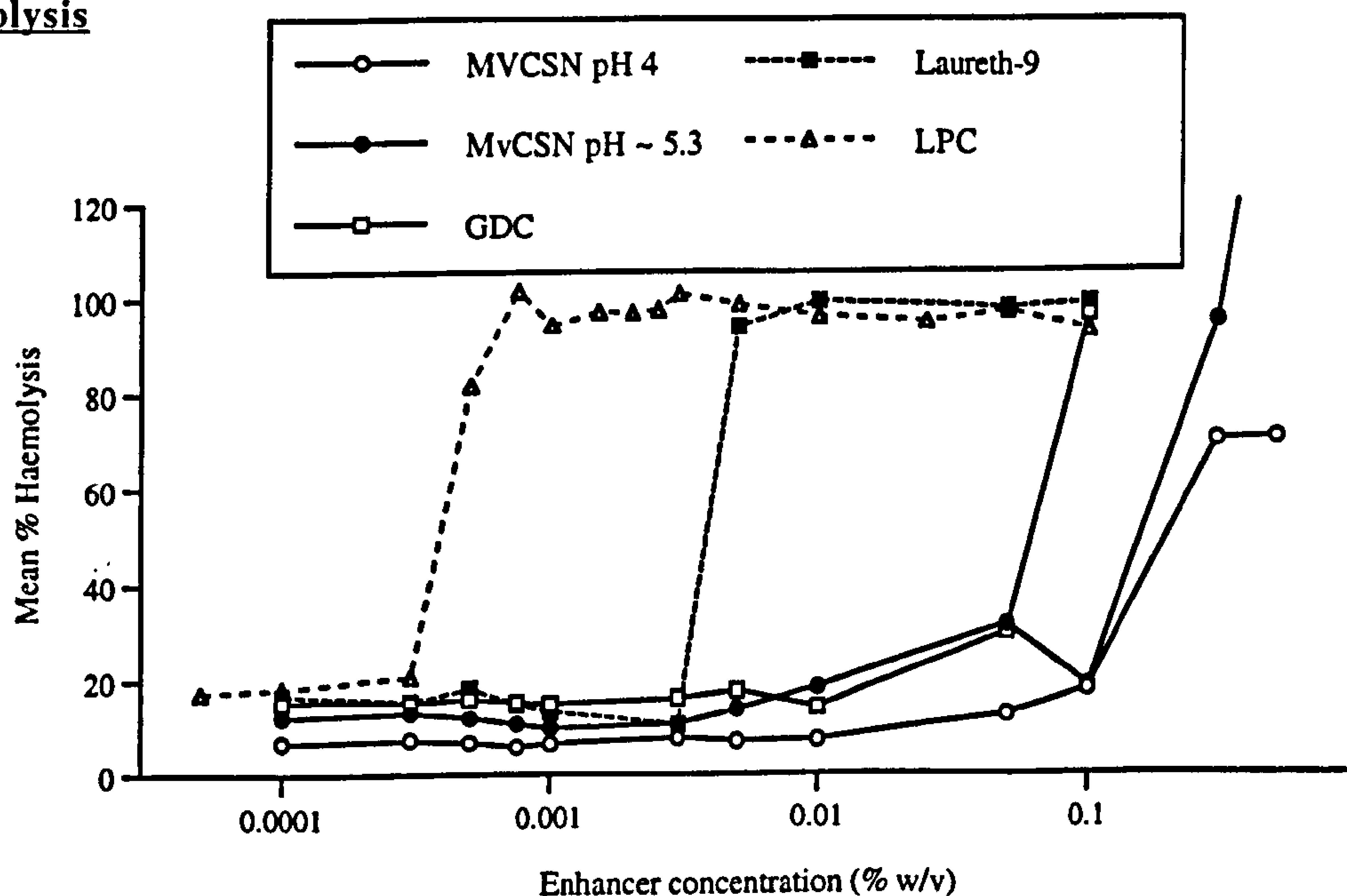
Lysate	Mean % haemolysis (± SD)									
water	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
Enhancer conc. (mg/ml)	MVCSN pH 4	SD	MVCSN pH 5.3	SD	GDC	SD	Laureth-9	SD	LPC	SD
0.0000	7.9	0.46	146	5.36	17.6	2.02	21.6	3.53	17.0	2.04
0.0001	-	-	-	-	-	-	-	-	17.3	1.54
0.0001	6.9	0.61	12.3	4.12	15.4	2.14	16.9	0.53	18.4	2.56
0.0003	7.4	1.52	13.1	4.84	15.3	2.35	15.5	1.00	20.7	4.46
0.0005	6.8	0.80	12.1	3.84	16.0	3.11	18.2	3.42	81.7	10.41
0.0008	6.0	0.61	10.8	2.75	15.3	1.45	14.9	0.41	101.7	9.74
0.0010	6.6	1.05	9.9	1.39	14.9	2.35	13.3	0.81	94.6	3.53
0.0015	-	-	-	-	-	-	-	-	97.3	4.60
0.0020	-	-	-	-	-	-	-	-	97.1	4.05
0.0025	-	-	-	-	-	-	-	-	97.7	3.10
0.0030	7.8	0.25	10.7	2.42	16.0	1.23	10.5	0.89	101.0	3.51
0 0050	7.1	0.83	13.8	3.81	17.6	0.87	94.2	4.37	98.8	2.55
0.0100	7.3	1.08	18.3	6.05	14.1	0.50	99.5	1.02	96.4	5.66
0.0250	-	-	-	-	-	-	-	-	95.1	4.53
0.0500	12.5	1.87	31.3	5.71	29.7	2.06	98.0	0.45	97.4	3.24
0.1000	17.8	9.62	18.5	8.29	93.6	4.35	99.0	0.85	93.3	2.62
0.3000	70.1	2.33	95.3	20.86	-	-	-	-	-	-
0.5000	70.8	0.78	183.2	28.51	-	-	-	-	-	-

Table 9.3. Haemolytic activity (values of L_{50}) of MVCS and various other absorption enhancers

Enhancer	L_{50} (% w/v)
MVCSN pH 4	0.25
MVCSN pH 5.3	0.18
GDC	0.08
Laureth-9	4.5×10^{-3}
LPC	4.5×10^{-4}

* Concentration of MVCSN expressed as concentration of chitosan glutamate

Figure 9.3. Effect of concentration of MVCSN or absorption enhancer on values of % haemolysis



9.5 Effect of relative centrifugal field (RCF) and duration of centrifugation on the apparent haemolytic activity of MVCSN

9.5.1 Aims and objectives

The values of % haemolysis calculated following the incubation of rat erythrocytes with viscous solutions of MVCSN may have been anomalously elevated due to insufficient centrifugation during the assay procedure. This could result in intact erythrocytes remaining in suspension in the lysate medium thus, producing anomalously high values of optical density. In this study the effect of the relative centrifugal field (RCF) and the duration of centrifugation on the apparent haemolytic activity of MVCSN was investigated. For this purpose solutions of 2% MVCSN were tested under different conditions of RCF and periods of centrifugation. In each assay incubations with water and PBS served as the positive and negative controls, respectively.

9.5.2 Materials and Methods

The general materials used are as listed in Section 9.2. Additional materials are given in Section 3.3. A solution of 2% MVCSN, expressed as the concentration of chitosan glutamate, was prepared in PBS. During preparation the pH of the solution was adjusted to pH 4.0 with 1M HCl. Each assay consisted of incubations with water, PBS and 2% MVCSN and these were performed in triplicate. Following the incubations the samples were centrifuged for 2, 5, 10 or 20 minutes at approximately 581 x g (1800 rpm), 1122 x g (2500 rpm) and 2198 x g (3500 rpm) in a MSE Mistral 2000 centrifuge or 2900 x g (6500 rpm) and 11600 x g (13000 rpm) in a MSE Microcentaur centrifuge. Other details of the assay were as given in Section 9.2.

9.5.3 Results and Discussion

Results are shown in Table 9.4 and Figure 9.5. The results show that the conditions of centrifugation (RCF and duration of centrifugation) greatly influence values of % haemolysis calculated for MVCSN solutions (Figure 9.5A). However, these conditions did not appear to markedly affect values of basal haemolysis (Figure 9.5B) or values of optical density at 100% haemolysis (not shown). For each of the assays performed, following incubation with water, the variation in values of optical density were low (mean ± SD = 1.046 ± 0.0534, n=60, CV = 5.1%). Variation in values of basal haemolysis were also reasonably low (0.117 ± 0.0267, n=60, CV = 22.8%).

Following incubations with MVCSN solutions, values of mean % haemolysis generally decreased with increases in RCF and time of centrifugation indicating more efficient sedimentation of intact erythrocytes. Irrespective of RCF, centifugation for only 2 minutes did not provide satisfactory sedimentation of erythrocytes. Centrifugation for 5 minutes gave acceptable separation of erythrocytes from the supernatant at 11600 x g but not at any of the other values of RCF investigated. At 11600 x g, increasing the centrifugation time from 5 to 20 minutes did not significantly increase the values of mean % haemolysis obtained and these values were much lower than those obtained for all of

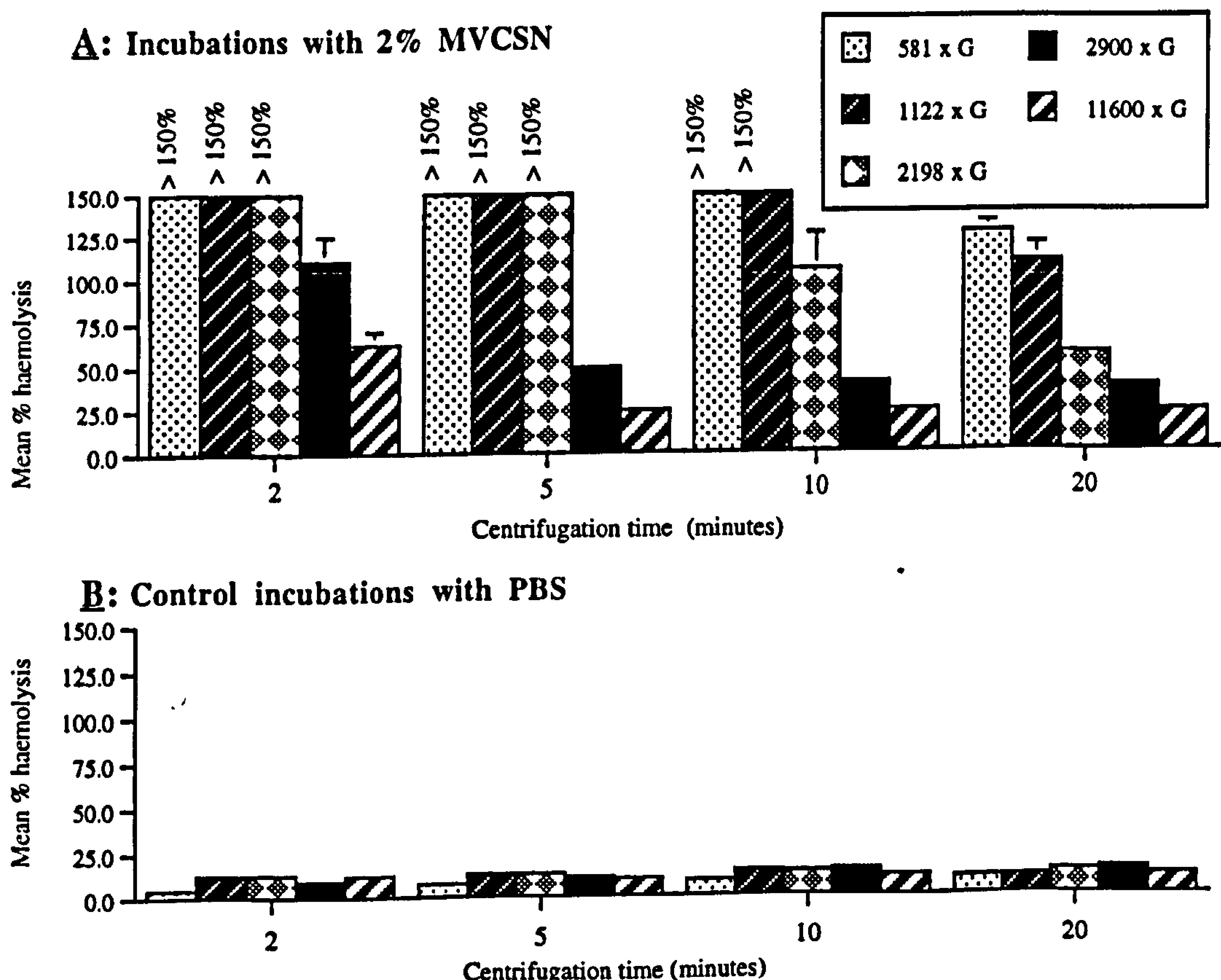
other combinations of RCF and centrifugation time. Thus, the study shows that without adequate centrifugation values of haemolysis of viscous solutions will be anomolously high. Centrifugation at 11600 x g for 5 minutes has been shown to be adequate for the sedimentation of intact erythrocytes in viscous solutions of MVCSN (2 % w/v) and thus, these conditions of centrifugation will be used to assess the haemolytic activity of chitosan solutions with greater accuracy.

Table 9.4. Summary of values of % haemolysis (lysis) following the investigation of the effect of relative centrifugal field (RCF) and duration of centrifugation on the haemolytic activity of MVCSN

Centrifugation time (minutes)	Lysate	Mean % haemolysis (± SD)									
		Centrifugal Field									
		581 x G	SD	1122 x G	SD	2198 x G	SD	2900 x G	SD	11600 X G	SD
2	water	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
	PBS	4.3	0.14	12.6	0.93	12.9	1.35	9.3	0.22	12.1	0.14
	MVCSN	220.8	21.45	184.8	31.56	164.0	5.48	110.6	14.12	62.1	7.92
5	water	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
	PBS	7.4	0.79	12.8	0.72	12.9	0.94	11.0	0.06	10.1	0.34
	MVCSN	175.7	38.36	167.8	9.60	167.4	15.56	48.7	4.06	24.0	0.70
10	water	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
	PBS	8.9	0.62	13.8	0.37	13.1	0.22	14.4	0.35	11.1	0.23
	MVCSN	151.4	2.15	168.3	6.63	105.5	21.31	40.1	4.11	23.5	0.26
20	water	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
	PBS	9.7	0.99	10.0	0.45	12.8	1.47	14.3	0.29	10.8	0.58
	MVCSN	127.3	5.69	110.3	10.46	56.7	1.09	38.3	1.82	23.1	0.32

* MVCSN tested at 20 mg/ml (concentration expressed as concentration of chitosan glutamate)

Figure 9.3. Effect of relative centrifugal field (RCF) and duration of centrifugation on the haemolytic activity of MVCSN



9.6 Further investigations of the haemoloytic effects of chitosan

9.6.1 Aims and objectives

Based on the findings of the previous study (Section 9.5), the aim of this study was to re-evaluate the haemolytic activity of MVCSN. For comparison the haemolytic activities of CSN lactate, CSN HCl and HVCSN were also assessed.

9.6.2 Methods

The general materials used are as listed in Section 9.2. Additional materials are as given in Sections 4.5 and 7.7. Isotonic solutions of MVCSN, CSN lactate and CSN HCl were prepared at pH 4.0 in 0.9 % NaCl. There were problems with the solution of CSN lactate which contained a fine suspension of insoluble material (different batch used from that used in nasal absorption studies). Attempt to filter the stock solution of LVCSN (2% CSN lactate) on 0.45 µm membrane filters was unsuccessful since the membrane quickly became blocked and hence filtration was performed on 5 µm membrane filters although particles were still observed in the filtrate. Due to the insolubility of HVCSN in NaCl at pH 4.0, isotonic solutions of HVCSN were prepared in 0.1% acetic acid and for comparison a solution of MVCSN were also prepared in acetic acid. The concentration of MVCSN, CSN lacate and CSN HCL was expressed as the concentration of chitosan salt. The concentration of HVCSN was expressed as the concentration of chitosan base. During each assay centrifugation was performed at 13000 rpm (11600 x g) for 5 minutes.

9.6.3 Results and Discussion

The results are given in Table 9.5 and Figure 9.6. The haemolytic activity of MVCSN was shown to be extremely low at concentrations up to 2%. Values of % haemolysis up to concentrations of 1.0% MVCSN were less than those of the control incubations with PBS or with NaCl (0% MVCSN) which suggests that chitosan protects the cell membrane at these concentrations. At 2% MVCSN, values of haemolysis were comparable to those of the control incubations. CSN HCL was shown to have a higher haemolytic activity than that of MVCSN resulting in 50% haemolysis (basal haemolysis not taken into account) at a concentration of about 0.5%. CSN lactate was shown to be more haemolytic than MVCSN at all concentrations investigated and above a concentration of 0.5% the haemolytic activity of CSN lactate increased considerably. This was attributed to the suspension of insoluble material in the CSN lactate solutions having a direct effect on the integrity of the cell membrane and thus, it was concluded that the results of the haemolysis assay for CSN lactate were anomalously high. Since the assay of CSN lactate was not repeated (a different batch would have been used) then conclusions on the haemolytic activity of CSN lactate could not be made. Following the assay of the MVCSN and HVCSN solutions prepared in acetic acid the basal values of haemolysis were observed to be higher (approximately 30%) than those in previous studies. However, taking the basal haemolysis into account, the haemolytic activity of MVCSN was still shown to be low although results were marginally higher than those obtained previously (in 0.9% NaCl) which may reflect increased sensitivity of the cell

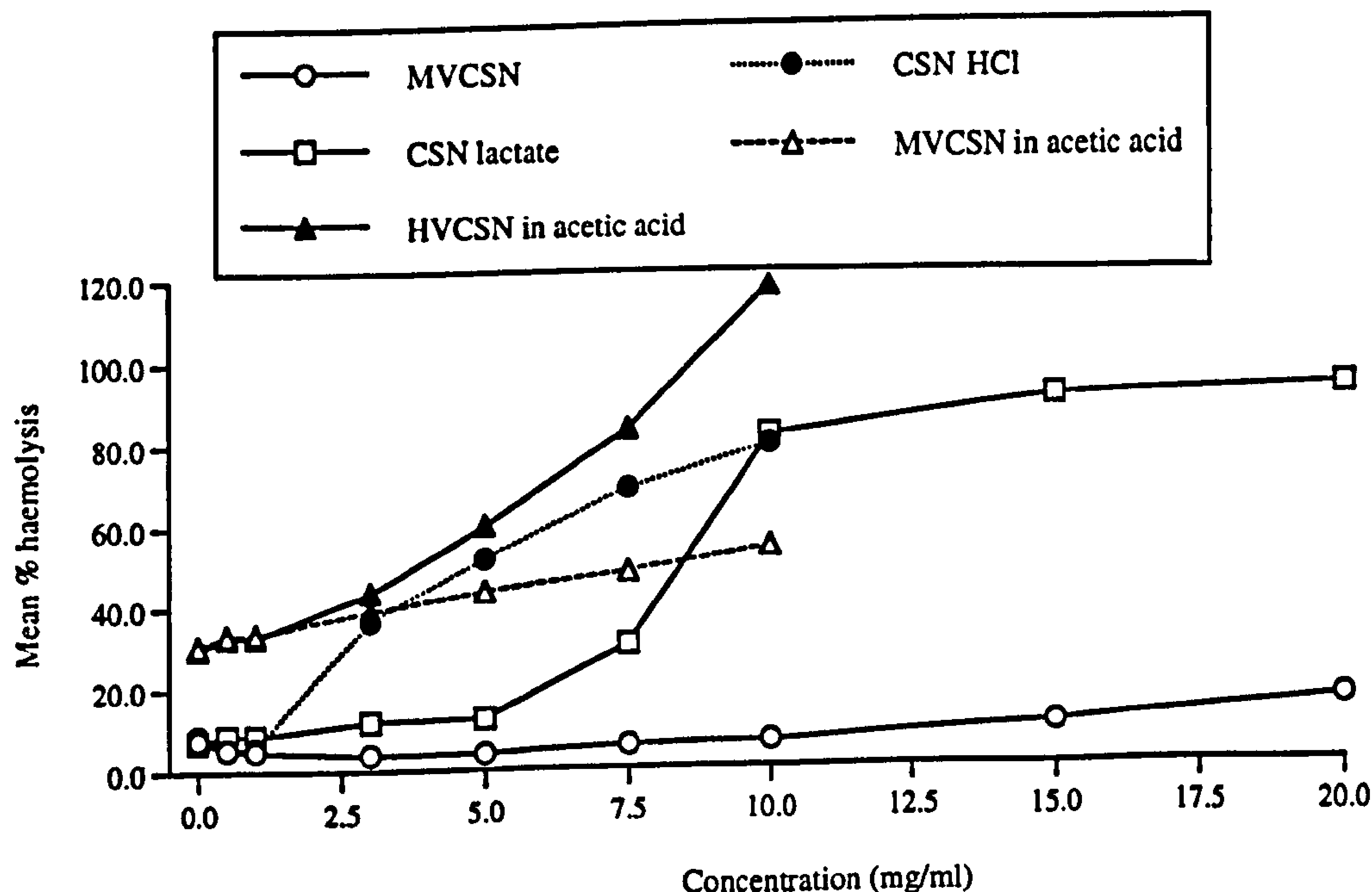
membrane to acetic acid. The haemolytic activity of MVCSN and HVCSN was shown to be comparable at concentrations up to 0.1% although above these concentrations HVCSN was apparently a more potent lytic agent. Judging from the degree of haemolysis of the 1.0% solution of HVCSN (values in excess of 100%), it is possible that the speed (RCF) and duration of centrifugation were insufficient to sediment intact erythrocytes in the high viscosity solutions and this should be kept in mind when reaching conclusions of the haemolytic activity of HVCSN.

Table 9.5. Summary of values of % haemolysis (lysis) following further investigation of the haemolytic activity of different grades of chitosan

Lysate	Mean % haemolysis (± SD)									
water	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00	100.0	0.00
PBS	8.3	0.31	11.1	2.09	8.9	2.21	11.3	1.26	14.7	3.21
chitosan conc (% w/v)	MVCSN	SD	CSN HCl	SD	CSN lactate	SD	MVCSN *	SD	HVCSN *	SD
0.0	7.9	0.50	8.9	0.73	7.4	0.23	30.9	0.21	30.0	0.47
0.05	5.5	0.33	5.7	0.35	8.8	0.07	33.3	0.80	32.9	0.46
0.1	5.1	0.28	5.9	0.48	8.7	0.38	33.7	0.18	32.9	1.07
0.3	3.9	0.19	36.7	3.00	11.9	0.20	39.0	1.41	43.5	2.95
0.5	4.0	0.69	51.8	2.58	12.5	0.24	43.8	2.10	60.0	1.16
0.75	5.6	1.66	68.8	3.27	30.4	1.47	48.4	0.48	83.4	2.71
1.0	6.2	0.70	79.4	13.82	81.7	2.99	54.2	0.76	117.9	3.6
1.5	10.2	0.73	-	-	90.5	2.60	-	-	-	-
2.0	16.0	1.42	-	-	92.9	4.62	-	-		

* Solutions prepared in acetic acid
Concentration of MVCSN, CVSN HCL and CSN lactate expressed as the concentration of chitosan salt
Concentration of HVCSN expressed as the concentration of chitosan base

Figure 9.6. Haemolytic activity of different grades of chitosan



9.7 Conclusions

Erythrocytes were shown to be a useful model for screening the membrane damaging effects of chitosan and various other compounds and based on studies reported in the literature, the haemolytic activity obtained correlated well with the degree of membrane damage of these compounds in vivo. The values of basal haemolysis of the rat erythrocytes was shown to be variable and thus improved accuracy may be obtained if human erythrocytes are used in future studies (Illum et al., Unpublished results). The haemolysis assay was shown to be stable over a wide range of values of osmolality (0.243-0.393 Osmol/kg) and pH (3.5-8.5). Thus, for test solutions within these limits of osmolality and pH, it was assumed that haemolysis above basal values, which are inherent for the haemolysis assay, is indicative of the haemolytic activity of the compound under investigation. For viscous chitosan solutions anomalously high optical density readings were obtained, hence corresponding values of % haemolysis were anomalously high, if both the relative centrifugal field (RCF) and duration of centrifugation were insufficient to sediment intact (un-haemolysed) erythrocytes. A RCF of 11600 x g (13000 rpm) for a minimum duration of 5 minutes was shown to be required when assaying most of the chitosan solutions.

The haemolytic activity of MVCSN was shown to be extremely low compared to the other absorption enhancers investigated. The compounds investigated ranked in order of haemolytic potency as follows: LPC >> Laureth-9 >> GDC > MVCSN. The haemolytic activity of a 0.5% solution of MVCSN was shown to be lower than that of basal values which implies that MVCSN protects the erythrocyte membrane, thus at this concentration MVCSN was shown to be non-toxic to the model membrane. In contrast, LPC, Laureth-9 and GDC caused maximal haemolysis at concentrations which were much lower than the concentration at which these compounds effectively enhanced nasal insulin absorption in the rat.

The haemolytic activity of MVCSN was less than that of CSN HCl and HVCSN although with HVCSN at the higher concentrations investigated the solution viscosity may have prevented the sedimentation of intact erythrocytes at the RCF and period of centrfiugation used in the assay. The studies with CSN lactate were inconclusive since although they indicated that CSN lactate had a much higher haemolytic potency than MVCSN this was attributed to insoluble impurities in the batch of CSN lactate used.

Overall, the studies demonstrated that MVCSN was relatively non-damaging to the erythrocyte model membrane up to concentrations of 2% w/v. From a safety perspective this further supports the potential application of MVCSN in nasal delivery systems.

CHAPTER 10

PHYSICAL CHARACTERISATION OF CHITOSAN

10.1 Introduction

The chitosan predominantly used in this project, medium viscosity chitosan glutamate (MVCSN), was characterised in terms of its partial specific volume, apparent molecular weight, intrinsic viscosity and apparent viscosity. For some of these techniques, the physical properties of other grades of chitosan were also determined. A brief description of the techniques used is given in the following sections:

10.2 Determination of partial specific volume, $\bar{v}$
10.3 Molecular weight determination by analytical ultracentrifugation
10.4 Determination of intrinsic viscosity
10.5 Determination of apparent viscosity

Measurements of partial specific volume, molecular weight and intrinsic viscosity were performed at the National Centre for Macromolecular Hydrodynamics, Department of Applied Biochemistry and Food Science, University of Nottingham, UK. Reference is made in this text regarding the general theory behind the measurement of these parameters to the work of Van Holde (1985). Additional references are given in the text.

10.2 Determination of partial specific volume, $\bar{v}$

10.2.1 Theory

Partial specific volume, $\bar{v}$, is the volume increment when 1 gram of solute is dissolved in an infinite volume of solution. Knowledge of the value of $\bar{v}$ is required in the calculation of molecular weight by ultracentrifugation where the term $(1-\bar{v}.\rho)$ is encountered (ρ is the solution density). Errors in the measurement of $\bar{v}$ will be magnified in the determination of molecular weight.

The value of v can be calculated from the equation given by Kratky et al. (1973):

$$\bar{v} = \frac{1 - d\rho/dc}{\rho_0}$$

Where $d\rho/dc$ is the change in solute density with concentration and ρ_0 is the solvent (buffer) density.

Density measurements were performed using a density meter which measures the period of oscillation, T, of a hollow glass oscillator filled with liquid sample. The principle of the measurement depends upon the variation of the natural frequency of the oscillator when filled with different liquids or gases. The mass and therefore density of the liquid

or gas changes this natural frequency because of the gross mass change of the oscillator caused by the introduction of the sample. For the difference of the densities of two samples, ρ_1 and ρ_2:

$$\rho_1 - \rho_2 = (1/A)(T_1^2 - T_2^2)$$

where A is a predetermined density meter constant and T_1 and T_2 is the period of oscillation of the two samples respectively. The density meter constant is determined from calibration measurements with samples of caesium chloride of known density and is a function of the volume and the spring constant of the apparatus. The volume of liquid in the oscillator, which will influence its frequency, can always be accurately defined, as long as the oscillator is properly filled, since it is the volume which is between two fixed points.

10.2.2 Method

An Anton Paar digital DMA O2C precision density meter (Anton Paar, Graz, Austria) with a Techne thermostatically controlled water bath (TU-16A Tempunit) set at 25.00°C (± 0.05) was used to determine the densities of solvent or chitosan solutions. Water was circulated from the water bath to the density meter using a water pump.

Chitosan solutions in the range 2.0 to 10.0 mg/ml were prepared in 0.05 M acetate buffer of pH 4.0. The chitosan solutions, the solvent (acetate buffer) and a sample of de-ionized distilled water were incubated at approximately 28°C in a water bath prior to injection into the density meter since this slight elevation of temperature helped to prevent air bubbles in the oscillator. An excess of sample (1.0 ml) was injected into the oscillator using a 1.0 ml disposable syringe. The capacity of the ocillator was about 0.7 ml. The oscillator was then viewed through a window in the instrument to check for complete filling and to ensure that it was free of air bubbles. After 10 minutes, allowed for thermal equilibration, the time lapse to complete 10,000 oscillations was measured and displayed by the instrument. This was repeated several times to obtain a reproducible value. In between samples the oscillator was washed with distilled water followed by absolute ethanol and dried with an air line.

Using the equation given previously, ($\rho_1 - \rho_2 = (1/A)(T_1^2 - T_2^2)$), the density of the chitosan solutions were calculated. The slope of a plot of density versus chitosan concentration was used to determine $d\rho/dc$ from which $\bar{v}$ was calculated ($\bar{v} = (1 - d\rho/dc)/\rho_0$). The units of $\bar{v}$ are ml/g.

10.2.3 Results and Discussion

The values of density at different solution concentrations of MVCSN are given in Table 10.1 and Figure 10.1. The density of the buffer (ρ_0) was 0.9988 g/ml and the slope

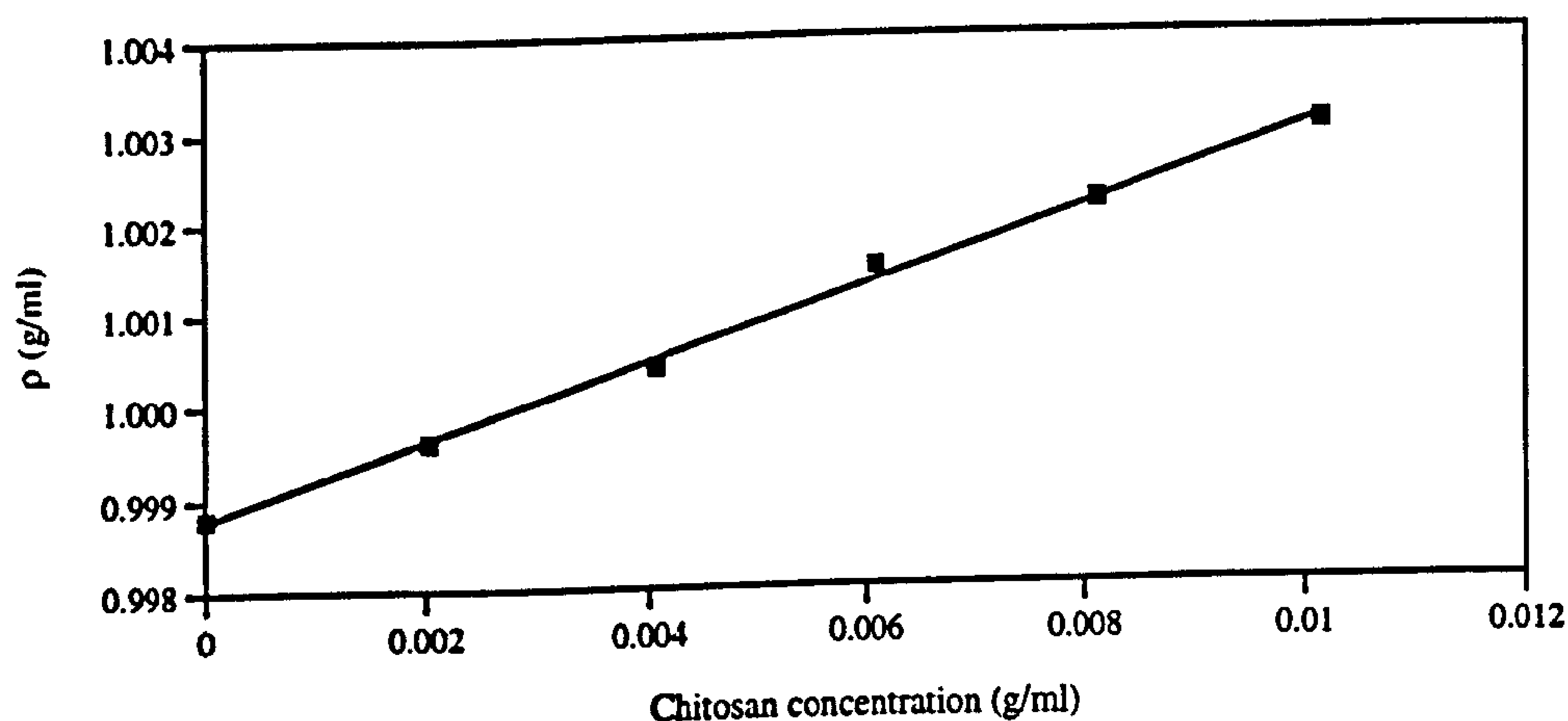
$d\rho/dc$ (± SE) was 0.4203 (± 0.01127). The value of partial specific volume, $\bar{v}$, (± SE) was 0.58 ml/g (± 0.011) as given in Table 10.1.

The value of $\bar{v}$ obtained was in excellent agreement with that reported by Errington (1993) and Errington et al. (1993) using a similar grade of chitosan (0.580 ± 0.011 ml/g). This value of $\bar{v}$ was subsequently used in the calculation of molecular weight of MVCSN by the technique of sedimentation equilibrium.

Table 10.1. Values of density (ρ) of different solutions of MVCSN and value of $\bar{v}$

MVCSN solutions	
Concentration (mg/ml)	ρ (g/ml)
0.00	0.9988
2.03	0.9996
4.07	1.0004
6.10	1.0015
8.13	1.0022
10.16	1.0030
$\bar{v}$ (± SE) = 0.58 ml/g (± 0.011)	

Figure 10.1. Plot of density (ρ) versus solution concentration of MVCSN used in the calculation of $\bar{v}$



10.3 Determination of molecular weight

10.3.1 Theory

A macromolecular solution will often be polydispersed i.e. will not contain molecules of the same molecular weight, M, and hence average molecular weights are often considered. Sedimentation equilibrium in the analytical ultracentrifuge is an absolute method to determine the weight-average molecular weight, M_w, which can be defined as:

$$M_w = \Sigma\, c_i.M_i \,/\, \Sigma\, c_i$$

in which c_i is the weight concentration (g/ml) of all molecules having molecular weight M_i.

The technique of sedimentation equilibrium uses relatively low speeds of rotation (typically 2000 to 15000 revs/min) so that a balance or equilibrium is established between the sedimentation of the molecules and their diffusion in the aqueous medium which is housed in a rotor cell. Sedimentation will tend to decrease the concentration of molecules at the meniscus and increase them at the cell base whereas diffusion tends to maintain the same concentration of molecules throughout the cell. In practical terms the molecules will sediment outwards from the centre of rotation. At equilibrium the concentration of the molecules will be lower at the meniscus and increased towards the cell base but there will be no net transport of matter i.e. sedimentation and diffusion are balanced. At equilibrium in an ideal two component system the concentration gradient for a single solute component can be given by:

$$1/c.(d\,c/\,d\,r) = \omega^2 r M\,(1-\bar{v}\rho)/\,R\,T$$

where: c = solute concentration (mass/volume), r = distance from the centre of rotation, ω = angular velocity, M = molecular weight, $\bar{v}$ = partial specific volume, ρ = solution density, R = Gas constant (8.31×10^7 ergs/degree/mol.), T = Absolute temperature.

For a polydisperse and heterogeneous system M will be a weight average molecular weight, M_w. For a non-ideal system and at finite concentration, c, M will be an apparent molecular weight, $M_{w,app}$, although as $c \rightarrow 0$, $M_{w,app} \rightarrow M_w$ (Tanford, 1961). The events during sedimentation are most accurately followed by Rayleigh interference optics which detect the changes in the refractive index (n) of the sample and the solvent which are loaded into different compartments of a double sector cell (Harding and Rowe, 1988). The resultant fringe pattern produced can be readily observed and recorded photographically. Each fringe traces a curve of the refractive index versus radial distance in the cell. The displacement of the fringes can be related to the solute concentration (c) increments in the cell since dn/dc is assumed to be constant. Although the measured solute concentration increments are relative, absolute concentrations, in terms of fringe numbers, at the meniscus of the solute column can be determined and hence absolute

concentrations determined at all other radial positions in the cell.

The absolute solute concentrations, in terms of fringe numbers, can be given by J such that J(r) is the absolute fringe concentration at radial position r and J(a) the absolute fringe concentration at the meniscus (a is the radial position at the meniscus). Mathematical processing of the fringe data can be used to determine weight average molecular weights (refer to Creeth and Harding, 1982, Harding et al., 1991). The (whole cell) apparent weight average molecular weight, $M_{w,app}$, measured over the whole distribution can be obtained from the limiting value at the cell base of the plot of M* (r) versus ξ (r) where M* (r) is an operational point average molecular weight, known as the "star" average, and ξ (r) is a function of the radial position as given below.

$$\xi(r) = (r^2 - a^2) / (b^2 - a^2)$$

where r = distance from centre of rotation, a = cell meniscus and b = cell base.

At the cell base ($\xi \rightarrow 1$) and M* (r)$\rightarrow M_{w,app}$(r) (Creeth and Harding, 1982). Alternatively, apparent point (local) weight average molecular weights, $M_{w,app}$(r), can be extrapolated to zero (fringe) concentration, J (r), by plotting $M_{w,app}$(r) versus J (r) to give $M_{w,\ app}$ (J$\rightarrow$0).

10.3.2 Method

Sedimentation equilibrium investigations were performed using a Beckman Model E analytical ultracentrifuge (Beckman Instruments UK Ltd) with an electonically controlled drive. Rayleigh interference optics were employed using a 5 mW helium-neon laser and a mercury arc light. A solution of MVCSN of 0.6 mg/ml was prepared in 0.05 M acetate buffer of pH 4, filtered on 0.45 µm syringe filters and dialysed overnight against the acetate buffer. A volume of 0.15 ml of the solution was loaded into the right-hand side and 0.15 ml of the dialysate (acetate buffer) loaded into the left-hand side of a 30 mm centrifuge cell (B II-8). Centrifugation was performed in an evacuated chamber at approximately 10,000 rpm and 25°C. Rotor temperature throughout the procedure was maintained at 25°C. The photographic images of the Rayleigh interference pattern were recorded throughout the procedure in the form of photographic negatives on 35 mm black and white film. When superimposable images were obtained, indicating equilibrium of the system, final images were obtained and the centrifuge run stopped. The fringe pattern obtained on one of the final photographic negatives was enlarged and processed using a LKB 2202 laser densitometer (LKB Instruments, Bromma, Sweden). The resultant digitised pattern was then processed using a Fourier cosine series Pascal alogarithm 'Analyser' to obtain data in the form of fringe concentrations, relative to the meniscus, versus radial dístance in the centrifuge cell. Full molecular weight analysis of the data was performed using the mainframe IBM 3081 computer at Cambridge. $M_{w,app}$ was obtained from the limiting value at the cell base ($\xi \rightarrow 1$) of the plot of M* (r) versus ξ (r). $M_{w,app}$(r) (J$\rightarrow$0) was obtained by extrapolation to zero (fringe) concentration of a plot of

$M_{w,app}(r)$ versus J (r). At zero concentration the effects of non-ideality $\rightarrow 0$ and hence the apparent $M_{w,app} \rightarrow$ the ideal Mw.

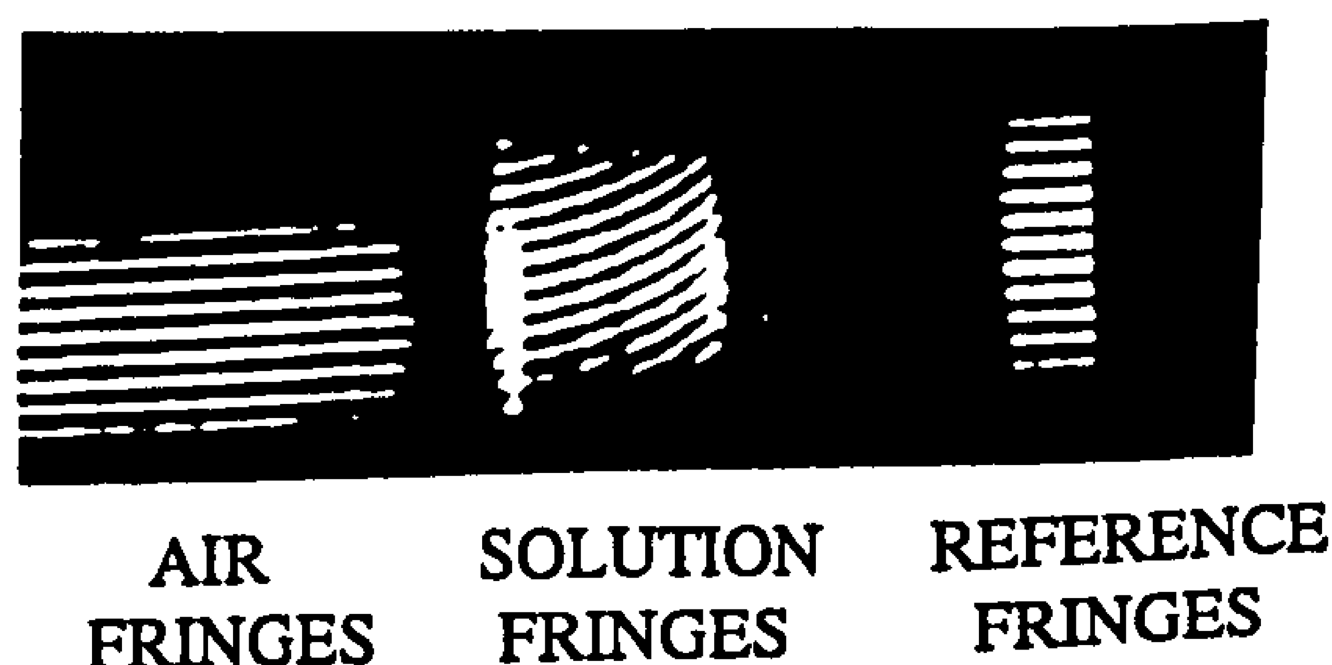
10.3.3 Results and Discussion

Plots of M* (r) versus ξ (r), $M_{w,app}(r)$ versus J (r) and Ln J (r) versus ξ (r) are shown in Figures 10.2-10.4. Values of $M_{w,appp}$ and $M_{w,app}(r)$ ($J \rightarrow 0$) for MVCSN, at a loading concentration of 0.6 mg/ml, are given in Table 10.2. The plot of Ln J (r) versus ξ (r) exhibited considerable downward curvature which was indicative of strong thermodynamic non-ideality through exclusion volume and charge effects (Harding et al., 1991). Thus, the thermodynamic effects of non-ideality outweigh the effects of polydispersity which would otherwise tend to produce upward curvature of the plot of Ln J (r) versus ξ (r). The values obtained for M_w are much lower than expected for this grade of chitosan based on values quoted by Pronova Biopolymer A/S in the region of 150,000-250,000. Errington (1993) and Errington et al. (1993) using the same batch of chitosan to the one used in this project reported a M_w of 162,000 g/mol (± 10,000) using sedimentation equilibrium. However, this was obtained by extrapolation, to zero concentration, of a plot of $1/M_{w,app}$ versus chitosan concentration. Also, lower concentrations of chitosan were also tested thereby minimising the effects of thermodynamic non-ideality (which tends to diminish measured molecular weights and mask heterogeneity (Harding et al., 1991)). Although the alternative method employed here of extrapolating point weight average molecular weights, $M_{w,app}$ (r), to zero fringe concentration, ($J \rightarrow 0$), effectively eliminates non-ideality, it could possibly have the effect of biasing the results towards the lower end of the molecular weight distribution in the sample if there were any re-distribution of sample at the rotor speed used. Attempt was made to reanalyse MVCSN solutions at lower concentrations (0.1-0.3 mg/ml). However, the resultant fringe deviations were too small to be adequately interpreted.

Table 10.2. Values of $M_{w,app}$ and $M_{w,app}$ (r) ($J \rightarrow 0$) for MVCSN at a loading concentration of 0.6 mg/ml

	$M_{w,app} \pm$ SE	$M_{w,app}$ (r) ($J \rightarrow 0$) $\pm$ SE
LVCSN	45000 ± 4000	65000 ± 4000

Figure 10.2. Example of Rayleigh interference optical fringe pattern



AIR FRINGES SOLUTION FRINGES REFERENCE FRINGES

Figure 10.3. M* (r) versus ξ (r) for MVCSN (loading concentration 0.6 mg/ml)

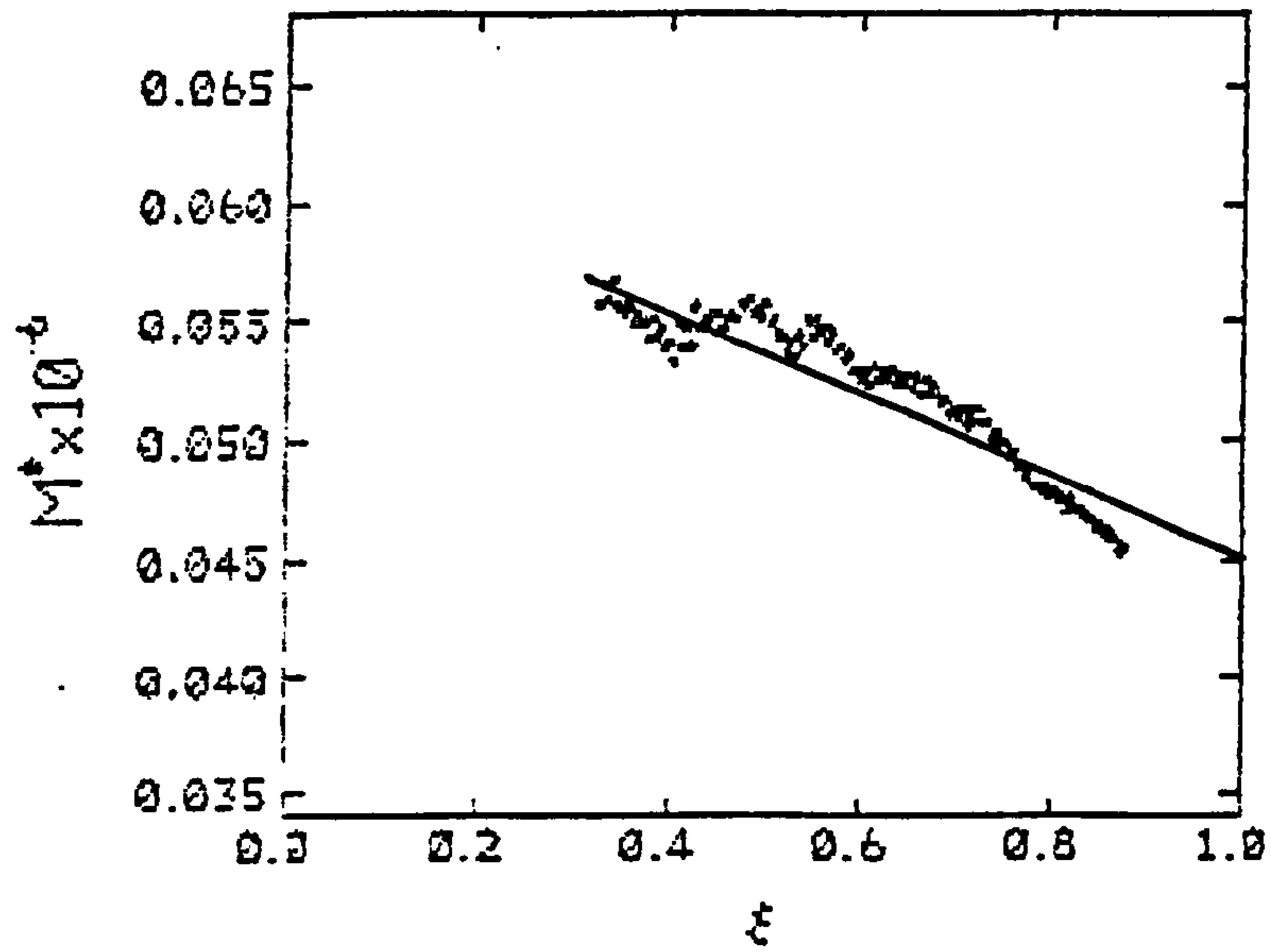


Figure 10.4. $M_{w,app}$ (r) versus J (r) for MVCSN (loading concentration of 0.6 mg/ml)

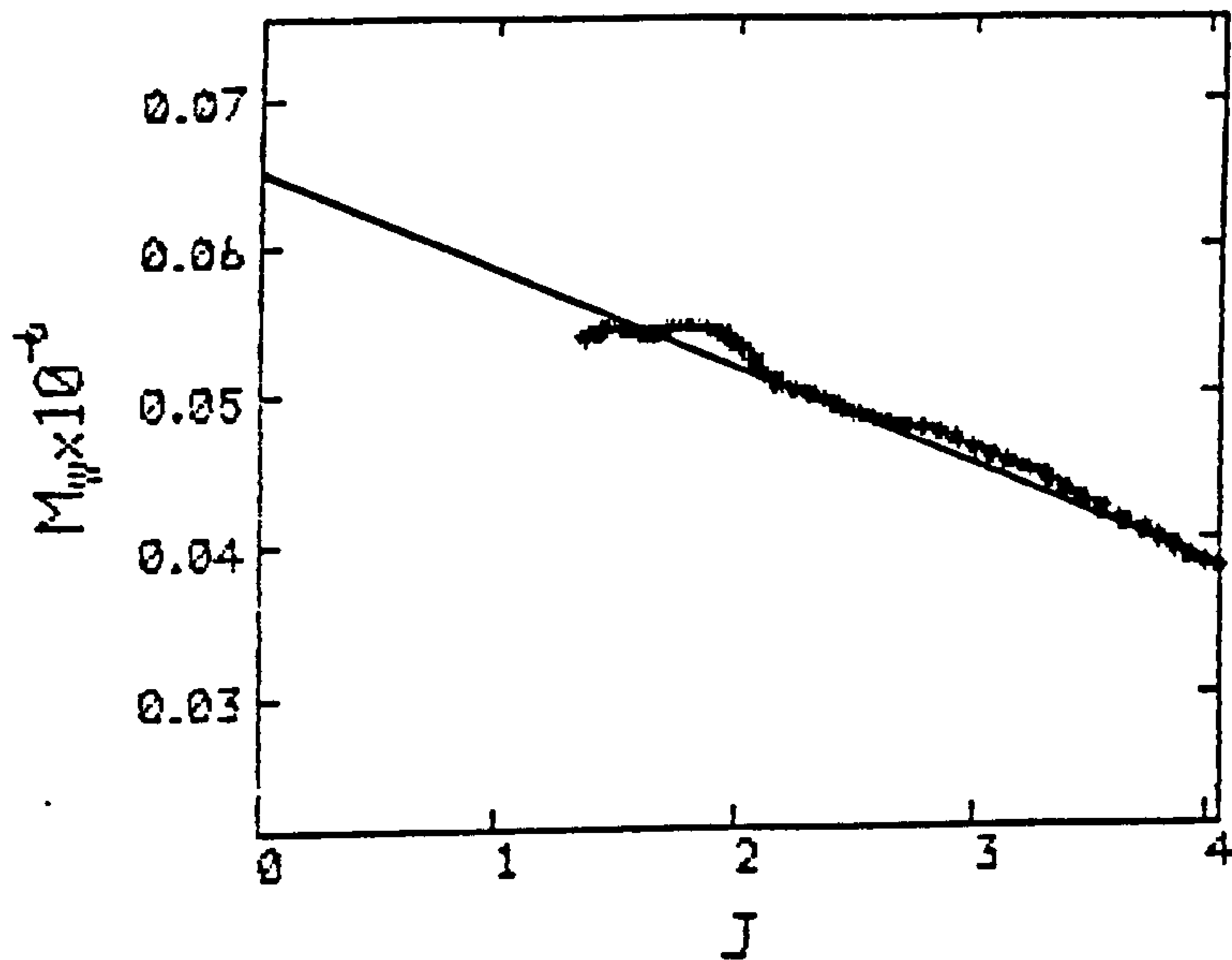
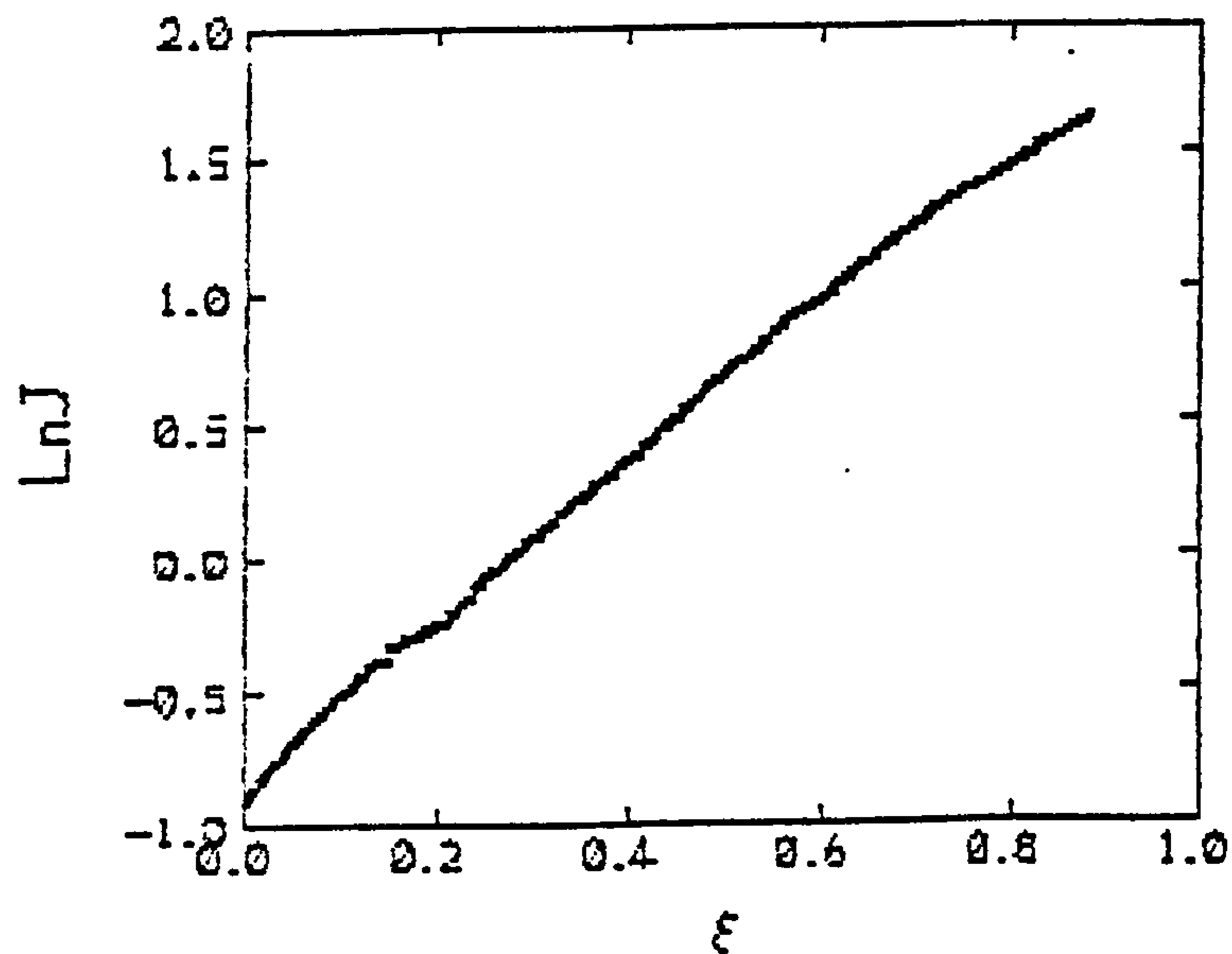


Figure 10.5. Ln J (r) versus ξ (r)for MVCSN (loading concentration of 0.6 mg/ml)



10.4 Determination of Intrinsic viscosity, [η].

Additional sources of reference are Schott (1995)

10.4.1 Theory

Rheology is a term which describes the deformation and movement of matter under the influence of stresses and covers the topics of viscosity, elasticity and plasticity. In physiology, the rheology of blood and mucus is vitally important in blood circulation and mucus transport, respectively. In the development of pharmaceutical products for nasal delivery, rheology will influence the spray properties of the liquid drug formulation. Viscosity is a measure of the internal friction of a fluid (i.e. a measure of the resistance of a liquid to flow). The greater the friction, the greater is the shear or force required to move the liquid, thus, high viscosity fluids require more force to move than less viscous fluids. Shearing occurs whenever a liquid is physically moved or distributed. Values of viscosity are 'absolute' when the value can be traced to the measurement of the test results in the absolute units of physics (unit of viscosity of: Pascal . second). Absolute values of viscosity are independent of the type or make of viscometer used. Capillary- and rotational cone and plate viscometers, both of which have been used in this project, can be used to measure values of absolute viscosity. The use of a cone and plate viscometer to measure the viscosity of a non-Newtonian fluid will yield 'apparent' values of viscosity since values obtained will depend on the shear rate applied to the sample. Since the speed of revolution of the cone (rpm) will govern the shear rate, values of viscosity obtained will apply only to the particular rpm used. Thus, to directly compare the viscosity of two or more samples requires that the samples were 'run' under identical conditions. Values of apparent viscosity will vary depending on the equipment used as well as on the run conditions.

Intrinsic viscosity, $[\eta]$, is a function of viscosity, η measuring the resistance of a liquid to flow. A macromolecular solution will have a viscosity, η, which will be higher than that of the solvent alone, η_0. The ratio of viscosities η/η_0 yields the relative dynamic viscosity, η_r, which can also be determined with knowledge of the flow times and densities of the solution (t and ρ, respectively) and solvent (t_0 and r_0, respectively) as follows:

$$\eta_r = \eta / \eta_0 = (t \cdot \rho) / (t_0 \cdot \rho_0)$$

(For very dilute solutions this approximates to t / t_0). Calculation of $[\eta]$ using t/t_0 gives the kinematic intrinsic viscosity $[\eta']$ which can later be corrected to dynamic viscosity $[\eta]$. An advantage of this method is that tedious determination of the density for each concentration of sample is not required. The following functions of η can then be calculated:

$$\text{kinematic relative visosity} = \eta'_r = t / t_0$$

$$\text{kinematic specific viscosity} = \eta'_{sp} = \eta'_r - 1$$

$$\text{kinematic reduced specific viscosity} = \eta'_{red} = \eta'_{sp} / c$$

where c is the solute concentration

$$\text{kinematic intrinsic viscosity} = [\eta'] = \text{limit}_{(c \to 0)} \, \eta'_{sp} / c$$

where limit $_{(c \to 0)}$ is the limit of zero concentration

[η'] can be corrected to [η] using the correction factor of Tanford (1955) as follows:

$$[\eta] = [(1 - \bar{v}. \rho_0) / \rho_0)] + [\eta']$$

where v is the partial specific volume of the macromolecule as described previously. In practice [η'] is determined by measuring η'_{sp} at several solute concentrations, plotting η'_{red} versus c (Huggins plot) and extrapolating to zero solute concentration. By extrapolation the intermolecular interactions in solution are removed and [η'] will depend on the properties of the isolated macromolecule. Alternatively, [η'] can be determined from a plot of ln η'_r / c versus c (Kraemer plot) and extrapolating to zero solute concentration. If dilute solutions are used then the density corrections for the various solute concentrations are assumed to be negligible and hence the kinematic intrinsic viscosity [η'] will be equal to the dynamic intrinsic viscosity [η]. The Huggins constant (k') can be determined from the equation:

$$\eta_{sp}/c = [\eta'] + k'[\eta]^2 c$$

10.4.2 Method

Chitosan solutions (LVCSN and MVCSN) were prepared in the range 0.2 to 1.0 mg/ml in 0.05 M acetate buffer of pH 4.0. Determinations of [η'] were performed using a Ubbelohde type micro-viscometer of capacity 3 ml (ref no. 537 13) in conjunction with a viscosity measuring system comprising an AVS 310 viscosity measuring unit, a CT 050/2 transparent thermostatic water bath and an AVS/S measuring stand (Schott-Geräte, Hofheim, Germany). A 3.0 ml volume of solvent or chitosan solution was dispensed into the lower reservoir of the viscometer using a pipette to deliver 3 x 1.0 ml volumes into the barrel of a 1 ml syringe, with its plunger removed, fitted with a 30 cm polypropylene cannula (Portex). The syringe plunger was then re-fitted and any droplets of sample in the syringe or cannula dispelled into the viscometer reservoir. Measurement of the flow times of sample liquids were fully automated. The viscosity measuring system was programmed to allow 10 minutes for the sample to reach thermal equilibrium following which it would perform and store 10 flow times per sample. The basis of the measurement is a follows:

The sample is pumped up past two measuring levels (light barriers) M1 + M2 in the

timing bulb. When liquid has passed the upper level M1, the unit switches off the pump, the viscometer is vented and as the sample runs back time measurement starts when the meniscus of the sample passes M1. The change in light intensity is converted to a digital electrical signal to start the internal crystal controlled clock with readout display. The acquisitioned flow time is then shown on the display and stored internally. In between samples the micro-viscometer was washed with 5% decon, 5% acetic acid, three times with de-ionized distilled water and finally with ethanol. The viscometer was dried with a vacuum line and then with nitrogen.

For each chitosan, $[\eta']$ was determined by plotting η'_{red} versus c (Huggins plot) and extrapolating to zero solute concentration. Since the concentration of chitosan in the solutions was low then it was assumed that corrections for the density of the solutions was negligible (thus $[\eta']$ was assumed to be equal to $[\eta]$).

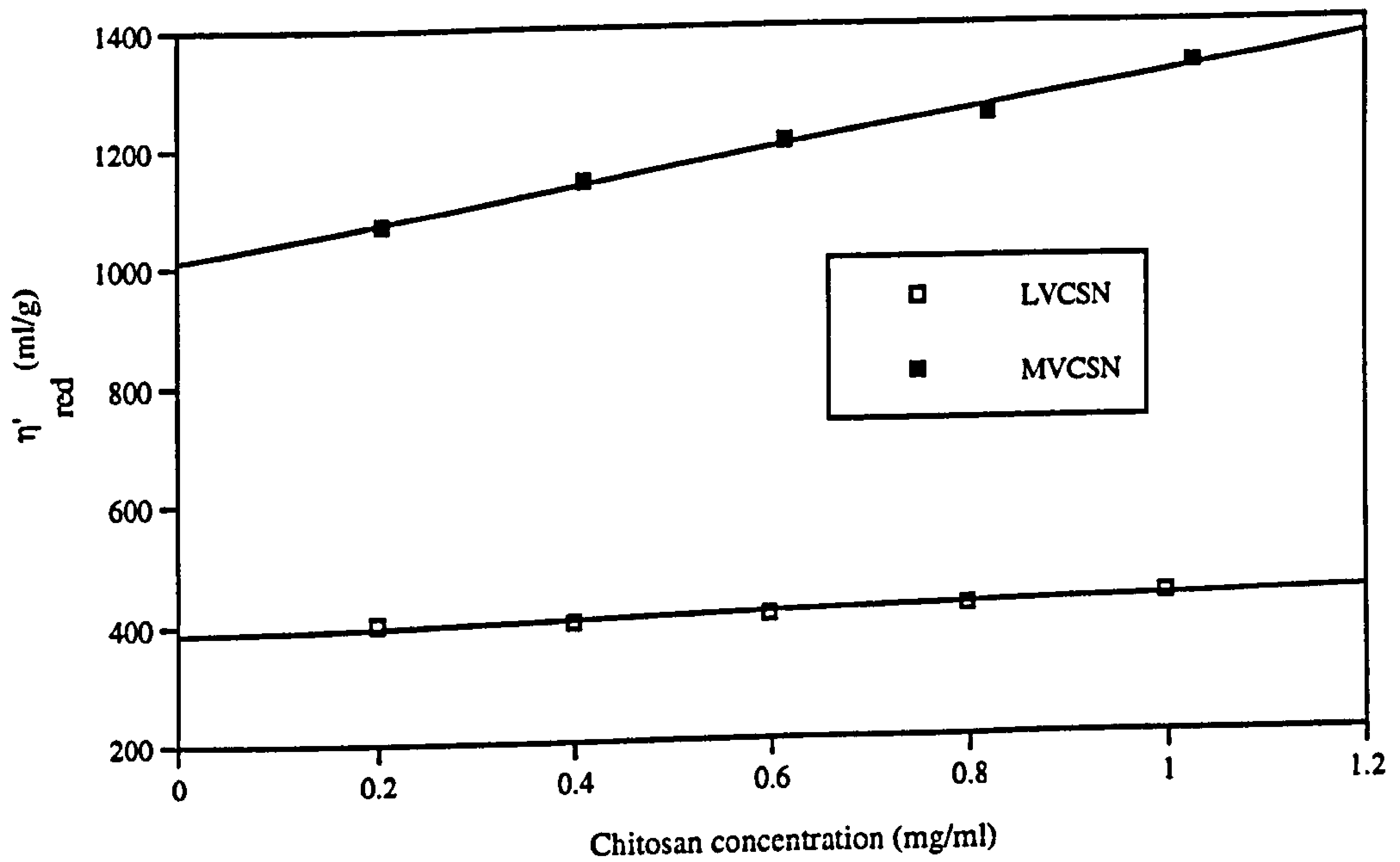
10.4.3 Results and Discussion

The plot of η'_{red} versus c (Huggins plot) is given in Figure 10.6. Values of $[\eta']$ and k' for each chitosan are given in Table 10.3. The intrinsic viscosity of the medium viscosity grade of chitosan (MVCSN) was, as expected based on product specifications, higher than that of the low viscosity grade (LVCSN). The Huggins constants (k') for each chitosan system was low which is indicative of high asymmetry of the respective chitosans (Errington, 1993). For MVCSN, the value of intrinsic viscosity, $[\eta']$, obtained (1010 ± 11.2 ml/g) was considerably higher than the value reported by Errington et al. (1993). Errington et al. (1993) reported that the intrinsic viscosity of a SeaCure +210 medium viscosity grade of chitosan glutamate (Pronova Biopolymer A/S), similar to the grade used in this study was 540 ± 20 ml/g. Errington (1993) later reported that values of intrinsic viscosity of medium viscosity chitosan glutamate (SeaCure +210) varied with variations in the ionic strength of the acetate buffer solution. Increasing buffer ionic strength resulted in a decrease in values of intrinsic viscosity (ranging from 401 ± 25 ml/g at 0.12 M to 590 ± 69 ml/g at 0.05 M. The composition of the buffer used by Errington at an ionic strength of 0.05 M was similar to that used in this study. There were slight differences in the pH of the buffers prepared in the respective studies with Errington using buffers at pH 4.3 compared to buffers of pH 4.0 used in this study. However, the discrepancies in the values of intrinsic viscosity do not appear to be explained by differences in the ionic strength and pH of the respective buffer systems. Aspden (1996) using a pH 4.3 acetate buffer of ionic strength 0.1 M reported a value of intrinsic viscosity of 930 ml/g for medium viscosity chitosan glutamate (SeaCure +210) and quoted a value obtained from Pronova Biopolymer for this batch of chitosan of 976 ml/g. The values reported by Aspden (1996) are comparable with values obtained in this study taking into account differences in the ionic strength of the buffers used. The relatively large variations in results is most likely to be ascribable to the large variation in the materials. Degradation of chitosan samples could also account for wide variations in the values of intrinsic viscosity obtained.

Table 10.3. Intrinsic viscosity data for LVCSN and MVCSN

Reduced viscosity (ml/g)			
MVCSN		LVCSN	
Concentration (mg/ml)	[η'_{red}] (ml/g)	Concentration (mg/ml)	[η'_{red}] (ml/g)
0.205	1074.32	0.199	401.18
0.410	1142.83	0.399	399.38
0.615	1204.59	0.598	406.36
0.820	1246.76	0.798	415.72
1.025	1331.54	0.997	430.04
Intrinsic viscosity ± SE (ml/g) [Huggins' constant, k']			
1010 ± 11.2		388 ± 5.7	
[0.301]		[0.251]	

Figure 10.6. Plot of η_{red} versus chitosan concentration, c



10.5 Measurement of values of apparent viscosity of chitosan solutions

10.5.1 Theory

Measurement of values of apparent viscosity using a rotational type cone and plate viscometer is a relatively rapid means of comparing the viscosities of different solutions. As mentioned in Section 10.4, the viscosity of non-Newtonian fluids can only be compared at a particular shear rate or rpm. The cone and plate viscometer consists of a rotating obtuse angled cone mounted on a spindle in close proximity to a stationary lower flat plate which is maintained at constant temperature. Liquid sample, filling a narrow triangular gap between the cone and plate, will cause viscous drag on the rotation of the cone which exerts a torque, proportional to the shear stress, on a dynamometer (calibrated spring). The low angle formed by the cone and plate and the narrow gap-width ensures that the shear rate throughout the sample is uniform.

10.5.2 Methods

In these studies, measurement of values of intrinsic viscosity was performed to investigate the effect of a number of formulation factors on the rheological behaviour of chitosan solutions and also to compare the values of viscosity of chitosan formulations. Formulation factors such as osmolality, pH and chitosan concentration were investigated. The effect of filtering chitosan solutions and the rate of shear applied during measurement of viscosity was also evaluated. The influence of temperature on the viscosity of chitosan solutions was not examined. However, viscosity measurements are highly temperature dependent. The apparent viscosity of chitosan solutions will tend to decrease with an increase in temperature although as shown below other factors will contribute to the viscosity of chitosan solutions.

A Brookfield DV-III programmable rheometer with CP40 cone and plate (cone angle 0.8°) and RHEOCALC software (Brookfield Engineering laboratories, Inc., Massachusetts, USA) was used to measure viscosity. The CP40 cone and plate had a sample volume of 0.5 ml. A Grant thermostatic bath / circulator (Grant Instruments, Cambridge, UK) equilibrated at 25°C was used to circulate water throught the plate to maintain a constant temperature. The equipment was calibrated prior to use with a (Newtonian) 50 centipoise (CPS) (± 1%) Brookfield viscosity standard solution. After dispensing 0.5 ml of chitosan solution into the plate and assembling the plate at the pre-set, fixed, level, a period of 5 minutes was allowed for the chitosan to reach thermal equilibrium. The instrument was operated over a torque range of 10-100%. Wherever possible, viscosity measurements were performed towards the upper limits of the torque range to improve the accuracy of the instrument, hence the speed of revolution of the cone was appropriately adjusted during the studies. However, to directly compare the viscosities of two or more solutions required that measurements were performed at the same rpm.

10.5.2.1 Effect of filtering on the viscosity of chitosan solution

Solutions of chitosan prepared for use in the preparation of formulations for testing in

nasal absorption studies were filtered on 0.45 μm syringe filters prior to use. However, it was not known if filtering would alter the viscosity of the chitosan solutions. In this study, the viscosity of solutions filtered on 0.45 and 0.2 μm syringe filters and unfiltered solutions were compared. Solutions containing 0.5, 1.0 and 2.0% MVCSN were prepared. All solutions were prepared at pH 4.0 in 0.9% NaCl (pH adjusted during formulation preparation using 1M HCl). Measurements of viscosity were performed in triplicate. Viscosity measurements of the 0.5, 1.0 and 2.0% chitosan solutions were performed at 35.0, 10.0, and 1.4 rpm, respectively.

10.5.2.2 Effect of concentration of NaCl on the viscosity of chitosan solutions

Solutions containing 0.5% MVCSN and different concentrations of sodium chloride (NaCl) were prepared at either pH 4.0 in water or at pH 3.4 in 1% acetic acid. The concentration of NaCl of each system was adjusted between 0-3.0 %. The osmolality of each solution was measured prior to use. All measurements were performed at 35.0 rpm.

10.5.2.3 Effect of pH on the viscosity of chitosan solutions

Adjustments of the pH of chitosan solutions used in this project had been made using HCl or acetic acid. In this study, the effect of adjusting the pH of chitosan solutions using either 1M HCl or 1M acetic acid on solution viscosity were evaluated. All solutions were prepared in 0.9% NaCl to which was added a fixed volume (0-150 μl) of the appropriate acid and filtered prior to use (0.45 μm). The pH of each solution was measured prior to use. All measurements were performed at 10 rpm.

10.5.2.4 Effect of shear rate on the viscosity of chitosan solutions

The effect of the shear rate applied to the chitosan solution, adjusted by altering the rpm of the cone, during measurement of viscosity was evaluated. Two solutions of 0.5% MVCSN at pH 4.0, one prepared in water and the second prepared in 0.9% NaCl, were used. Both solutions were filtered through 0.45 μm syringe filters prior to use. Shear rate was varied between 75.0-262.5 sec^{-1} by altering rpm between 10.0-35.0 rpm.

10.5.2.5 Effect of chitosan concentration

The viscosity of MVCSN, CSN lactate and HVCSN solutions at different concentration were measured at 5 rpm. All solutions were prepared at pH 4.0 in 0.05 M acetate buffer and filtered (0.45 μm) prior to use.

10.5.3 Results and Discussion

10.5.3.1 Effect of filtering on the viscosity of chitosan solution

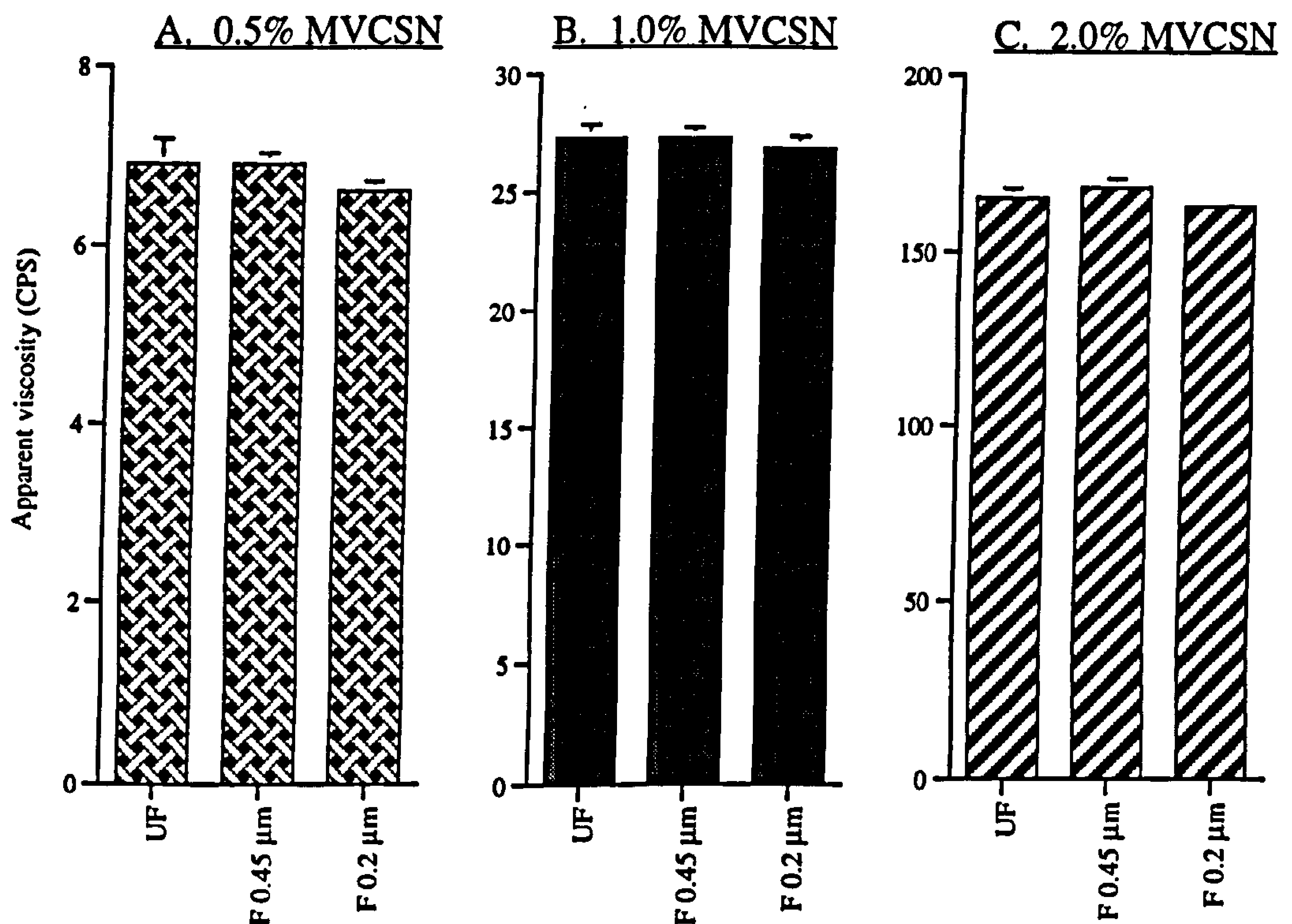
The results showed that there was a marginal decrease in viscosity when solutions were filtered through 0.2 μm syringe filters (Table 10.4, Figure 10.7). Filtering through 0.45 μm syringe filters had no effect on the viscosity of 0.5% and 1.0% MVCSN solutions although there was a marginal increase in viscosity with 2.0% MVCSN solution. However, a one-way ANOVA showed that there was no significant different between values of apparent viscosity of solutions filtered through 0.45 or 0.2 μm filters and

unfiltered solutions.

Table 10.4 Effect of filtering on the apparent viscosity of MVCSN solutions

	Mean apparent viscosity (± SD) (CPS)					
	0.5% MVCSN		1.0% MVCSN		2.0% MVCSN	
	5 rpm	35 rpm	2 rpm	10 rpm	0.5 rpm	1.4 rpm
Unfiltered	6.9 (± 0.29)	6.7 (± 0.08)	27.3 (± 0.56)	26.9 (± 0.15)	165.1 (± 2.76)	166.8 (± 2.44)
Filtered 0.45 μm	6.9 (± 0.13)	6.7 (± 0.07)	27.3 (± 0.44)	26.9 (± 0.21)	168.1 (± 2.54)	169.4 (± 2.45)
Filtered 0.2 μm	6.6 (± 0.11)	6.6 (± 0.08)	26.9 (± 0.50)	26.6 (± 0.30)	162.9 (± 1.60)	165.3 (± 1.78)

Figure 10.7 Effect of filtering on the apparent viscosity of MVCSN solutions



10.5.3.2 Effect of concentration of sodium chloride

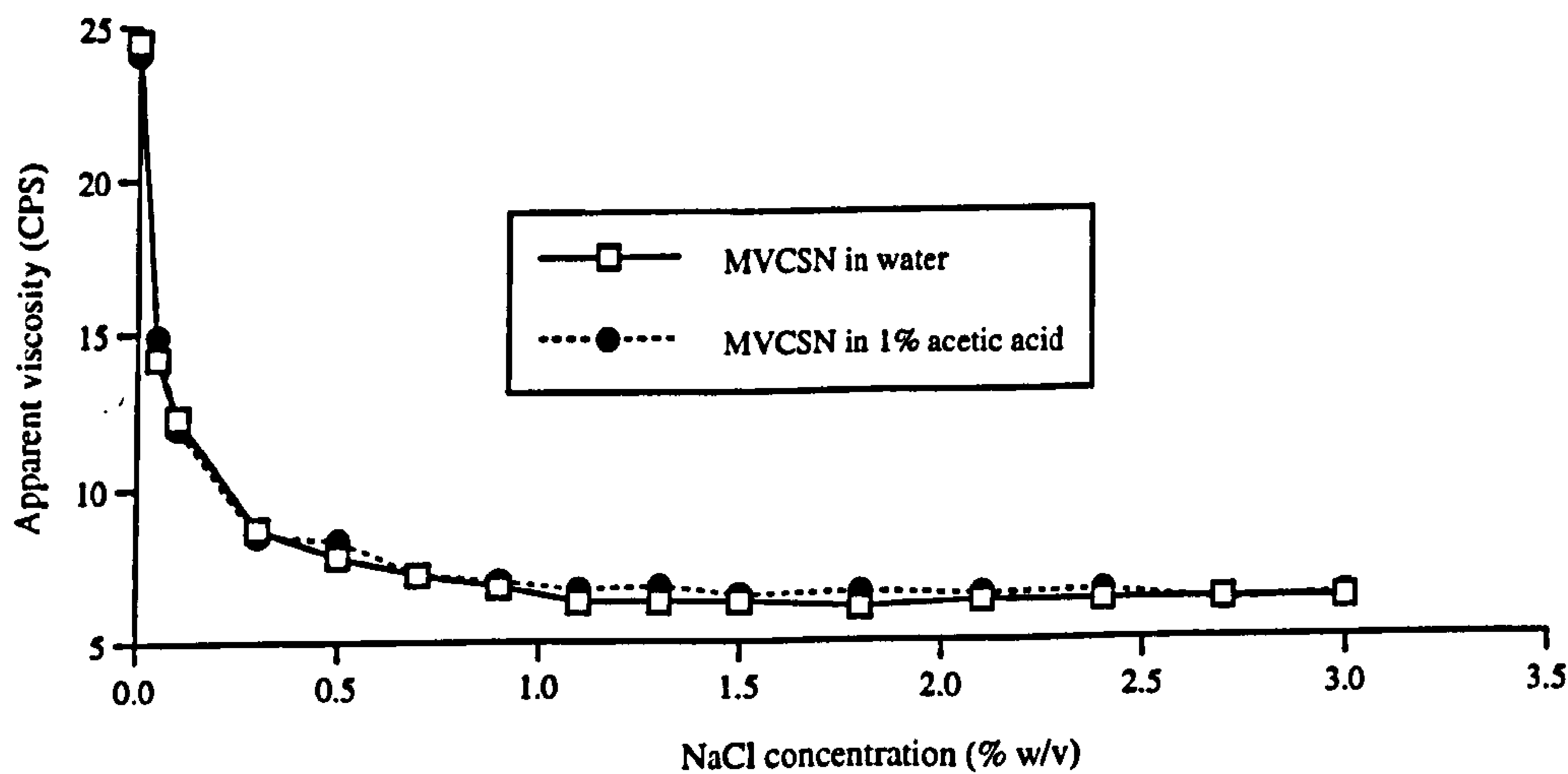
Increasing the sodium chloride concentration, hence osmolality, of solutions of MVCSN was shown to result in a dramatic decrease in values of apparent viscosity over the range 0 to 0.3% NaCl (Table 10.5, Figure 10.8). Further increases in the concentration of NaCl produced only a small decrease in values of apparent viscosity between 0.3 to 1.1% NaCl. Subsequent increases in NaCl concentration, between 1.1 to 3.0%, had very little effect on the apparent viscosity of MVCSN solutions. The apparent viscosity versus NaCl concentration profiles of MVCSN solutions prepared in water and 1% acetic acid were very similar (Figure 10.?) despite considerable differences in the osmolalities of these solutions (Table 10.5). Thus, it appears that it is the ionic concentration of the solution, rather than solution osmolality, which influences the viscosity of chitosan solutions.

In solution at very low ionic strength, the chitosan molecule will tend to adopt an extended conformation due to the repelling effect of each positively charged deacetylated unit on neighbouring glucosamine units (Pronova Biopolymer A/S, 1992). An increase in ionic strength will tend to reduce the charge repelling effects in the chitosan molecule thus favouring a random coil conformation. This will significantly decrease solution viscosity due to a reduction in hydrodynamic volume (Muzzerelli, 1977) At low ionic strength the conformation of chitosan in solution, hence solution viscosity, is highly dependent on ionic strength. Above a certain ionic strength with the chitosan molecule in the 'full'random coil confirmation, further increase in ionic strength will have little effect on molecular conformation, hence on solution viscosity. However, at higher concentrations of electroytes, a 'salting-out' effect will occur due to the precipitation of chitosan out of solution resulting in a decrease in solution viscosity.

Table 10.5 Effect of NaCl concentration on the apparent viscosity of MVCSN solutions

	MVCSN in water (pH 4.0)		MVCSN in 1% acetic acid (pH 3.4)	
NaCl conc. (% w/v)	Viscosity at 10 rpm (CPS)	Osmolality (Osmol/kg)	Viscosity at 10 rpm (CPS)	Osmolality (Osmol/kg)
0	24.5	0.018	24.1	0.181
0.05	14.2	0.035	14.9	0.200
0.1	12.3	0.051	12.0	0.216
0.3	8.7	0.116	8.5	0.284
0.5	7.8	0.180	8.3	0.340
0.7	7.2	0.240	7.2	0.413
0.9	6.8	0.304	7.0	0.477
1.1	6.3	0.364	6.7	0.539
1.3	6.3	0.426	6.8	0.601
1.5	6.3	0.488	6.5	0.666
1.8	6.1	0.582	6.6	0.764
2.1	6.3	0.676	6.5	0.855
2.4	6.3	0.770	6.6	0.951
2.7	6.3	0.865	6.2	1.043
3.0	6.3	0.958	6.4	1.141

Figure 10.8 Effect of NaCl concentration on the apparent viscosity of MVCSN solutions



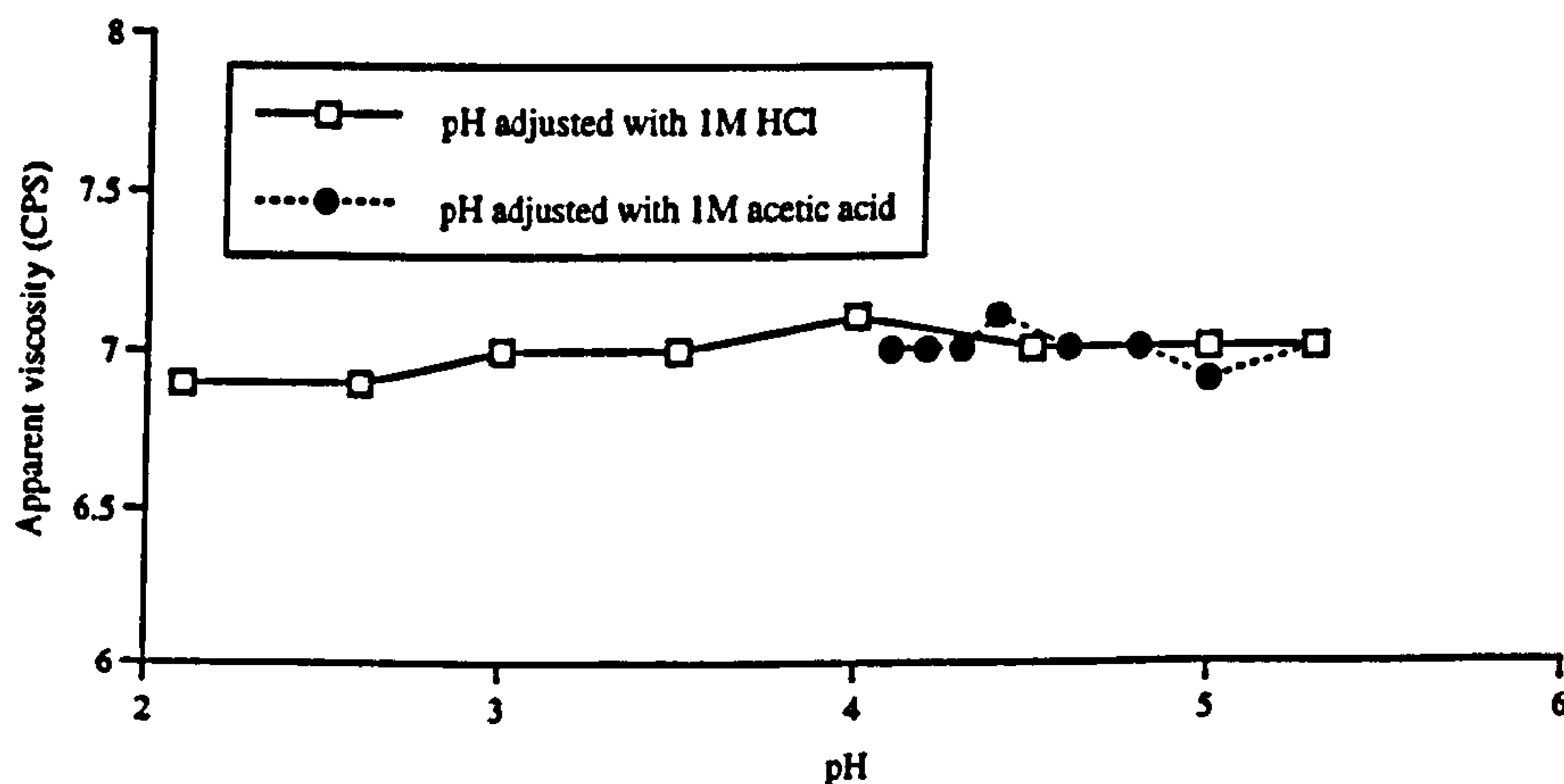
10.5.3.3 Effect of pH on the viscosity of chitosan solutions

Altering solution pH, over the range pH 2.1 to 5.3 with 1M HCl or pH 4.1 to 5.3 with 1M acetic acid, did not effect the apparent viscosity of MVCSN (Table 10.6, Figure 10.9). However, the solution contained a relatively high concentration of NaCl which could have masked the effects on viscosity of changing solution pH. In the previous study, looking at the effects of NaCl concentration, at 0% NaCl there was very little difference between the MVCSN solutions at pH 4.0 and pH 3.4 (Table 10.5). Aspden (1996) showed that at lower NaCl concentration, increasing the pH of a solution of chitosan glutamate between pH 3-5 resulted in a decrease in apparent viscosity. Thus, the study should be repeated in media containing different concentrations of NaCl to establish the relationship between ionic strength, pH and apparent viscosity.

Table 10.6 Effect of pH on the apparent viscosity of MVCSN solutions

	pH adjusted with 1M HCl		pH adjusted with 1M acetic acid	
Volume of acid added (µl)	Viscosity at 10 rpm (CPS)	pH	Viscosity at 10 rpm (CPS)	pH
0.0	7	5.3	7.0	5.3
10.0	7	5.0	6.9	5.0
20.0	7	4.5	7.0	4.8
40.0	7.1	4.0	7.0	4.6
60.0	7	3.5	7.1	4.4
80.0	7	3.0	7.0	4.3
100.0	6.9	2.6	7.0	4.2
150.0	6.9	2.1	7.0	4.1

Figure 10.9 Effect of pH on the apparent viscosity of MVCSN solutions



10.5.3.4 Effect of shear rate on the viscosity of chitosan solutions

The apparent viscosities of MVCSN solutions prepared in water was shown to decease with an increase in shear rate which demonstrated the pseudoplastic or shear-thinning nature of chitosan (Table 10.7, Figure 10.10). However, a solution of MVCSN in 0.9% NaCl exhibited negligible pseudoplastic behaviour over the range of shear rates investigated. Pseudoplasticity, an example of non-Newtonian flow behaviour of fluids, results from the competition between the external forces, i.e. shear rate, and the intermolecular and intramolecular forces which influence the behaviour of chitosan in solution. At low shear rate, the rate of entanglement and randomisation of chitosan polymer chains and the rate of aggregation of particles which occur due to Brownian motion far outweigh the rate of disentanglement and alignment of polymer chains and the

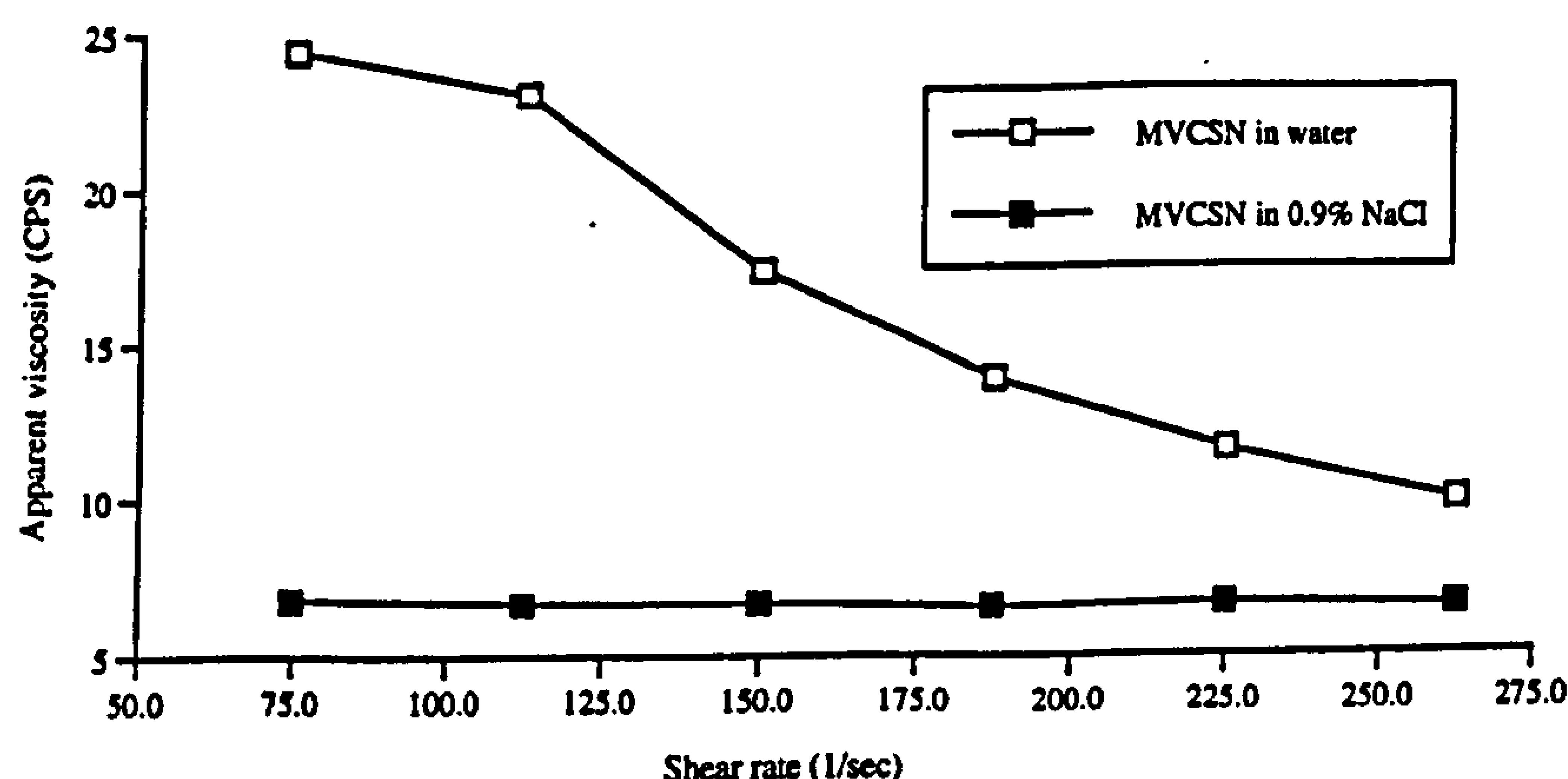
breaking-up of aggregates and particles due to the application of shear. At very low shear rate (usually below 1sec^{-1}) the fluid will behave as a Newtonian liquid. However, as shear rate increases, progressive breakdown of the polymer structure in solution occurs and when this exceeds the rebuilding effect due to Brownian motion the viscosity will be dramatically reduced. At an optimal shear rate, the polymer chains will be completely disentangled and aligned in the direction of the applied shear and aggregates of particles will be fully broken up. Thus, since the residual structure of the polymer is fully broken, further increase in shear rate will not result in further decrease in solution viscosity.

In this study at relatively low shear rate (sec^{-1}), the observed differences in the apparent viscosity of the two chitosan solutions was attributable to the differences in the apparent viscosities of chitosan solutions at relatively low and high ionic strength (i.e. in water and 0.9% NaCl, respectively). For chitosan solution in water, the decrease in viscosity will occur due to the progressive breakdown of the chitosan structure as outlined above. For chitosan in 0.9% NaCl, the random coil conformation of chitosan will significantly reduce values of apparent viscosity compared to solutions at low ionic strength. For chitosan solutions in 0.9% NaCl progressive increase in shear rate did not result in a shear thinning effect. This was presumably due to the viscosity reducing effects attributable to ionic strength reaching a maximum such that increase in shear rate did not cause further breakdown of chitosan structure.

Table 10.7 Effect of shear rate on the apparent viscosity of MVCSN solutions

		Apparent viscosity (CPS)	
RPM	Shear rate (1/sec)	MVCSN in water	MVCSN in 0.9% NaCl
10.0	75.0	24.5	6.8
15.0	112.5	23.2	6.7
20.0	150.0	17.5	6.7
25.0	187.5	14.0	6.6
30.0	225.0	11.7	6.7
35.0	262.5	10.0	6.6

Figure 10.10 Effect of shear rate on the apparent viscosity of MVCSN solutions



10.5.3.5 Effect of chitosan concentration

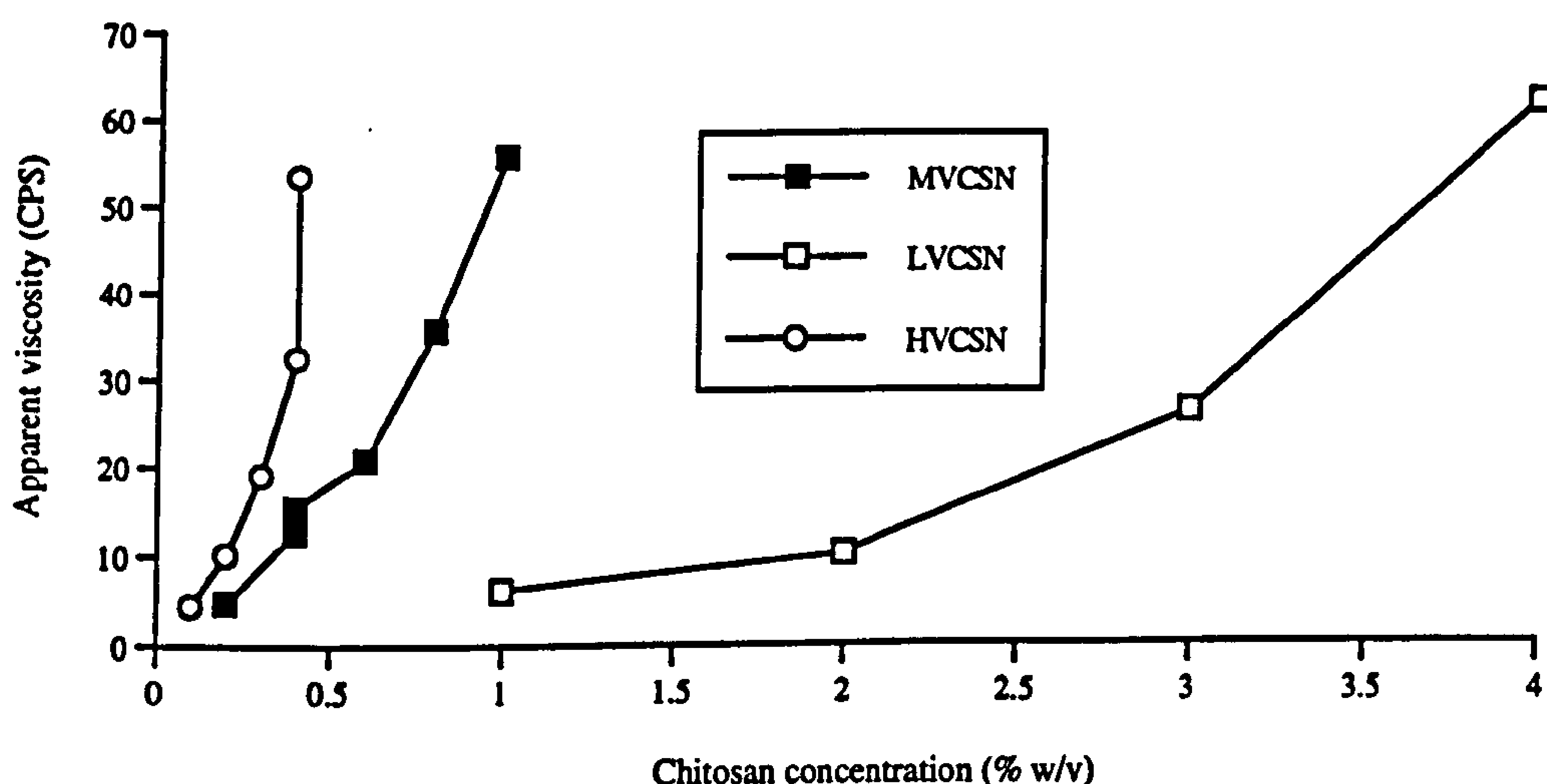
The concentration and viscosity grade of the chitosan used were shown to strongly influence the viscosity of chitosan solutions (Table 10.8, Figure 10.11). At equal concentration, as expected (refer to Table 2.1), the viscosity of HVCSN was higher than

that of MVCSN which in-turn was higher than that of LVCSN. An increase in the solution concentration of each grade of chitosan resulted in a non-linear increase in values of apparent viscosity. An increase in chitosan concentration will increase the hydrodynamic volume of chitosan molecules in solution chains thus increasing solution viscosity.

Table 10.8 Effect of chitosan concentration on values of apparent viscosity

	Apparent viscosity (CPS) at 5 rpm		
Chitosan conc. (% w/v)	MVCSN	LVCSN	HVCSN
0.1	-	-	4.4
0.2	4.7	-	10.1
0.3	-	-	19.1
0.4	12.6	-	32.5
0.5	15.4	-	53.5
0.6	20.8	-	-
0.8	35.7	-	-
1.0	55.8	6.1	-
2.0	-	10.2	-
3.0	-	26.4	-
4.0	-	62.2	-

Figure 10.11 Effect of chitosan concentration on values of apparent viscosity



10.6 Conclusions

The values of $M_{w,\ app}$ and $M_{w,\ app}(r)$ $(J \rightarrow 0)$ of 45,000 and 65,000, respectively have drammatically underestimated the molecular weight of MVCSN. Errington (1993) reported a molecular weight of 162,000 for the batch of MVCSN used in this project. Thus, any future measurement of molecular weights of chitosan should follow the procedures of Errington (1993). Intrinsic viscosity measurements showed that the intrinsic viscosity of MVCSN was much higher than that of LVCSN (1010 and 388 ml/g, respectively). It would have been useful to measure the intrinsic viscosity of each grade of chitosan used in this project. Measurement of the apparent viscosities of chitosan solutions generally supports claims made in the literature regarding the effect of ionic concentration, pH, shear rate and chitosan concentration on values obtained. They also highlight the different viscosities of the grades of chitosan employed in this project.

CHAPTER 11

PROJECT OVERVIEW AND SUGGESTED FURTHER STUDIES

The findings of this project have added to the pool of information reported in the literature about the application of the nasal route for the delivery of insulin and other peptide drugs. The preliminary studies reported in this project were apparently the first studies performed to investigate the potential use of chitosan in nasal delivery systems and some of the results generated here were reported by Illum et al. (1994).

The efficacy of chitosan in enhancing the nasal absorption of insulin was demonstrated in rat and sheep models. In the rat model, insulin / chitosan formulations at pH ~4 were more effective than formulations at pH ~7 in enhancing intranasal insulin absorption. The reduced absorption in the latter formulation which was in the form of a suspension was attributed to complex formation between insulin and chitosan. Further investigation of the effect of pH on absorption enhancement was not evaluated since pH had to be lowered to below pH 5 to obtain solutions formulations of insulin and chitosan. However, this could be investigated using an alternative peptide such as salmon calcitonin where there does not appear to be a problem with complex formation. This may in part be due to the much higher potency of salmon calcitonin compared to insulin, thus solution formulations contain much lower concentrations of salmon calcitonin. At low ionic concentration, reduction in pH will increase the polycationic behaviour of chitosan. Thus, investigation of formulations at different pH would allow the influence of the polycationic properties of chitosan on nasal absorption enhancement to be further evaluated provided that the formulation components were in solution.

In the rat model, the performance of chitosan, in terms of the degree of hypoglycaemia following dose administration, was second only to that of LPC. However, in the sheep model, a formulation incorporating chitosan was much more effective than a formulation containing LPC in promoting nasal insulin absorption. These differences were attributed to the animal models used to investigate nasal absorption. In the rat model, animals were anaesthetised which is likely to impair mucociliary clearance mechanisms. Thus, both the chitosan and LPC formulations remain pooled in the nasal cavity thereby increasing the residence time of both formulations in the nasal cavity and increasing the intimate contact between the formulation and the nasal mucosa. However, in the conscious sheep model, where mucociliary clearance mechanisms are not impaired, the relatively poor performance of the LPC formulation may be due to faster clearance of the LPC formulation from the nasal cavity compared to the clearance of the viscous / bioadhesive chitosan formulation. Thus, improved efficacy of chitosan formulations compared to LPC formulations in the sheep model are probably attributed to the increased residence time of the chitosan formulations in the nasal cavity. Gamma scintigraphic studies using radiolabelled chitosan or possibly insulin, would be useful to confirm the increased residence time of the formulation in the nasal cavity.

Further investigation of nasal delivery systems employing chitosan in the rat and sheep models showed that the degree of nasal absorption enhancement was improved by increasing the solution concentration of chitosan (MVCSN grade) until an optimal concentration was attained. In the rat studies, an optimal range of chitosan concentrations was demonstrated. A reduction in insulin absorption in rats at chitosan concentrations above the optimal range could be due to the increased viscosity of the formulation thereby preventing the spread of the formulation in the nasal cavity and/or reducing the rate of release of insulin from the formulation. *In vitro* studies to investigate the rate of insulin release from the insulin / chitosan formulations would be useful to evaluate the latter. It would also be useful to assess higher formulation concentrations of chitosan in the sheep model to establish if there was an upper limit to the optimal range of chitosan concentrations. Further evaluation of nasal insulin / chitosan formulations in sheep, suggested that the formulation concentration of chitosan was important for its absorption enhancing efficacy and at optimal chitosan concentration nasal insulin absorption was limited by the dose concentration of insulin. In both rat and sheep models, the nasal administration of hypotonic or isotonic formulations of insulin with chitosan did not influence the degree of nasal absorption enhancement attained. However, in rats, a hypertonic formulation was shown to further improve nasal insulin absorption which was attributed to the combined effects of the chitosan and the increased tonicity of the formulation on the nasal membrane. It would be useful to establish if a similar effect was observed in the sheep model.

The grade of chitosan used in the nasal absorption studies appeared to influence the degree of absorption enhancement obtained. The rat model appeared to be of limited use for these investigations since differences in the absorption enhancing efficacy of a low viscosity grade of chitosan lactate and a medium viscosity grade of chitosan glutamate were not apparent. In contrast, in the sheep model differences between the two grades were observed with the medium viscosity grade performing better than the low viscosity grade. There was no difference in the performance of medium viscosity grades of glutamate and hydrochloride salts of chitosan (both tested at 0.5% w/v of chitosan salt) in sheep although considering the chitosan content of these salt forms (55-65 and 80-90% respectively), the glutamate salt form may potentially be the more effective. Aspden (1996) showed that in the sheep model, medium viscosity chitosan glutamate performed better than low, medium and high viscosity grades of chitosan hydrochloride, at concentrations of 0.25% w/v chitosan base. Increasing chitosan concentration to 0.5% w/v did not further improve the performance of chitosan glutamate but improved the performance of the hydrochloride salts. However, the glutamate salt still appeared to possess the greatest absorption enhancing efficacy.

An elaborate study would be required to evaluate the relationship between the grade of chitosan and absorption enhancing efficacy. The use of different molecular weight, hence viscosity, fractions of chitosan prepared from the same parent material should be considered since it is difficult to directly compare different chitosan salts forms. Although the chitosan content of the different salt forms may be equated in terms of the

respective content of chitosan base, as in the studies by Aspden, the salt form used is likely to influence the behaviour of the chitosan in solution. Aspden (1996) reported that compared to medium viscosity chitosan hydrochloride, the intrinsic viscosity of medium viscosity chitosan glutamate was much greater than that predicted from its molecular weight. This was attributed to the greater influence of the glutamate moiety on molecular conformation and hence on solution viscosity than the hydrochloride moiety. Also the differences in viscosity observed could be attributed to the higher content of glutamate compared to hydrochloride. The importance of viscosity of the formulation should be further evaluated since the lower viscosity of the solutions prepared from low viscosity grades of chitosan may be the reason for the lower efficacy of nasal absorption enhancement. The effect of the degree of deacetylation of the chitosan on nasal absorption enhancement should also be fully evaluated. Aspden (1996) reported that there was no difference in the absorption enhancing efficacy of different grades of chitosan hydrochloride which were either 50% or approximately 85% deacetylated. However, these results would seem inconclusive in view of the high variability in values of intrinsic viscosity between the grades of chitosan used. Investigation of the effect of the degree of deacetylation on nasal absorption enhancing efficacy would best be achieved by preparing batches of chitosan of different degree of deacetylation from the same parent material. Careful control of the experimental conditions would be required since deacetylation can lead to depolymerisation of the chitosan. Depolymerisation should be avoided since this will decrease chitosan molecular weight, hence solution viscosity, thereby introducing another experimental variable. By optimisation of the degree of deacetylation and grade of chitosan employed in the nasal formulation, it may be possible to prepare stable formulations of chitosan at higher values of pH. This may be favourable if chitosan is to be used in nasal delivery systems for other peptide and non-peptide drugs.

The degree of nasal absorption enhancement was shown to be reasonably reproducible in rat and sheep models, in terms of the hypoglycaemic responses following insulin / chitosan dose administration. The sheep model is probably better suited for these investigations since animals can be reused, thus intra- and inter-animal variability can be elucidated. The sheep model is also more likely to allow subtle differences between the performance of nasal delivery systems to be evaluated, thus, is better suited for optimisation studies. The study could be widened to investigate once daily or multiple daily dosing of the insulin /chitosan formulation in the same animals over a longer time period. Measurement of both plasma glucose and insulin concentrations should also be performed. It is important to establish if the nasal route for intranasal insulin delivery remains viable over longer time periods since the treatment of insulin dependent diabetes mellitus by this route would require chronic therapeutic application.

The demonstration of the transient effect of chitosan on the permeability of the nasal membrane in the rat model and the results of the *in vitro* haemolysis studies are highly encouraging and further support the view that chitosan is non-toxic and safe to use in nasal delivery systems. The transient nature of chitosan on nasal absorption

enhancement should be further evaluated in the rat model. A suitable positive control, such as Laureth-9 or LPC solutions which have both been shown in the literature to cause membrane damaging effects, should be included to allow the transient effects of potentially membrane damaging solutions to be compared with those of chitosan. Investigations could also be performed in the sheep model. In sheep, use of nasal sprays may be required to overcome difficulties in the delivery of chitosan and insulin formulations to the same region of the nasal cavity. However, pre-evaluation studies would be required to ensure that the distribution patterns of the viscous chitosan and non-viscous insulin solutions in the nasal cavity were similar from the spray devices. The sheep could also be anaesthetised during the dosing period and the dosing device left in-place in the nasal cavity to ensure that chitosan and insulin solutions were delivered to the same region of the nasal cavity. In conscious rats and /or sheep it would be interesting to assess the transient effect of chitosan on the nasal membrane following chronic administration. The haemolysis studies showed that MVCSN was non-damaging to rat erythrocyte membranes at concentrations which were higher than the concentrations used in nasal absorption studies. This was encouraging since most erythrocyte haemolysis studies reported in the literature (e.g. Chandler, 1992, Jabbal-Gill et al, 1994a) have demonstrated severe membrane damage by compounds at concentrations which were much lower than the concentrations which were effective for nasal absorption enhancement. The haemolysis assay appears to be effective for screening the membrane damaging effects of compounds. However, the studies performed in this project indicate that values of basal haemolysis are fairly high and thus the use of human erythrocytes is recommended (Illum et al., Unpublished data).

It would be useful to further investigate toxicological issues in parallel to the nasal absorption studies in animal models during the optimisation of nasal delivery systems. The appropriate negative (e.g. buffer solution) and positive (e.g. Laureth-9 or LPC solution which are known to cause cell damage) controls should be included in the studies. Perfusion studies could be performed in the rat to assess damage to the nasal mucosa indicated by the detection of concentrations of cellular markers (specific membrane bound and cytosolic enzymes) in the perfusate. Nasal washings could also be collected in sheep at pre-determined time intervals after dose administration and again concentrations of cellular markers measured. However, validation work would be required to establish if the concentrations of cellular markers can be detected in each system. Histological examination of the nasal mucosae of the rat and sheep models following chitosan dose administration should be performed on selected formulations. Histological examinations after both acute and chronic administrations of chitosan should be performed. Studies reported by Illum et al. (1994) and Aspden (1996) have investigated the acute effects of chitosan administration by histological examination but have not considered the effects after chronic application. Cilia beat frequency (CBF) studies would also be useful. Merkus et al. (1993) suggested that measurement of CBF is a valuable tool for evaluating the safety of nasal absorption enhancers. In frog palate studies, chitosan was shown to cause a transient slowing of mucociliary transport (Aspden et al., 1995a). This would also be expected following nasal administration of

chitosan to conscious animal models or humans and may account for the increased residence time of these formulations in the nasal cavity. Acute and chronic CBF studies are recommended. Toxicity studies in animal models or human subjects should also be supported by cell culture models. Artursson et al. (1994) suggested that chitosan affected the integrity of epithelial tight junctions in Caco-2 cell monolayers. Dodane et al. (1996), proposed that chitosan caused a transient modulation of the permeability and structure of the Caco-2 cell layer. Thus, extensive investigations are required to establish the precise effect on modulation of the permeability and structure of cell monolayers. Again, it is important to establish the effects of chitosan after acute and chronic application. The above toxicological investigations are useful for evaluating potential problems which may be associated with nasal delivery systems employing chitosan and also for evaluating the potential mechanisms of action of chitosan. The topical irritancy of chitosan applied to the nasal cavity should be assessed in human volunteers since irritant actions of compounds applied nasally may not correlate with the toxicological studies.

Although this project was primarily concerned with the investigation of chitosan as a nasal delivery system for insulin, insulin could also be considered as a model peptide. Thus, investigation of the nasal absorption of insulin can be used as a general indicator of the nasal absorption of other peptide drugs. It was shown that MCSN enhanced the nasal absorption of salmon calcitonin (S-CT) in the rat and sheep models. However, the nasal absorption of S-CT was assessed indirectly from the hypocalcaemic response following nasal dose administration. These studies should be repeated and both plasma or serum S-CT and calcium concentrations measured to further evaluate nasal S-CT absorption. The nasal absorption of other peptides and proteins, and indeed other drugs, could also be investigated.

This project has demonstrated that chitosan enhances the nasal absorption of insulin in rat and sheep models. In the sheep model the bioavailability of nasal insulin, relative to the subcutaneous route, was generally less than 5%. However, the hypoglycaemia which followed nasal insulin / chitosan dose administration was encouraging and a similar degree of efficacy in humans could be feasible for the therapeutic application of nasal insulin therapy. A nasal insulin / chitosan formulation could replace or complement subcutaneous insulin therapy. In insulin-dependent diabetics, nasal insulin could be used to replace injectable insulin before meal times for the control of post-prandial rises in glucose blood concentrations. In Type II diabetics, nasal administration of insulin could replace the absent early peak of insulin secretion.

Clearly, full human clinical trial, toxicological and formulation stability evaluation would be required and furthermore economic factors associated with scaling-up procedures would have to be considered before nasal delivery systems became commercially viable.

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Appendix 1

Constituents of reagents used during this project

14.65 mM phosphate buffer of pH 7.4

1.904 g $Na_2HPO_4.2H_2O$ (M Wt 178.05)+ 0.616 g $NaH_2PO_4.2H_2O$ (M Wt 156.01) made upto 1000 ml with water.

Sodium chloride (NaCl) solutions

Required concentrations prepared by dilution of 0.9% NaCl (details given in the relevant chapter)

Phosphate buffered saline (PBS)

Prepared by mixing solutions A and B (given below) until the appropriate pH was achieved

A.	In 250 ml water	0.39 g $NaH_2PO_4.2H_2O$ 2.25 g NaCl
B.	In 500 ml water	0.89 g $Na_2HPO_4.2H_2O$ 4.5 g NaCl

Citrate phosphate dexrose adenine buffer (CPD-A)

	(mmol/l)	(Gram % (w/v)
Citric acid (monohydrate)	16	0.327
Sodium citrate (dihydrate)	89	2.630
Sodium acid phosphate (dihydrate)	16	0.251
Dextrose (anhydrous)	129	2.320
Adenine	2	0.027

Acetate buffer for hydrodynamics

Prepared by mixing solutions of 13.609 g/l CH3COONa.3H2O and 5.728 ml/l glacial acetic acid to achieve a pH of 4.0