Uptake and Transport of Orally-Deliverable Drugs Across Caco-2 Cell Monolayers; the Effect of Lipid Formulations

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Doctor of Philosophy

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Thesis Summary

The aim of this thesis is to investigate the physicochemical parameters which can influence drug loading within liposomes and to characterise the effect such formulations have on drug uptake and transport across *in vitro* epithelial barrier models. Liposomes composed of phosphatidylcholine (PC) or distearoyl phosphatidylcholine (DSPC) and cholesterol (0, 4, 8, 16 μM) were prepared and optimised in terms of drug loading using the hand-shaking method (Bangham *et al.*, 1965). Subsequently, liposomes composed of 16 μM PC or DSPC and cholesterol (4 μM) were used to monitor hydroxybenzoate release and transport from liposomes. The MTT (3[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and crystal violet assays were employed to determine toxicity of the liposome formulations towards the Caco-2 cell line, employed to model the epithelial barrier *in vitro*. Uptake and transport of mannitol, propranolol, glutamine and digoxin was measured in the presence and absence of liposome formulations to establish changes in absorption resulting from the presence of lipid formulations.

Incorporation of the four hydroxybenzoates was shown to be influenced by a number of factors, including liposome composition and drug conformation. Methyl hydroxybenzoate (MP) was incorporated into the bilayer most effectively with percentage incorporation of 68% compared to 45% for butyl hydroxybenzoate (BP), despite its increased lipophilicity. This was attributed to the decreased packing ability of BP within the hydrocarbon core of the lipid bilayer compared to MP. Release studies also suggested that the smaller MP was more strongly incorporated within the lipid bilayer with only 8% of the incorporated solute being released after 48-hours compared to 17% in the case of BP. Model transport studies were seen to reflect drug release profiles from the liposome bilayers with significantly ($p < 0.01$) higher amounts of BP partitioning from the liposome compared to MP.

Caco-2 cell viability was maintained above 86% in the presence of all liposome formulations tested indicating the liposome formulations are non-toxic towards Caco-2 cells. Paracellular (apical-to-basolateral) transport of mannitol was significantly increased in the presence of DSPC, PC / DSPC:Cholesterol (16:4 μM; 1000 μg). Glutamine uptake and transport *via* the carrier-mediated route was significantly ($p < 0.01$) increased in the presence of PC / DSPC:Cholesterol (16:0; 16:4 μM). Digoxin apical-to-basolateral transport was significantly increased ($p < 0.01$) in the presence of PC / DSPC:Cholesterol (16:0; 16:4 μM), thus reducing digoxin efflux *via* P-glycoprotein. In contrast, PC:Cholesterol (16:0; 16:4 μM) significantly ($p < 0.01$) decreased propranolol uptake *via* the passive transcellular route. Bi-directional transport of propranolol was significantly ($p < 0.01$) decreased in the presence of PC / DSPC:Cholesterol (16:0; 16:4 μM).

The structure of a solute is an important determinant for the incorporation and release of a solute from liposome formulations. PC, DSPC and cholesterol liposome formulations are non-toxic towards Caco-2 cell monolayers and improved uptake and transport of mannitol, glutamine and digoxin across Caco-2 cell monolayers; thus providing a potential alternative delivery vehicle.

Key words: Liposomes, LogP, paracellular transport, transcellular transport, efflux.

Dedication

For Uncle Paul: You were always there to offer your support and encouragement - Your Memory has provided me with the inspiration to complete my studies.

"*Everybody's a mad scientist, and life is their lab. We're all trying to experiment to find a way to live, to solve problems, to fend off madness and chaos*" - David Cronenberg.

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Abbreviations

A – B	Apical-to basolateral
ABC	ATP-binding cassette
ATTC	American Type Tissue Culture Collection
ATP	Adenosine tri-phosphate
AZT	3'Azido-3'deoxythymidine
B – A	Basolateral-to-apical
BCA	Bicinchoninic acid
BCRP	Breast cancer resistance protein
BCS	Biopharmaceutics drug classification system
BNF	British National Formulary
BP	Butyl 4-hydroxbenzoate
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
Caco-2	Adenocarcinoma colon cell line
CFTR	Cystic fibrosis transmembrane regulator protein
Ci	Curie
Cl^-	Chloride ion
C_{max}	Maximum concentration
cMOAT	Canalicular multispecific organic anion transporter
CPM	Counts per minute
CMs	Chylomicrons

CsA	Cyclosporin A
CYP	Cytochrome
CYP3A	Cytochrome P3A
Da	Daltons
DBPC	Dibehynoyl phosphatidylcholine
ddH_2O	Double-distilled water
DDRG	Drug Delivery Research Group
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DMPC	Dimyristoyl phosphatidylcholine
DPH	1,6-Diphenyl-1,3,5-hexatriene
DPM	Disintegrations per minute
DPPC	Dipalmitoyl phosphatidylcholine
DSPC	Distearoyl phosphatidylcholine
DSC	Differential scanning calorimetry
DTS	Di-tri-peptide transporter
E_a	Activation energy
ECACC	European Collection of Animal Cell Cultures
EDTA	Ethylene diamine tetraacetic acid
EP	Ethyl 4-hydroxbenzoate
Epo	Human recombinant erythropoietin
FDA	Food and Drugs Agency
FaSSIF	Fasted state simulated intestinal fluid
FBS	Foetal bovine serum

FeSSIF	Fed state simulated intestinal fluid
g	Gravitational - centrifuge
g	Gram
GI	Gastrointestinal
Gly-Phe-Leu	Glycine-Phenylalanine-Leucine
Gly-Try	Glycine-Tryptophan
GSH	Glutathione
G3	Generation 3 (dendrimer)
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric acid
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethansulphonic acid
HL60	Human Promyelocytic Leukaemia Cells
HSM	Hand-shaking method
HUVEC	Human Umbilical Vein Endothelial Cells
i.m.	Intramuscular
i.v.	Intravenous
IVIVC	*In vitro In vivo* correlation
kDa	kilo-Daltons
K_m	Michaelis-Menten constant
LDH	Lactate dehydrogenase
LogP	Log Partition coefficient
LUV's	Large unilamellar vesicles
M	Molar
MES	2-[*N*-morpholino]ethanesulphonic acid

MLogP	Moriguchi LogP
MLV's	Multilamellar vesicles
MP	Methyl 4-hydroxbenzoate
MDR	Multidrug resistance
mdr	P-glycoprotein gene
mg	Milligram
ml	Millilitre
mRNA	Messenger ribonucleotide acid
MRP1	Multidrug resistance-associated protein
MTT	3[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide
N_2	Nitrogen
Na^+	Sodium ion
NaCl	Sodium chloride
NEAA	Non essential amino acids
ng	Nanogram
PAMAM	Polyamidoamine
P_{app}	Apparent permeability
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PC	Polycarbonate
PEG	Polyethylene glycol
PET	Polyethyleneterephatalate
P-gp	P-Glycoprotein
p*Ka*	Negative logarithm of the ionisation constant

PLA_2	Phospholipase A_2
PMSF	Phenylmethylsulfonyl fluoride
PP	Propyl 4-hydroxbenzoate
PS	Phosphatidylserine
RES	Reticuloendothelial system
rhEGF	Recombinant human epidermal growth factor
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEDDS	Self-emulsifying drug delivery systems
SGF	Simulated gastric fluid
SUV's	Small unilamellar vesicles
T_c	Liquid-crystalline transition temperature
T_m	Phase transition temperature
TEER	Transepithelial electrical resistance
TMA-DPH	1-(4-trimethylammonium)-6-phenyl)-1,3,5-hexatriene
TPGS	D-alpha-tocopheryl polyethylene glycol 1000 succinate
U	Unit
µg	Microgram
µl	Microlitre
UWL	Unstirred water layer
UV	Ultraviolet
V_{max}	Maximum rate of uptake / transport
% v/v	Percentage volume per volume

% w/v	Percentage weight per volume
% w/w	Percentage weight per weight

Chapter 1

General Introduction

1. General Introduction

1.1 Introduction

The oral route is the most common, convenient, economical and socially accepted route for administration for drug delivery (Hunter and Hirst, 1997; Daugherty and Mrsny, 1999). However, the success of this route can be limited due to the existence of barriers that drugs must negotiate in order to reach their desired destination and become effective. These include both the physical anatomy of the gastro-intestinal (GI) tract and physiological / biological barriers including enzymes and pH (Table 1.1), (Borgstrom *et al.*, 1957; Ovensen *et al.*, 1986; Evans *et al.*, 1988; Dressman *et al.*, 1991; Russell *et al.*, 1993). The different mechanisms of drug absorption are examined in detail below.

Location	Average pH in a Fasted State	Average pH in a Fed State
Stomach	1.3	4.9
Duodenum	6.5	5.4
Jejunum	6.6	5.2 – 6.0
Ileum	7.4	7.5

Table 1.1 – Average pH values in healthy humans in the fasted and fed state at various sites in the upper GI tract (Hörter and Dressman, 2001).

1.2 Physiology of the Gastro-Intestinal Tract

It is vital to have an in-depth knowledge and an extensive understanding of the GI tract when attempting to improve delivery and bioavailability. The GI system consists of the GI tract and associated glandular organs that produce secretions (Berne and Levy, 1993). The GI tract comprises the mouth, pharynx, oesophagus, stomach, small intestine, large intestine, rectum and anus (Figure 1.1). The GI tract is a hollow tube approximately 9 metres in length. The salivary glands, liver, gall bladder and pancreas are the associated glandular organs. Digestion

of food into smaller molecules that can be absorbed into the bloodstream is the major function of the GI system.

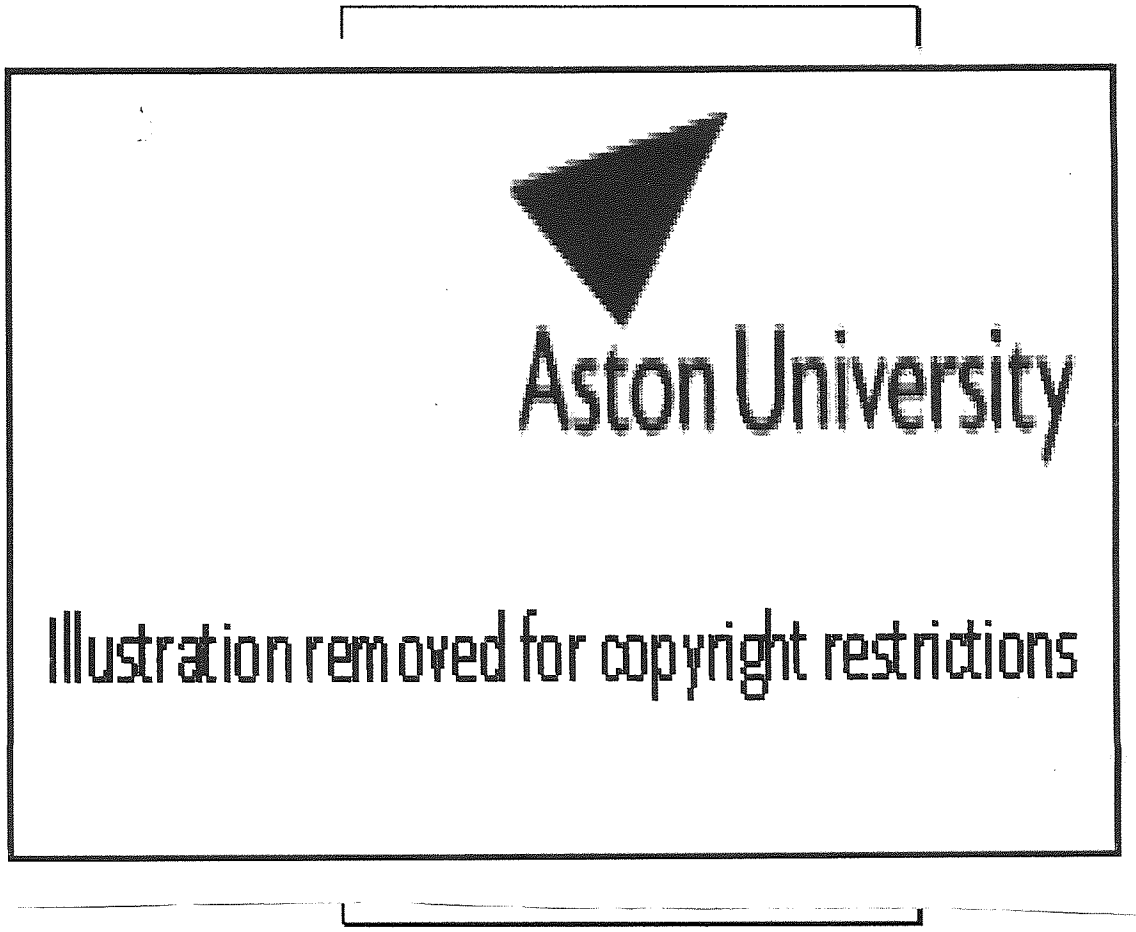

Figure 1.1 – The human gastro-intestinal (GI) tract. The pharynx, oesophagus, stomach, small intestine, large intestine, and rectum are clearly shown (Raven and Johnson, 1996).

Absorption occurs following digestion and occurs primarily in the small intestine; however, some absorption occurs in the stomach, for example aspirin is absorbed directly by the stomach (Sherwood, 1997). The process of absorption allows the products of digestion, along with water, vitamins, and electrolytes to be transferred from the digestive tract of the lumen into the blood or lymph.

The intestinal lymphatics are a specialised absorption pathway through which dietary lipids, fat soluble vitamins and lipophilic drugs can gain access to the systemic circulation. Exogenous compounds absorbed *via* the intestinal lymph are generally transported in association with the

lipid core of intestinal lipoproteins, chylomicrons are the major transport lipoprotein (Porter and Charman, 1997; Nordskog *et al.*, 2001). Water / octanol coefficient (LogP) and lipid solubility are important determinants for intestinal lymphatic transport (Bibby *et al.*, 1996; Porter and Charman, 1997; Edwards *et al.*, 2001). A LogP value ≥ 5 (highly lipophilic) and a lipid solubility of 50 mg / ml are required, increased lymphatic transport is observed with higher lipid solubility (Porter and Charman, 1997; Porter and Charman, 2001). Absorption *via* the intestinal lymphatics has been shown to contribute to the absorption of a number of highly lipophilic drugs such as cyclosporin, probucol, mepitiostane and lipophilic vitamins (Porter and Charman, 1997).

1.2.1 Structure and Function of the Gastro-Intestinal Tract

The GI tract has a common structure throughout its length, from the oesophagus to the anus, with areas of local specificity. The common structure is three layers of smooth muscle, two longitudinal and one circular (Figure 1.2).

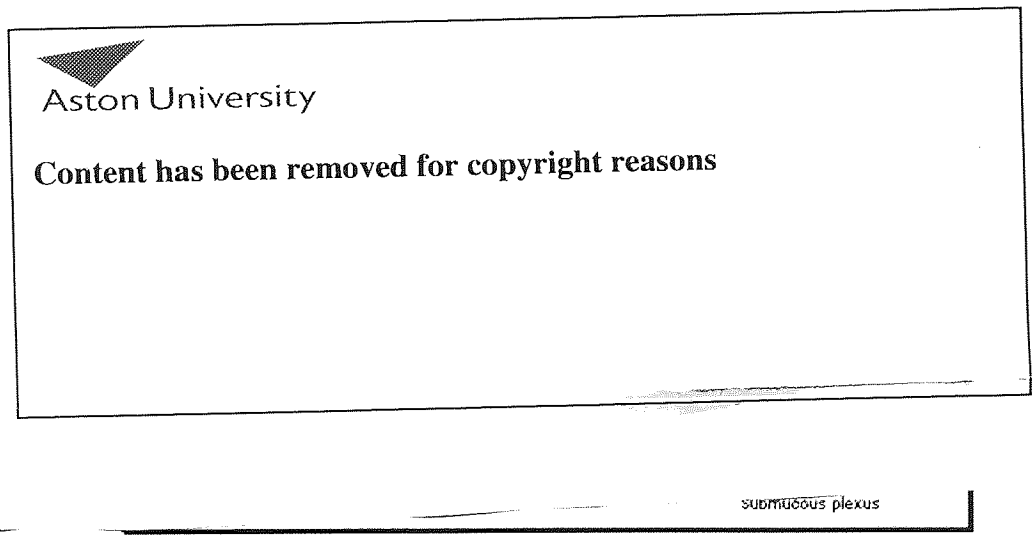

Figure 1.2 – Physiology of the GI tract illustrating the four major tissue layers of the digestive tract wall – the mucosa, submucosa, muscularis externa and serosa (Sherwood, 1997).

The efficiency of absorption varies along the GI tract with maximum absorption occurring in the small intestine (Ungell, 1997). In contrast, minimal drug absorption occurs in the stomach;

however, as stated previously some drugs, for example, aspirin are absorbed in the stomach (Sherwood, 1997). The stomach can influence drug absorption in a number of ways. Acid hydrolysis can cause degradation due to the pH of the stomach ranging from pH 2 to pH 4.

Following ingestion, drugs mix with the acidic contents of the stomach; however, there is relatively limited absorption from this organ, partly due to the surface area and contact area. Although, small amounts of certain lipid-soluble compounds can be absorbed, including aspirin, other non-steroidal anti-imflammatory drugs and ethanol. The mixing of food with gastric secretions to produce chyme is known as gastric mixing, gastric emptying follows this process. Two litres of gastric juice are produced everyday (Sherwood, 1997). Gastric emptying enables the contents of the stomach to move from the stomach into the small intestine, the major site of absorption for both food and drugs (Kararli, 1989). Gastric emptying is an important physiological event that significantly influences the uptake of drugs from the intestine.

The gastric mucosa exclusively contains secretory cells, *e.g.* mucous, chief, parietal, and endocrine. Gastric fluid contains electrolytes, pepsin, gelatinase, intrinsic factor, water, and mucus; approximately three litres of gastric fluid are secreted daily by gastric glands. Glands in the pyloric region are mainly responsible for secreting mucus. Mucus is composed of mucopolysaccharides and plays a vital role in the lubrication of food (Kararli, 1989); however, mucus can limit drug absorption (Section 1.2.5).

1.2.2 Small Intestine

The small intestine is the major site of food and drug digestion and absorption; consequently a detailed understanding of the anatomical structures is required to understand the different mechanisms of drug absorption.

1.2.2.1 Composition of the Small Intestine

The small intestine consists of three sections: duodenum (0.2 metres), jejunum and ileum (2.5 metres combined). The transit time through the small intestine is 3.5 – 4.5 hours (Wilson, 1989). The duodenum and the jejunum are the major sites of absorption with very little absorption occurring in the ileum. The ileum has absorptive capacity; however, the majority of absorption has already been achieved in the duodenum and jejunum. The duodenum is the site where bile and pancreatic juices are secreted. There are no macroscopic differences between the jejunum and the ileum; however, the ileum has more Peyer's patches. Peyer's patches are collections of sub-epithelial lymphoid follicles burgeoning amongst the villi and distributed throughout the small intestine (Hussain *et al.*, 2001). The inside of the intestinal wall is covered by mucosa and the serosa covers the outside.

The small intestine is well adapted for its absorptive function. An important morphological feature concerned with the efficient absorption of food and drugs is a large surface area; in humans, the total surface area of the small intestine is approximately 200 m^2 and the epithelial cells possess a variety of specialised transport mechanisms. The surface area is large due to the presence of villi and microvilli. The presence of the brush-border membrane – circular folds, villi (finger-like projections) and microvilli (hair-like projections) increases the surface area of the small intestine 600-fold compared to a tube of the same length and diameter lined with a flat surface (Sherwood, 1997; Ganong, 1997).

Villi are folds in the surface of the intestine (mucosa) that are 0.5 to 1 mm in length and have a diameter of 0.1 mm. There are 20 – 40 villi per square millimetre of mucosa (Ganong, 1997). Both the finger and leaf-shaped villi are found in humans. Enterocytes (or columnar absorptive cells) make up 90 per cent of the epithelial cell population found on the mucosa; hence, enterocytes form the greatest anatomical barrier against drug absorption (Kararli, 1989). Epithelial cells are occasionally interspersed with mucous cells that cover the surface of the each villus.

The most distinctive feature of the enterocyte (Figure 1.3) is its apical or brush-border membrane, composed of densely packed microvilli; approximately 1 μm in length and 0.1 μm in diameter. Each epithelial cell has as many as 3000 to 6000 microvilli. Exterior to the microvilli is the glycocalyx or 'fuzzy coat'. The glycocalyx extends 0.1 μm from the tips of the microvilli. Enterocytes continuously secrete glycocalyx that is made from glycoprotein. The apical membrane with its 'fuzzy coat' contains digestive enzymes including sucrase, oligoaminopeptidases, maltase, and alkaline phosphatase (Kararli, 1989). The small intestine enzymes perform their function within the membrane of this brush-border (Sherwood, 1997). The brush border enzymes form a barrier to drug absorption, refer to Section 1.2.5 and Section 1.5. Additionally, the glycoprotein coat serves to protect the intestinal mucosa (MacAdam, 1993). Adjacent to the surface of the cell membrane is an unstirred water layer (UWL). To reach the mucosal cells (enterocytes) solutes must first diffuse across the unstirred water layer (Ganong, 1997).

Enterocytes are produced through mitotic division and maturation of the cells occurs in the Crypts of Lieberkuhn that are located at the base of the villi. The epithelial cells have a rapid rate of turnover because of the harsh luminal conditions; hence, there is a high mitotic activity in the crypts, the crypts function as 'nurseries' (Sherwood, 1997). The rapid turnover rate of epithelial cells does not cause inflammation because the epithelial cells undergo apoptosis (programmed cell death) consequently; cells die without causing an inflammatory response (Ganong, 1997). Division and migration of the precursor cells in the crypts is a continuous process, replenishing daily losses, and repairing the damage incurred to the epithelium during the digestive processes (Berne and Levy, 1993). The maturing enterocytes continuously migrate up to the tips of the villi; the migrating cells push off older cells at the tips of the villi into the lumen. The average life of these cells is three to five days; approximately 50-million cells are sloughed per day (Kararli, 1989). The mature cells forming the villi and microvilli are the most effective at absorption and have active uptake mechanisms present that other cells will not possess until maturity. As the cells progress up the villus several changes occur, including an increase in brush border enzyme concentration and the capacity for absorption improves. The resultant being, that the cells at the tip of the villus have the greatest digestive and absorptive

capabilities. The old cells that are sloughed off into the lumen are absorbed into the blood and reclaimed for synthesis of new cells (Sherwood, 1997). The high rate of cell division in the crypts results in these cells being very sensitive to damage by radiation and anticancer drugs, both of which inhibit cell division (Sherwood, 1997).

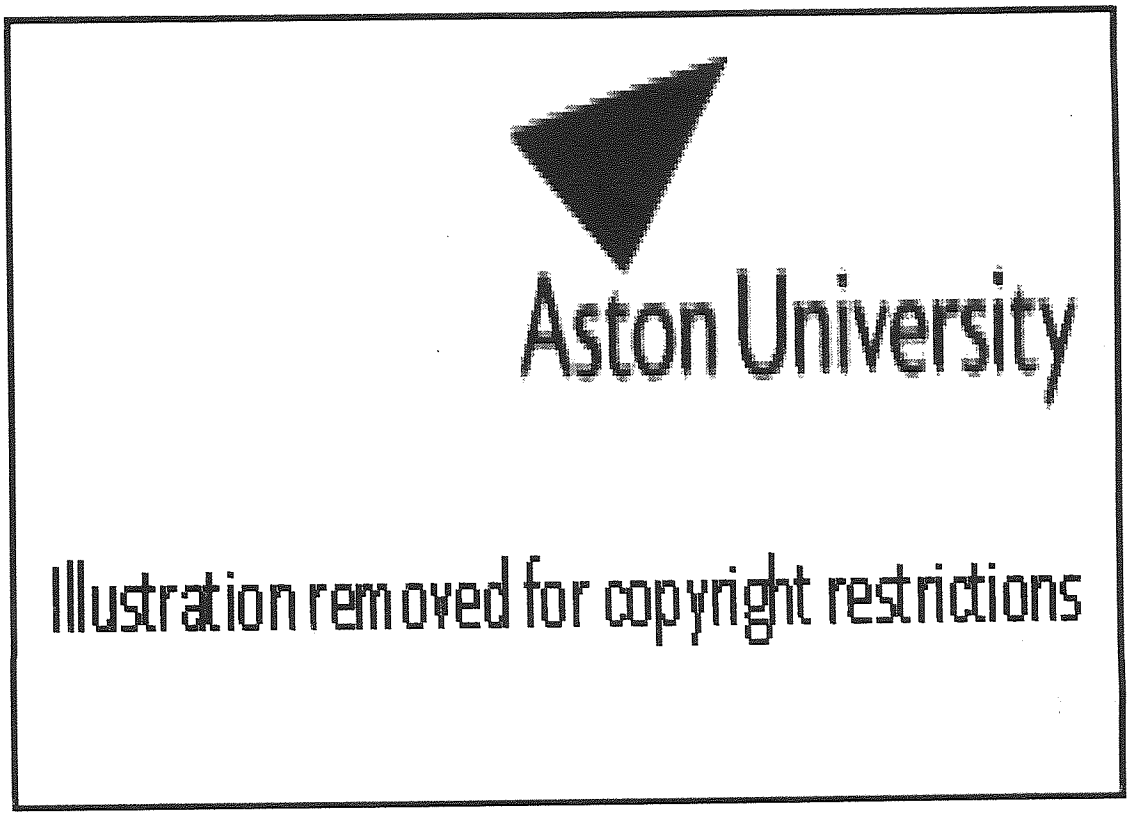

Figure 1.3 – Diagram of an enterocyte from the human small intestine, showing the microvilli that make up the brush border (Ganong, 1997).

The core of the microvilli consists of parallel filaments of actin. These filaments are anchored to the cell membrane and connected to the network of filaments that form the terminal web and lateral plasma membrane. The terminal web extends across the apical cytoplasm just beneath the microvilli. The presence of these structures allows the microvilli to have a contractile capacity that plays a role in the rapid diffusion of the absorbed substances away from the apical portion of the enterocyte. The filaments of the terminal web are involved in the formation of junctional complexes, tight junctions (zonula occludens), zonula adherens, and desmosomes (macula adherens). Tight junctions circle the apical end of each epithelial cell and separate the contents of the intestinal lumen from the intracellular spaces. They separate the apical

membrane from the basolateral membrane. Mechanical stability of the epithelium is provided by the zonula adherens and the desmosomes (Kararli, 1989).

1.2.2.2 Cellular Composition of the Small Intestine

The four main cell types of the intestinal epithelium are absorptive (columnar) epithelial cells, goblet cells, enteroendocrine cells, and Paneth's cells originate from the stem cells at the base of the crypts, the latter two types of cells occur in smaller numbers (Artursson, 1991). Paneth's cells are protein-secreting cells and are only found in the bases of the crypts. After feeding, secretion of the enzyme lysozyme increases. Lysozyme digests bacterial cell walls; hence, Paneth's cells may be involved in regulating the microbial flora of the intestine. The renewal rate of Paneth's cells is slow at about 30 days. Goblet cells are found scattered among the enterocytes in the villi. Goblet cells excrete an acidic glycoprotein that forms a protective layer on the glycocalyx of the microvilli of the enterocytes. Goblet cells have an average lifespan similar to enterocytes of about three days. Enteroendocrine cells are found in the crypts and on the villi and secrete peptides that regulate gastric secretion, and gall-bladder contraction. Additionally, a number of migrating cells can be identified, predominately lymphocytes, however, other leukocytes have been identified. Finally, M cells are found in the epithelium overlying Peyer's patches. M cells are specialised cells whose basolateral surface is closely related to lymphocytes lying in the epithelium. M cells are responsible for transporting macromolecules from the lumen of the intestine to these lymphocytes, where responses to foreign antigens can be undertaken (Leeson *et al.*, 1988). Immunoglobulin G (IgG) and immunoglobulin A (IgA) are associated with M cells (Ganong, 1997; Clark *et al.*, 2001).

Divisions exist between cells because the epithelium is not a continuous layer. Tight junctions are present at the apical region of the cells, below the brush-border membrane, forming a tight 'seal' between neighbouring epithelial cells. Tight junctions separate the contents of the intestinal lumen from the contents of the intercellular spaces. Tight junctions limit passive diffusion through the intercellular spaces; therefore, controlling the interchange between the luminal contents and the circulation. They play a crucial role in maintaining the selective barrier

function of cell. Functionally, the tight junctions are not sealed but are permeable to water and electrolytes and other molecules. The size of the pores varies along the length of the GI tract; the usual upper limit is 200 Daltons (Da) (Hochman and Artursson, 1994).

1.2.3 Large Intestine

The large intestine consists of the colon, caecum, appendix, and rectum. The colon makes up most of the large intestine, and is divided into three sections, the ascending colon, the traverse colon, and the descending colon. In humans the colon is about 1.1 metres in length (Kararli, 1989; Sherwood, 1997). After passing through the small intestine, the remaining food and drugs will enter the colon, in the form of chyme. The colon receives approximately 1 – 2 litres of chyme from the small intestine, the contents are indigestible food residues, *e.g.* cellulose, unabsorbed biliary components, the remaining fluid, only 200 ml *per* day is eliminated (Bieck, 1993; Ganong, 1997).

After the luminal contents have left the small intestine and entered the large intestine, no further digestion or absorption of nutrients occurs. The large intestine does not secrete digestive enzymes because digestion has already occurred; hence, unlike the small intestine, the large intestine is not designed for absorption, there are no villi present in the colon reducing the surface area. Colonic enterocytes differ from enterocytes found in the small intestine, the number of microvilli is greatly reduced and the glycocalyx (or 'fuzzy coat') is less compact. Additionally, there are no specialised transport mechanisms in the large intestine for absorption to take place. The mucosal surface of the colon is a flat mucosa with deep crypts. However, the large intestine is capable of absorbing water, electrolytes and short-chain fatty acids formed during fermentation of carbohydrates by colonic bacteria. The colon extracts water and salt from chyme and then converts chyme into faeces to be excreted from the body (Kararli, 1989).

The residence time of food in the colon can vary from hours to days depending on the individual dietary habit and level of physical activity. Movement in the large intestine is slow and non-propulsive, consequently bacteria have time to grow and accumulate in the large intestine, the

majority of colonic micro-organisms are harmless. An estimated 500 different species of bacteria reside in the colon the majority being anaerobic and are involved in reductive reactions, *e.g. Escherichia coli* and *Enterobacter aerogenes* (bacilli) and *Bacteroides fragilis* (pleomorphic organisms) (Sherwood, 1997; Ganong, 1997). The colonic bacteria digest some of the cellulose for their own metabolism.

1.2.4 Gastro-Intestinal Tract Epithelium

The human body has many specialised organs that perform specific and vital functions; for the body to achieve these functions, the organs that perform them are highly specialised in their structure, morphology and presence of enzymes *etc.* – the GI tract is no different. The GI tract epithelium has a specialist structure maximising absorption, as previously described (Section 1.2.1 and 1.2.2).

The components and composition of the cellular membrane determines the fluidity and function of the membrane. Diet and disease states can alter the lipid composition of the GI tract epithelium (Ungell *et al.*, 1998).

For orally administered drugs to be effective, they must first traverse through the GI epithelium and reach their target tissue or organ; methods utilised to traverse the epithelium are discussed in (Section 1.3). Successful drug delivery can only be accomplished if the physical and biological barriers presented by the GI can be circumvented. The epithelial cells and tight junctions located between the epithelial cells pose a permeability barrier to drug absorption. The intestinal epithelium acts as a physical barrier impeding the movement along the paracellular and transcellular routes. Efflux mechanisms return the drugs back to the GI tract lumen. In addition, enzymatic metabolism and degradation is a biochemical barrier (Pauletti *et al.*, 1996).

To penetrate the GI epithelium, drug molecules must pass through the brush-border, the intracellular space and the basolateral membrane. In addition to the digestion and absorption of

food, the intestine must protect the body from systemic exposure and absorption from various toxins, micro-organisms and antigens. The bioavailability of orally administered drugs can be affected by a number of factors. Aqueous solubility and dissolution rates are important. The poor solubility of griseofulvin (15 μg/ml at 37°C) means that griseofulvin has a limited oral bioavailability (Hörter and Dressman, 2001). Instability in acidic pH or metabolism by luminal digestive enzymes, or by luminal micro-organisms all lead to degradation. Another major problem is the efflux of drugs after they have penetrated the epithelium (Anderle *et al.*, 1998). Digoxin, erythromycin and ritonavir are all substrates for efflux *via* P-glycoprotein (Spatzenegger and Jaegar, 1995; Ambudkar *et al.*, 1999; Matheny *et al.*, 2001).

1.2.5 Physicochemical Barriers to Oral Drug Delivery

As with any route of drug delivery, there are barriers to drug absorption (Figure 1.4); these can be general (*e.g.* solubility and dissolution) or specific (*e.g.* enzymatic degradation and efflux). General barriers to drug delivery *via* the oral route are discussed within this section.

For an orally administered drug to reach its intended destination, the drug must first overcome several steps or barriers (Figure 1.4). For a drug to be successful it must be soluble in the aqueous environment, solubility is a major concern with lipophilic drugs. A slow dissolution rate may lead to incomplete absorption because the drug might be too far down the GI tract by the time the drug has dissolved into solution. Additional physiological factors include GI motility, gastric emptying, the surface pH of the mucosa, the unstirred water layer, intestinal blood and lymph flow, and colonic microflora.

When gastric emptying occurs, the acidic contents of stomach enter the small intestine (duodenum). The acidic contents are neutralised by the secretion of a bicarbonate-rich alkaline fluid from the pancreas along with other alkaline secretions. The pH progressively becomes alkaline. Weak acidic and basic drug absorption can be affected by the changing pH.

In addition to the changing pH, an acidic microlayer approximately 20 μm thick exists next to the intestinal mucosa, (Kakemi *et al.*, 1969); the pH of the microlayer is about 0.5 lower than the bulk phase (pH 6.2) (Fawcus *et al.*, 1997). The acidic microlayer will have an effect on the fraction of ionised drug present at the site of absorption.

An unstirred water layer (UWL) is found adjacent to the gastro-intestinal wall; the UWL has a thickness of approximately 300 μm. Incomplete mixing of the luminal contents near the intestinal mucosal surface form the UWL. Transport across the UWL can be a rate-limiting step for drug absorption, due to the UWL not being in equilibrium with the bulk phase and thus, helps to preserve the acidic microlayer (Wilson and Dietschy, 1972). In order to be able to reach the hydrophobic phospholipid membrane, drug molecules must navigate through the UWL where the molecule may undergo binding to cell surface components, electrostatic attraction or repulsion, consequently for lipophilic molecules the UWL is an especially rate-limiting step.

Transport across the apical membrane is the rate-limiting step in the transmucosal uptake of low permeability compounds opposed to diffusion through the unstirred water layer. Intestinal absorption in humans for both low and high permeability compounds would appear to be membrane controlled. For highly permeable drugs (*e.g.* testosterone), the resistance to intestinal absorption provided by the UWL has been overestimated (Wilson and Washington, 1989); efficient stirring in the Caco-2 cell model doubled the permeability of testosterone, at the same time mannitol transport remained unaffected (Kuhfeld and Stratford Jr., 1996).

After successful navigation of the UWL, drug molecules face the epithelium with a number of different transport mechanisms for transport across the membrane. The basolateral membrane must be crossed for the drug to enter the systemic blood circulation. Metabolic barriers against absorption can mean that the drug is metabolised and / or excreted back across the apical membrane. Enzymes present include peptidases that break peptide bonds, esterases that can degrade esters and the cytochrome P450 family of enzymes that oxidise atoms to form new functional groups that can then be used to conjugate the drug metabolite to larger hydrophilic

groups (such as sulphates). These enzymes are located at the villus tips of enterocytes (Wacher *et al.*, 2001) (Section 1.5.1). Efflux transporters, *e.g.* P-glycoprotein (P-gp), pump the drugs back across the apical membrane (See Section 1.4).

The majority of successful drugs that are absorbed from the GI tract enter the portal blood supply and enter the liver. In the liver, the drugs may undergo extensive first-pass metabolism, these include metoprolol and diltiazem (Sirsuth and Eddington, 2002). Disadvantages of first-pass metabolism include inactivation of drugs; whereas, pro-drugs can exploit first pass metabolism. α-Methyldopa and Enalaprilat are examples of pro-drugs (Amidon and Lee, 1994).

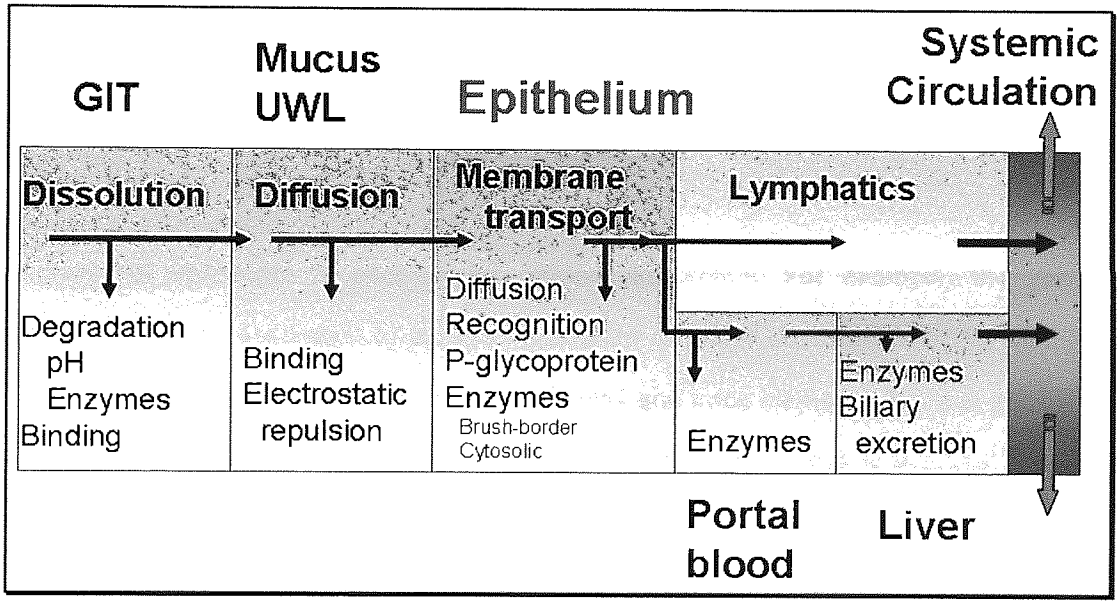

Figure 1.4 – Illustrating the barriers to absorption of oral drugs.

A number of absorbed drugs enter the systemic circulation *via* the intestinal lymphatic system, thereby avoiding potential first pass metabolism. Lymphatic transport is limited to highly lipophilic drugs (Section 1.2) that become associated with the triglyceride core of lipoproteins and gain entry to the lymphatic system; chylomicrons are the major transport lipoprotein. (Bibby *et al.*, 1996; Porter and Charman, 1997; Nordskog *et al.*, 2001). B-lymphocytes and T-lymphocytes play a major role in maintaining the immune system and they circulate through the lymphatics in relatively high concentrations compared with systemic blood making intestinal

lymphatic transport an important drug delivery route (Porter and Charman, 1997; Edwards *et al.*, 2001). To improve HIV treatment attempts have been made to target anti-HIV drugs for lymphatic transport (Bibby *et al.*, 1996; Edwards *et al.*, 2001). Prodrugs of 3'azido-3'deoxythymidine (AZT, zidovudine) have been synthesised to promote AZT transport through the intestinal lymph (Bibby *et al.*, 1996).

1.3 Mechanisms of Absorption

For a drug to reach its destination and be effective, it must traverse the intestinal epithelium and enter the blood stream and lymph systems. Drug absorption principally occurs *via* two methods, paracellular transport (between cells), and transcellular transport (through the cells). Transcellular transport can be further subdivided into three mechanisms, passive (Section 1.3.2.1), active carrier-mediated (Section 1.3.2.2) and endocytotic (Section 1.3.2.3) (Figure 1.5). These mechanisms are examined below in Section 1.3.1 and 1.3.2.

The intestinal epithelium is adapted for efficient absorption. For example, the intestinal epithelium facilitates absorption of nutrients and other essential constituents of the diet; these include amino acids, small peptides, sugars, vitamins and trace elements. Several transporters are located on the apical and basolateral membranes of the absorptive enterocyte; hence, the nutrients can be transported efficiently.

Many drugs administered orally are lipophilic with a high epithelial permeability. The cellular membrane is permeable to lipophilic drugs that are distributed into membrane lipids. Therefore, these drugs are generally rapidly absorbed. In contrast, hydrophilic and charged drugs are not distributed into the cell membranes; they are passively absorbed through water-filled canals between the cells – the paracellular route (Artursson, 1991).

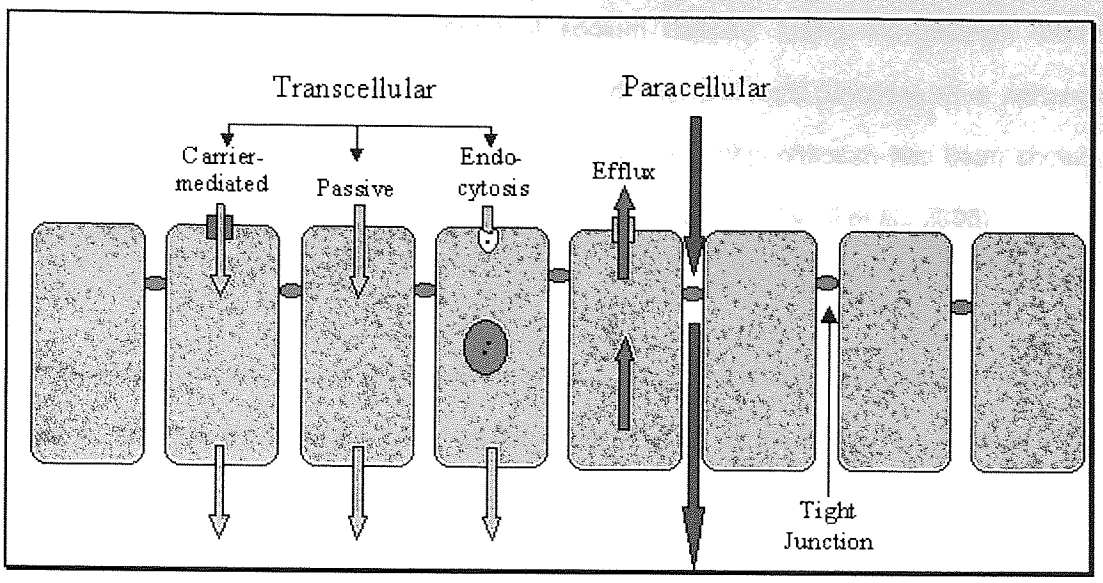

Figure 1.5 – The different routes a compound may use to traverse the epithelium membrane.

1.3.1 Paracellular Mechanisms

Paracellular diffusion, by definition is the movement of molecules between the tight junctions in the intercellular spaces and is a passive process. Paracellular transport across a healthy intestinal membrane is small compared to drugs that are transported passively or transcellular permeability (Hunter and Hirst, 1997). For example, the paracellular permeability of mannitol is 150 times less than the transcellular permeability of metoprolol.

Transport between the tight junctions immediately implements a strict size limit on molecules that can fit between the junctions (Hunter and Hirst, 1997). Only molecules with a molecular weight less than 200 Daltons (Da) are transported (Kingham and Loehry, 1976). Significant paracellular transport only occurs with very small ions, *e.g.* sodium ions (Na^+), chloride ions (Cl^-) and small molecules, *e.g.* urea. Disruption of the tight junctions affects the paracellular route; therefore, the route is sensitive to chelating agents (*e.g.* EDTA) that disrupt the tight junctions by binding with calcium or magnesium ions (Erlij and Martinez-Palarmo, 1972; Kararli, 1989). One method to improve absorption of hydrophilic drugs is to modulate the tight junctions, as they are not static (Hunter and Hirst, 1997). Sodium caprate has been shown to enhance cefotaxime sodium bioavailability *via* modulation of tight junctions (Sharma *et al.*, 2005). *In vivo* cefotaxime sodium is poorly absorbed (up to 30 %); however, in Male Sprague-Dawley rats, bioavailability

was increased to 70 % in the presence of sodium caprate. Using transmission electron microscopy sodium caprate was shown to induce dilations in tight junctions thus enhancing paracellular permeability (Sharma *et al.*, 2005). *N*-diethyl methyl chitosan has been shown to modulate tight junctions and increase *in vivo* absorption of insulin (Avadi *et al.*, 2005).

The intercellular spaces occupy only approximately 0.01 per cent of the total surface area of the epithelium; additionally, they are gated by the closely fitting tight junctions on the apical side. The mean pore radii of the intercellular spaces in the duodenum, ileum and colon in humans are 0.8, 0.3 and 0.4 nm, respectively; this allows only very small molecules to pass through the tight junctions. The duodenum is more permeable than the ileum and colon (Artursson, 1991).

1.3.2 Transcellular Mechanisms

1.3.2.1 Passive Transcellular

Passive transport is the movement of a solute along a concentration or electrical gradient, *e.g.* propranolol, or testosterone. Passive diffusion occurs through non-specific permeability pathways (Daugherty and Mrsny, 1999) and the solute does not interact with the structural elements of the membrane. The rate of transport is directly proportional to the gradient; it is not satuarble and cannot be inhibited by the presence of other drugs. The chemical potential difference of a drug across the membrane is the driving force of passive transport (Kararli, 1989).

LogP (logarithm of the partition coefficient) of a parent drug is related to the rate of absorption. Small molecules with a low LogP cannot penetrate the lipid bilayer; in contrast, small molecules with a high LogP value, are too lipophilic so that other factors become the rate-limiting step including diffusion through the unstirred water layer (Kararli, 1989; Daugherty and Mrsny, 1999). Increasing lipophilicity of a compound results in increasing permeability across the intestinal epithelium until a plateau is reached at a LogP value of approximately 2 (Martin, 1981). Compounds with a LogP of about 2 display the highest absorption and are generally predicted to be completely absorbed in humans. Permeability begins to decrease for compounds with a

LogP value greater than 4 (Wils *et al.*, 1994; Artursson *et al.* 2001). However, drugs that have a high LogP value (> 5) and lipid solubility > 50 mg / ml are able to enter the intestinal lymphatic system opposed to the systemic blood circulation (Section 1.2 and 1.2.5). Prodrugs of anti-HIV drugs have been researched to target the drugs to the lymphatic system to improve efficacy (Bibby *et al.*, 1996) (Section 1.2.5). Prodrug modifications have been used to mask charged hydrophilic regions to improve passive diffusion. For peptides, this index cannot be used to predict transport across the membrane. Transport of peptides with varying LogP values (-2.2 to +2.8) was measured using Caco-2 cell monolayers and there was no correlation between lipophilicity and transport; however, there was a strong correlation between the total number of hydrogen bonds that must be broken and transport (Conradi *et al.*, 1991; Conradi *et al.*, 1992). Consequently, the number of hydrogen bonds that must be broken to traverse the membrane is used in replacement (Daugherty and Mrsny, 1999).

Hydrophobic drug molecules can gain access into a cell by passive transcellular diffusion by incorporating into the lipid membrane and then partitioning into the cell, this is known as lipid-partitioning. Charged molecules, due to their high solubility and low partition coefficient, are impermeable to the lipid bilayer (Daugherty and Mrsny, 1999).

The pH-partition theory states that there is an inter-relationship between the extent of drug absorption, pK_a of the diffusing drug, and the pH of the site of absorption. The relationship between pH, pK_a (the ionisation constant) and the ratio of ionised and unionised forms of the drug are defined using the Handerson-Hasselbach equation (Equation 1.1, and Equation 1.2) (Kararli, 1989).

$$pH = pKa + log\frac{[unionised]}{[ionised]}$$

Equation 1.1 – The Handerson-Hasselbach equation for a weak acid.

$$pH = pKa + log \frac{[ionised]}{[unionised]}$$

Equation 1.2 – The Handerson-Hasselbach equation for a weak base.

The pH at any site will determine fraction of the drug that is unionised and consequently the extent of absorption because drugs have a higher lipid solubility in the unionised form and thus increased absorption. Drugs in the ionised form have limited absorption because of limited entry to the lipid bilayer (Kararli, 1989). Very weak bases and acids are essentially unionised at physiological pH and absorption is rapid and independent of pH. In contrast, strong bases and acids are completely ionised at physiological pH so there absorption is slow and pH-independent. Sensitivity to pH is likely to be seen with drugs whose unionised fraction changes substantially within the normal physiological range of pH values have sensitivity to pH. Such drugs include acids within the pK_a range 3 to 7.5 and bases in the pK_a range 7 to 11.

Ungell *et al.*, (1998) showed that there are regional variations of absorption and permeability. For low permeability drugs, *e.g.* mannitol, terbutaline and atenolol, the intestinal permeability decreased in the order, jejunum > ileum > colon. For high permeability drugs, *e.g.* gemfibrozil, metoprolol, propranolol, the intestinal permeability order is reversed, jejunum < ileum < colon.

Carrier-mediated transport can be either facilitated or active (discussed within Section 1.3.2.2). Facilitated transport differs from passive transport because the solute interacts with membrane proteins. Membrane proteins bind reversibly with specific substrates and aid the transport of the solutes across the membrane. As with passive diffusion, solutes move along a concentration gradient so there is no energy expenditure; however, the system can be saturated. Glucose transport is one of the few examples of facilitated diffusion (Kararli, 1989).

1.3.2.2 Active Transport Mechanisms

Active transport is similar to facilitated diffusion because membrane proteins specific to substrates are required; however, in contrast to facilitated diffusion, active transport, transports

solutes against concentration gradients and an energy source is needed. The energy can be supplied in two ways; firstly, hydrolysis of ATP (adenosine tri-phosphate), or another high energy compound on the surface of the protein serving as the porter. These are known as pumps and the hydrolysis of ATP leads to phosphorylation of the pumps, resulting in a conformational change exposing the active site with its ligand to the other side of the membrane, and thus, translocation into the cell. An example of active transport is Na^+ by the Na^+ / K^+ ATPase pump. Co-transporters transport two or more solutes at any one time. One solute is transported along its concentration gradient and so has a large and negative free energy transfer, *e.g.* Na^+-dependent amino acid transporter. Transport of the second molecule (that needs energy) can be in the same direction (symport) or in the opposite direction (antiport) (Kararli, 1989).

Active transport can only occur at intact, closed membranes. The membrane does not have to be the cellular membrane but can be membranes of cell compartments. Enabling ions and metabolites to be concentrated within the respective compartment of the cell and the steady state of metabolism can be kept constant despite large fluctuations in the composition of the external medium.

Active transport mechanisms are sensitive to metabolic inhibitors, *e.g.* sodium azide. Substrate analogues compete for the active site inhibiting transport, this can be either competitive or non-competitive. Competitive inhibitors compete with solute for the active site, both the competitor and solute bind to the membrane proteins reversibly; increasing the solutes concentration can reverse inhibition. Several active uptake transporters can be found along the GI tract, *e.g.* amino acid transporters and di-tri-peptide transporters (Kararli, 1989). The transport of compounds through the transporters follows Michaelis-Menten kinetics (see Appendix 1).

1.3.2.3 Endocytosis

Endocytosis is the uptake of material into a cell by inclusion within the plasma membrane. Endocytosis occurs when the cytoskeleton of the plasma membrane extends outwards towards

macromolecules, the plasma membrane completely encircles the macromolecule and the plasma membranes fuse together when they meet forming a vesicle around the macromolecule. The vesicle is then free to merge with all cellular membranes, *e.g.* lysosomes (the contents will be degraded) or the basolateral membrane (the contents will enter the blood stream) (Raven and Johnson, 1996; Daugherty and Mrsny, 1999).

Different variants of endocytosis occur: phagocytosis brings particulate or organic material into the cell, pinocytosis is the process of fluid uptake in a cell, and receptor-mediated endocytosis allows large molecules including epidermal growth factor, immunoglobulins, transferrin and vitamin B_{12} to enter cells *via* specific receptors. (Raven and Johnson, 1996; Daugherty and Mrsny, 1999). Endocytosis is minimal in the small intestine, it is an energy-intensive process and, except for receptor-mediated endocytosis, is not a selective process thereby enabling unwanted material to enter the cell (Raven and Johnson, 1996). Transcytosis is an important pathway for specialised intestinal cells such as M cells of the immune system (Hunter and Hirst, 1997). Macropinocytosis is a form of endocytosis that accompanies cell surface ruffling. Macropinocytosis provides an efficient route for non-selective endocytosis of solute macromolecules and may facilitate antigen presentation by dendritic cells (Swanson and Watts, 1995). Membrane rafts in cellular membranes are clusters enriched with cholesterol and sphingolipids; proteins partition preferentially into membrane rafts. They form because lipids do not always mix uniformly in membranes and form microdomains (Brown, 2002).

1.4 Efflux Mechanisms and Barriers to Drug Absorption

When a drug has crossed the apical surface and entered a cell, it has to cross the basolateral membrane to leave the cell and enter the blood. In addition to physicochemical barriers, metabolic barriers against absorption can exist. Instead of leaving the cell, the drug may be metabolised into a different compound or excreted back across the apical surface. Several cellular enzymes can potentially metabolise the drug, including peptidases (breaks peptide bonds) and esterases (degrades esters). The cytochrome P450 family of enzymes oxidise atoms within the drug creating a new functional group that can then be used to conjugate the

drug metabolite to larger hydrophilic groups (such as sulphates). Many efflux transporters have been identified. The most well known are P-glycoprotein (P-gp), multidrug-resistance protein (MRP) and the organic anion transport protein (OATP). These transporters are examined in more detail below. Enzyme metabolism and efflux form a combined barrier see Section 1.5.1 for a more detailed explanation.

One of the main obstacles to drug delivery is the process of efflux. Efflux is an active secretory mechanism that occurs once the 'drug' has successfully traversed the apical membrane. Once inside the cell, the 'drugs' are subject to transporters known collectively as efflux pumps, that work against the transporters that transport drugs into the cells – the efflux pumps transport the drugs back out of the cell; hence, decreasing drug absorption and bioavailability.

1.4.1 P-Glycoprotein (P-gp)

1.4.1.1 Structure and Expression of P-Glycoprotein

In 1976, Juliano and Ling first discovered P-glycoprotein; since 1976 P-glycoprotein has been rigorously researched. The human gene encoding P-glycoprotein, *mdr1*, was cloned and sequenced in 1986. The *mdr1* gene encodes a 1280 amino acid protein (Chen *et al.*, 1986). P-glycoprotein is a 170 kDa protein (Figure 1.6). P-glycoprotein is composed of twelve putative transmembrane regions and two ATP binding sites (Germann, 1993). The polypeptide can be split into two similar halves each containing six putative transmembrane regions and one intracellular ATP binding site. The first extracellular loop is heavily N-glycosylated (Schinkel and Jonker, 2003).

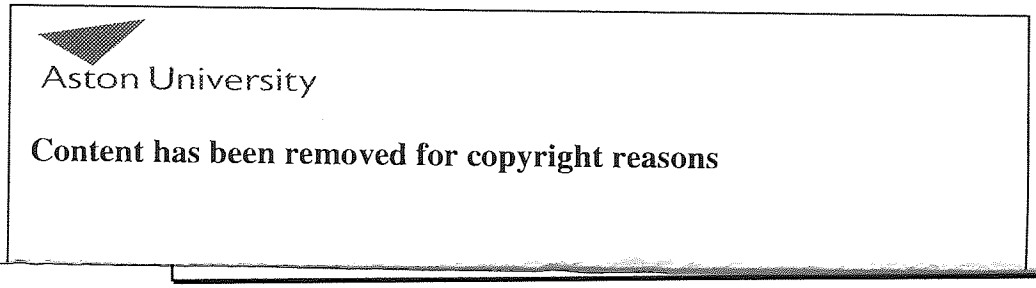

Figure 1.6 – Predicted secondary structure of the efflux transporter P-glycoprotein (Schinkel and Jonker, 2003).

Expression of P-glycoprotein is linked to the villus epithelium of the small intestine and displays complimentary expression with a fellow member of the ABC gene family, the cystic fibrosis transmembrane regulator protein (CFTR). CFTR is expressed in the crypts, as the enterocytes differentiate and move up the crypt-villus axis the expression is switched to P-glycoprotein so expression is maximal at the villus tips (Hunter and Hirst, 1997). P-glycoprotein function can be modulated by excipients, refer to Section 1.9.2 for a more detailed explanation.

1.4.1.2 Function of P-Glycoprotein

P-glycoprotein is a member of the ATP-binding cassette (ABC) super-family of membrane transporter proteins. The ABC super-family is extensive and diverse; other examples include the multidrug resistance proteins (MRPs) 1 – 5, and breast cancer resistance protein (BRCP). The ABC transporters are primarily located on the plasma membrane (Schinkel and Jonker, 2003). The ABC super-family can act as importers, exporters, receptors and channels capable of mediating an abundance of physiological phenomena (Kerr, 2002). Consequently, they pose a major obstacle to drug delivery and absorption, as they transport drugs back out of the cell, a process known as efflux.

P-glycoprotein is an active, ATP-dependent efflux pump that can transport compounds against substantial concentration gradients (Schinkel and Jonker, 2003). As with active transport processes described in Section 1.3.2.2, hydrolysis of ATP provides the energy required for P-glycoprotein to function.

The pharmacological behaviour of many drugs used today is altered by P-glycoprotein and fellow members of the ABC family of proteins, altered behaviour includes oral bioavailability, hepatobiliary, direct intestinal, and urinary excretion of drugs and drug-metabolites and drug-conjugates (Schinkel and Jonker, 2003).

The mechanism of P-glycoprotein action remains unknown and different theories have been put forward, these include aqueous pore, hydrophobic vacuum model, and flippase model (Hunter and Hirst, 1997). The simplest model is the aqueous pore model, with P-glycoprotein forming a transmembrane pore through which substrates from within the cell are pumped out into the aqueous environment on the outside of the cell (Higgins *et al.*, 1997). P-glycoprotein has been described as a 'hydrophobic vacuum cleaner', identifying drugs in the plasma membrane opposed to the aqueous phase and removing the drugs from cells (Hunter and Hirst, 1997). Currently, it is thought that P-glycoprotein operates as a 'flippase'. The drug is recognised within the inner leaflet of the membrane and 'flips' into the outer leaflet or directly into extracellular space (Hunter and Hirst, 1997; Ueda *et al.*, 1997; Schinkel and Jonker, 2003). The flippase model is supported by human *mdr2* P-glycoprotein, a phosphatidylcholine-specific translocase in the bile canalicular membrane hepatocytes. Human *mdr1* P-glycoprotein and human *mdr2* P-glycoprotein share 80 per cent identity at the amino acid level. All of the amino acids reported to alter substrate specificity of *mdr1* were conserved between *mdr1* and *mdr2*. Therefore, based on these results, *mdr1* may work as a flippase like *mdr2*, so that *mdr1* interacts with a substrate in the inner leaflet of the lipid bilayer and flips them to the outer leaflet (Ueda *et al.*, 1997). The 'flippase model' may explain why P-glycoprotein exhibits specificity for a broad range of substrates. Further to this, the substrate must intercalate into the lipid bilayer, amphipathy of the substrate being important, the substrate must interact with the binding-site(s), molecular volume and structure determining whether the substrate can fit into the binding-site (Ueda *et al.*, 1997). Many substrates of P-glycoprotein are hydrophobic and in theory they should diffuse passively across the lipid bilayer with ease. The P-glycoprotein transporter is saturable at high intestinal concentrations; therefore, if a drug has a high passive diffusion the effect of P-glycoprotein will be relatively low because the transporter will be saturated. In contrast, P-glycoprotein will have a significant effect on compounds that have a low passive diffusion rate (Schinkel and Jonker, 2003). Efflux transporters like P-glycoprotein may begin to explain why observed permeabilities differ from drug permeabilities calculated from water-octanol partition coefficients.

1.4.1.3 Natural Function of P-Glycoprotein

In addition to the efflux mechanisms of P-glycoprotein, there appears to be a natural function within the human body as a secretory detoxifying system. P-glycoprotein expression in the kidney, bowel and biliary tree facilitates the excretion and / or to minimise the absorption of toxic natural products present in our diet and environment. P-glycoprotein is present in the placenta and may function as part of the maternal-foetal barrier (Hunter and Hirst, 1997).

Evidence for a natural function of P-glycoprotein was provided by Panwala *et al.*, (1998) who studied knock-out mice deficient in *mdr1a* P-glycoprotein. These knock-out mice developed inflammation in the large intestine similar to the inflammatory bowel disease, colitis, occasionally observed in humans. The observed inflammation did not appear to be a consequence of an abnormal immune response; however, the inflammation was dependent on the presence of intestinal bacteria. One possible explanation is that toxins produced by the intestinal bacteria are normally removed by *mdr1a* P-glycoprotein, however, the inflammation occurs in the knock-out mice due to the accumulation of the toxins.

1.4.1.4 Tissue Distribution and Location of P-Glycoprotein

Three distinct types of normal human tissues express P-glycoprotein; a subset of columnar epithelial cells, endothelial cells of capillary beds in specific anatomical locations and placenta trophoblasts (Hunter and Hirst, 1997). A wide number of tissues express P-glycoprotein including adrenals, bladder, endothelial cells of the blood-brain barrier, kidney, liver, lungs, pancreas, rectum, spleen; and more importantly for oral drug delivery, the oesophagus, stomach, jejunum, and colon (Benet *et. al.*, 1999).

1.4.1.5 Substrates of P-Glycoprotein

One aspect of P-glycoprotein function has still to be answered; what properties does a compound require to be a substrate for P-glycoprotein? P-glycoprotein transports a vast number compounds with a varied structure, including digoxin, methotrexate, and vincristine (Table A4.1). There appears to be no distinct set or rules to define which compounds will be P-

glycoprotein substrates. Substrates tend to be hydrophobic, in the molecular weight range 300 to 2000 Da (Ueda *et al.*, 1997) are amphipathic, containing one or more aromatic rings (Benet *et al.*, 1999) and often have a positive charge at physiological pH. It has been proposed that substrates need to be amphipathic so they can be intercalated into the lipid bilayer. Uncharged or (weakly) basic drug molecules are transported most efficiently; however, acidic compounds, *e.g.* methotrexate or phenytoin, are transported at a low rate (Schinkel and Jonker, 2003). Although, P-glycoprotein recognises and transports substrates with a wide range of structural features, it would appear that there is specificity from P-glycoprotein in identifying substrates. This specificity of P-glycoprotein has been investigated using mutational analysis, it was concluded that amino acid substitution that affect the specificity were located throughout the transmembrane domains, cytoplasmic loop and in the nucleotide-binding fold (Ueda *et al.*, 1997).

1.4.1.6 Inhibition and Modulation of P-Glycoprotein Activity

Inhibitors of P-glycoprotein can modulate or reverse the effect of P-glycoprotein; these include verapamil (calcium channel blocker) and cyclosporin A (immunosuppressive agent) (Hunter and Hirst, 1997; Schinkel and Jonker, 2003) (Table A4.1). Many of the inhibitors of P-glycoprotein are substrates themselves implying that they function as competitive inhibitors. Inhibitors that are not P-glycoprotein substrates act *via* another mechanism (Schinkel and Jonker, 2003).

1.4.1.7 *In Vitro* Models

In vitro models used to study P-glycoprotein include cell lines; commonly used cell lines include the Caco-2 cell line and HT-29 cell line, further explained in Sections 1.6.3 and 1.7.

1.4.2 Multidrug Resistance (MDR)

P-glycoprotein is the underlying mechanism for the phenomenon termed multidrug resistance (MDR) and decreased cellular accumulation. MDR is defined as the ability of cells exposed to one form of a drug to develop resistance to a broad range of structurally and functionally unrelated drugs, due to enhanced efflux of drugs out of the cell. Cell lines that display MDR

have significant cross-resistance to other drugs, including natural products, *e.g.* anthracyclines (Hunter and Hirst, 1997). A membrane glycoprotein transporter mediates this process. Research in this field led to laboratories around the world isolating their own drug-resistant cell lines, from these cell lines a 190 kDa protein band was detected and named the multidrug-resistance associated protein (MRP); therefore, the *mdr* gene confers the MDR phenotype. MRP and P-glycoprotein have a similar tissue distribution, are both members of the ABC family of transporters, and have a wide range of overlapping substrates. MDR is a major clinical problem for administered chemotherapeutic drugs, with a reduced cellular accumulation and retention of drugs (Hunter and Hirst, 1997).

1.5 Enzymatic Metabolism

Metabolism is defined as the modification of a compound as a result of enzymatic attack. The metabolism of carbohydrates, proteins and fats provides the energy for life processes (Ganong, 1997). Metabolism removes foreign molecules that have been absorbed in the GI lumen (Thummel *et al.*, 1997). Metabolism can be detrimental to drug absorption; for example, converting a lipophilic compound that is easily absorbed into a more hydrophilic compound that is less easily absorbed. There are two phases of metabolism, phase I and phase II. Phase I metabolism is the formation of a functional group in the molecule and the conjugation of a hydrophilic group to the functional group is known as phase II metabolism (Spatzenegger and Jaeger, 1995). Phase I metabolism is primarily catalysed by the cytochrome (CYP) P450 mono-oxygenase system, comprising of CYP450s and several other enzymes.

Administered therapeutic drugs need to be eliminated once they have performed their function, these drugs are metabolised by cytochrome P450 enzymes. The highest concentration of CYP enzymes is found in the liver although they are found in almost all tissues. The kidney is responsible for the excretion of the metabolised drugs in urine, the kidney is far more efficient at excreting polar compounds, and consequently the CYP450 enzymes catalyse the biotransformation of lipophilic drugs to more polar compounds. Generally this biotransformation produces less active or inactive metabolites; however, in rare circumstances the metabolites

produced have an enhanced pharmacological activity or display toxic side effects (Spatzenegger and Jaeger, 1995).

1.5.1 P-Glycoprotein and Cytochrome P450

Figure 1.7 – Synergistic effects of CYP3A4 and P-glycoprotein on intestinal metabolism.

Drugs in the lumen (hexagon) enter the enterocyte across the apical membrane and are subject to P-gp (circle) mediated efflux back into the lumen, intestinal metabolism (triangle), or are absorbed into the systemic circulation. Repeated cycles of absorption and luminal efflux increase the net exposure of the drug to CYP3A4 prior to systemic absorption resulting in efficient intestinal metabolism (Lin, 2003).

The primary site for absorption of orally administered drugs is the villus enterocytes of the small intestine, where both P-glycoprotein the multidrug efflux pump and CYP3A4 the major phase I drug metabolising enzyme in humans, are both present at high levels. P-gp and CYP3A4 form a major obstacle to drug delivery, absorption and bioavailability; the same compounds induce both of these proteins, also they share similar substrate and inhibitor specificities. P-gp and CYP3A4 are a major determinant of oral drug bioavailability and variability (Wacher *et al.*, 1996). An example is cyclosporin, which is metabolised into many compounds that are actively transported out of the cell by P-glycoprotein (Benet *et al.*, 1996). Cyclosporin is not poorly absorbed; moreover, two-thirds of the oral dose is metabolised in the gut by CYP3A thus limiting bioavailability (Benet *et al.*, 1996). Without efflux, drugs are only be exposed to metabolism for a

relatively short time; however, with efflux the drugs are recycled leading to increased exposure resulting in substantial metabolism (Figure 1.7).

P-glycoprotein and CYP3A4 act synergistically against drug delivery and absorption whereby CYP3A4 metabolises compounds forming new modified compounds that are substrates for P-gp, the newly modified compounds / metabolites have an increased polarity making them less subject to passive reabsorption (Wacher *et al.*, 1996).

1.6 Absorption Models

An ideal way to study small intestine uptake and transport in humans would be to use humans; however, this is not ethically appropriate nor is the small intestine easily accessible. Therefore, replacement models are generally used; these fall into the following categories:

i. *In vivo*

ii. *In situ*

iii. *In vitro*

Models are far from ideal and they all have advantages and disadvantages; however, they are the best substitute for the 'real thing'.

1.6.1 *In Vivo* Models

In vivo models are defined as the use of intact animals. In the case of drug delivery, the bioavailability of the case compound or drug can be determined. The routes of administration used include oral and intravenous (i.v.). Numerous conditions can be controlled, for example, stomach contents, and fed or fasted state.

The main advantage of using *in vivo* models is that researchers are observing the effects in an alive intact model, where all potential interactions will be encountered. The disadvantages include that the animal models used are not of human origin and the model is not the species

for which the drug is intended; consequently the biological structure will differ and how the drugs are metabolised may not be exactly the same, forming different metabolites or producing bioavailabilities that are different than in a human body. There are ethical issues to be considered when using animal models. The use of animals can incur substantial monetary costs and time-consuming welfare care.

1.6.2 *In Situ* Models

In situ models are based on *in vivo* models; however, areas of interest are removed and the experiment is performed. For example, inverted loops, everted sacs or vascularly perfused intestine are isolated, the drug being investigated is administered into the loop, the disappearance of the drug from the loop or the appearance of the drug in the blood can be measured. Ussing chambers are an alternative *in situ* method, the intestinal segment acts as a semi-permeable membrane separating a donor and receiver chamber; the movement of drug between the two chambers can be measured.

Advantages to *in situ* models are that actual intestine tissue is being used; hence, the intended destination of the drug is being investigated. Disadvantages are that the intestinal tissue has been removed from its natural environment; therefore, the interactions with the natural environment no longer exist or are exerted upon the tissue, also the tissue will only have a limited viability duration allowing a limited number of experiments to be performed. As with the *in vivo* models, the tissue being used is not of human origin.

1.6.3 *In Vitro* Models

In vitro models commonly utilise cell lines to study drug absorption. Cell lines can only be used when they provide a representation of the tissue being studied. Caco-2 (Bailey *et al.*, 1996) and HT-29 (Philips, *et al.*, 1988; Wikman *et al.*, 1993) are both epithelial cell lines used to study intestinal absorption.

Advantages of using cell lines include that they can be derived from human origin. Cell lines can allow rapid assessment of numerous compounds for epithelial permeability and metabolism. Experiments can be performed simultaneously and over a long time period. A cell line can be characterised prior to experimental work; environmental control can be achieved, *e.g.* temperature, pH, CO_2 and O_2 levels.

Disadvantages of using cell lines include phenotypic characteristics of the tissue may be lost because the passaging process favours fast-growing sub-populations (Yu *et al.*, 1997). If this occurs it is difficult to relate results back to the original tissue of isolation. Cell culture techniques must be performed under strict aseptic conditions. There is also a lack of *in vivo* (*e.g.* permeability) *in vitro* (*e.g.* bioavailability) correlation (IVIVC), this being the basis of the usefulness of these models.

Many cell lines have been investigated for potential models of absorption; however, Caco-2 cells have proven to be the most useful (Artursson, 1990).

1.7 Caco-2 Cell Model

1.7.1 The Use of the Caco-2 Cell Model

Orally administered drugs are primarily passively absorbed in the intestinal mucosa. The most significant barrier against absorption of orally administered drugs is the single layer of epithelial cells; consequently, it should be possible to use monolayers of intestinal epithelial cells to study passive drug absorption *in vivo* (Artursson and Karlsson, 1991).

A cell line must first be isolated prior to use as a cell culture model. The following strategies have been tried to obtain intestinal epithelial cell cultures:

- Cultivation of normal intestinal cells.
- Cultivation of intestinal explants.

- Cultivation of neoplastic epithelial cells derived from intestinal adenocarcinomas.
- Human adenocarcinoma cell lines are being used because of problems with the other sources, for example small pieces of intact intestinal mucosa obtained from humans following a biopsy can only be maintained for 24 hours in culture (Artursson, 1991).

The Caco-2 cell line was isolated from a 72-year-old Caucasian male with a moderately well differentiated colon adenocarcinoma. The Caco-2 cell line was first cultivated and reported in 1977 by Fogh *et al.*, however, Caco-2 cells only became widely used in the 1980's.

Grown under standard conditions and in the absence of inducers, Caco-2 cells spontaneously differentiate like mature intestinal enterocytes. Caco-2 cells are used as a model of intestinal absorption because they exhibit morphological and functional characteristics similar to intestinal enterocytes, including tight junctions and dome formation. Caco-2 cells are polarised and the apical membrane is covered with brush-border microvilli and brush-border enzymes including alkaline phosphatase and sucrase isomaltase; cytochrome P450 (CYP3A) and P-glycoprotein transmembrane protein are also expressed (Pinto *et al.*, 1983; Grasset *et al.*, 1984).

Transmission and scanning electron microscopy were used to show that brush-border microvilli were present at confluency and that there was a morphological polarisation of the cells. Polarisation was defined as cell asymmetry; brush-border microvilli located on the apical side only and by the junctional complexes composed of tight junctions and desmosome (Pinto *et. al.*, 1983). Tight junctions characterise the morphology of epithelial cells and seal the epithelial layer (Delie and Rubus, 1997).

The Caco-2 model allows the study of presystemic drug metabolism because the differentiated Caco-2 cell line expresses various cytochrome P450 isoforms and phase II enzymes including UDP-glucuronosyltransferases, sulphotransferases and glutathione-*S*-transferases (Meunier *et al.*, 1995). Markers were used to determine the extent of cell differentiation; for Caco-2 cells

enzymes are used as markers. It has been shown that Caco-2 cells express the main enzymes involved in drug metabolism. CYP3A4 is the predominant isoform of the CYP3A family; however, the polyclonal population does not express CYP3A4 possibly explaining observed differences in permeabilities between the Caco-2 model and intestinal tissues for drugs that are a substrate for CYP3A4 (Delie and Rubus, 1997).

Caco-2 cell proliferation started after a lag time of 48 hours, on day five – six, confluency was reached and the stationary phase by day nine. Dome formation characterised confluency, the number of domes increased up until day nine and than began to decrease (Pinto *et al.*, 1983).

Caco-2 monolayers have a relatively low paracellular pathway permeability (Artursson, 1991). Enterocytes and other intestinal cells express transport proteins to aid transport of compounds; cell culture models therefore should express the same transport proteins. Caco-2 cells express peptide carriers, amino acid carriers, nucleoside carriers, bile acid transporters, acid-base transport systems, receptor-mediated and carrier-mediated transport, and efflux pumps (Delie and Rubus, 1997). The benefits of any model used to mimic intestinal absorption are determined by the ability to predict permeability across the intestinal tissues.

Caco-2 cells originate from the colon; however, they are used to model the small intestine. Why colon cells spontaneously differentiate into intestinal enterocytes is not known. One explanation may be that Caco-2 cells represent an example of the similarity that exists between cancer and foetal cells. Enzyme studies and glycogen storage studies have shown that Caco-2 cells correspond to colon cells of 15-week old foetuses (Pinto *et al.*, 1983; Delie and Rubus, 1997).

1.7.2 Passaging and Changing / Altering Characteristics

Cell lines are grown and maintained in plastic flasks; once confluency has been reached, (*i.e.* a monolayer), the cells are detached from the plastic flask and the cells can be seeded onto new plastic flasks. This cycling process is known as passaging and each cycle is known as a

passage. Cell characteristics change from passage to passage and cells at a high passage number are more likely to be phenotypically different from the parental cell line (Delie and Rubus, 1997).

Many cell lines have a range of genotypes that is constantly changing. Phenotypic variations result from genetic variation. Furthermore, intestinal epithelial cell lines are frequently heterogeneous populations of cells, *i.e.* they are polyclonal. Therefore, the properties of any given cell line is equal to the sum of the properties of the subpopulations. Consequently, the characteristics of a cell line will only be reproducible if the ratios between the different subpopulations are constant. Rapidly growing subpopulations increase in number at the expense of more slowly growing subpopulations, thus changing the characteristics of the cell line. One way to achieve a more stable cell line characteristic is to work within a limited passage window, thus limiting the number of passages will minimise genotypic variation (Artursson, 1991).

Yu *et al.*, (1997) investigated the affect of passaging by using low passage (28 – 36) and high passage (93 – 108) Caco-2 cells. High passage Caco-2 cells were characterised by less morphological heterogeneity, higher transepithelial electrical resistance, higher transcellular diffusion, lower paracellular diffusion, lower carrier-mediated transport and lower alkaline phosphatase activity (Yu *et al.*, 1997). The process of passaging significantly affects biological characteristics and transport properties of Caco-2 cell monolayers (Yu *et al.*, 1997).

Heterogeneity in Caco-2 cells has been observed both as a function of culture time but also from batch to batch; as a consequence, care must be taken when comparing results from different laboratories (Delie and Rubus, 1997).

1.7.3 Cell Culture Conditions Altering Characteristics

Cell culture conditions can play a role in determining the reproducibility of the Caco-2 system. Seeding Caco-2 cells on different membrane materials can alter the cell density and transepithelial resistance (Nicklin *et al.*, 1992). Walter and Kissel (1995), compared Caco-2 cells; all Caco-2 cell types were cultivated under identical conditions, the transport of hydrophilic markers such as mannitol, PEG 4000 and fluorescein were measured. From Table 1.2, it can be seen that the results are very different; moreover, the transepithelial electrical resistance across the monolayers seems to correlate with the permeability coefficients of the hydrophilic markers.

Table 1.2 – Permeability coefficients and transepithelial electrical resistance (TEER) in differently passaged Caco-2 cell monolayers (Walter and Kissel, 1995). PC – polycarbonate; PET – polyethyleneterephatalate.

Hashimoto and Shimizu (1993), cultured Caco-2 cells in serum (FCS)-free medium and compared them to Caco-2 cells cultured in medium containing FCS. They showed that Caco-2 cells grown in serum containing medium formed tight junctions and maintained cell polarity. In contrast Caco-2 cells grown in serum-free medium had poor tight junction formation as reflected by reduced TEER measurements. In addition, maintenance of cell polarity strongly depends on tight junctional barrier so there was a decrease in cell polarity. Addition of FCS to Caco-2 cells grown in the absence of serum resulted in an increase in TEER measurements. Caco-2 cells maintained in serum-free medium did resemble Caco-2 cells grown in serum-containing medium

in cell growth, morphological appearance, expression of brush-border enzymes and levels of Gly-Gly transport activity were similar (Hashimoto and Shimizu 1993).

The field of drug delivery has been revolutionised by the field of combinatorial chemistry resulting in an increased number of compounds to be assessed for their affinity for specific receptors, as well as their biopharmaceutical properties. Behrens and Kissel (2003) investigated the effects cell culturing has on Caco-2 cells. Factors affecting the morphology of Caco-2 cells, paracellular permeability and expression of transporters; including Pept1 and P-gp, were investigated:

i. Time in culture

ii. Type of membrane support

iii. Seeding density

PC, PET, and PE are all commercially available porous membrane supports. Caco-2 cells grown on PET and PE showed slight differences in carrier-mediated transporter expression as caused by an increase of tight junctions organisation. Also there will be different cell-support interactions between the basolateral cell surface with the extracellular filter matrix. Caco-2 cells monolayers grown on PE and PET were flat depicting poor differentiation. Microvillus structure was altered when Caco-2 cells were grown on PET. Collagen-coating removed these differences and appeared to enhance differentiation and induce expression of carrier-mediated transporters (Behrens and Kissel, 2003).

Seeding densities were shown to be important, multilayers formed after high seeding densities, reflecting cell layer disorganisation. Thin monolayers resulted from low seeding densities, Decreased differentiation at both high and low seeding densities was depicted by a significant decrease in mRNA expression levels of HPT1 and P-gp (Behrens and Kissel, 2003).

The conclusions obtained were that the following conditions should be considered:

i. Filter inserts PC (polycarbonate), since PE and PET filters strongly restrict the passive transcellular transport.

ii. Collagen-coating, since this significantly enhances carrier-mediated transporter expression without changing morphology and paracellular permeability.

iii. Average seeding density of 6 x 10^4 cells/cm^2, since this prevents changes in cell morphology, monolayer formation, and carrier-mediated transporter expression.

iv. Medium supplement for investigating substrate-transporter interactions, since this increases the expression levels.

Caco-2 monolayers are widely used as an *in vitro* model of small intestinal absorption despite different laboratories reporting large differences in permeability for actively transported substances (Behrens and Kissel, 2003). Therefore, following standardised conditions for example those described above and implementing appropriate controls, comparison of permeability data from different laboratories could be facilitated (Behrens and Kissel, 2003).

1.7.4 Monolayer Integrity

Transepithelial electrical resistance (TEER) is a measurement used to determine monolayer integrity. Transepithelial resistance measurement is a direct reflection of uniform presence of tight junctions (Grasset *et al.*, 1984). TEER is defined as the product of resistance (Ω) x surface area (cm^2). The resistance reflects the resistivity across cell membranes; TEER is constant for a homogenous tissue regarding the number and size of tight junctions (Delie and Rubus, 1997).

In addition to measuring TEERs, monolayer integrity can be determined by the flux of hydrophilic markers including mannitol and PEG 4000. Monolayer integrity was assessed using radiolabeled mannitol. Inserts with a flux greater than 0.5 per cent, per hour were excluded from this study (Yee, 1997). TEER is determined primarily by the ion flux through the paracellular

space, a range of values have been reported between 150 – 400 Ω cm^2 – Caco-2 monolayers can display the electrical properties of either small intestinal or colonic enterocytes. Therefore, the flux of an impermeable marker like mannitol is a more sensitive indicator of the size of the tight junction than the resistance assay (Yee, 1997).

1.7.5 Problems and Improvements with the Model

As described previously, there are differences found in Caco-2 cells reported from different laboratories. Expression level and substrate specificity of carriers and efflux systems found in Caco-2 cell monolayers tend to be variable in comparison to the *in vivo* situation. A cell line cannot express all barriers presented to administered drugs in the *in vivo* situation, *e.g.* absence of the mucus layer. To study drug transport and metabolism, the ideal cell-culture model would have passive transcellular and paracellular permeabilities comparable to those of the human small intestine. Expression of carriers and enzymes would also be similar to the levels expressed *in vivo* in the small intestine. Caco-2 cells are known to overexpress P-gp; the presence of this efflux pump may cause the absorption of some compounds to be under-estimated (Yee, 1997). The small intestine is not only composed of enterocytes, other cell populations are found including mucin-producing goblet cells; the ideal cell culture model would comprise of all cell types found it the small intestine. Mucus consisting of large glycoproteins containing oligosaccharide side-chains is secreted along the intestine. The primary function of mucus is to protect the cells from hydrolysis by gastric and pancreatic secretions. Mucus presents an important barrier to drug absorption, shown using HT-29 cells. In Caco-2 cells, mucus production is minimal after confluency, the period when cells are used for transport studies. Goblet cells are responsible for the mucus secretion and the goblet cells are interspersed among enterocytes. One method to produce a better model is to co-culture different cell types. In an attempt to develop a better representation of the intestine, co-culturing of the goblet cell line HT-29 and Caco-2 cells has been undertaken (Walter *et al.*, 1996). Co-culturing of Caco-2 and goblet cell lines (derived from HT-29 cell lineage), has been attempted; unfortunately, co-culturing has had limited success because cell lines seem to prefer to grow as mono-cultures opposed to co-cultures, possibly due to differences in their repertoires of cell adhesion molecules (Artursson and Borchardt, 1997; Delie and Rubus, 1997).

1.8 Liposomes

Bangham, Standish and Watkins first observed the formation of vesicles following the rehydration with excess water of phospholipids (water insoluble lipids) in 1965, these vesicles are now known as liposomes.

Liposomes are macroscopic spherical vesicles that form when phospholipids are hydrated above their transition temperature and agitated (Gregoriadis, 1976). Upon hydration of phospholipids, a highly ordered arrangement of concentric closed phospholipid bilayers form that can encapsulate part of the aqueous medium in their interior (Gregoriadis, 1976). The bilayer membrane formed is continuous. The bilayers formed are separated by the aqueous medium used to rehydrate the phospholipids.

Phospholipids comprise of a highly polar head group and usually two hydrocarbon tails. The amphipathic properties of phospholipids, the hydrophilic head group and hydrophobic nature of the hydrocarbon chains, are exploited in membrane formation. When surrounded by an aqueous environment, phospholipids orientate themselves so the hydrophilic head groups are in closest contact with the aqueous environment and the hydrophobic chains are furthest away from the aqueous environment; generally creating a bilayer (two hydrocarbon chains) or micelle formation (one hydrocarbon chain) (Raven and Johnson, 1996).

Cholesterol belongs to a group of compounds collectively known as steroids and is another important component of membranes. Unlike phospholipids, cholesterol is a weakly amphipathic molecule, due to the presence of a hydroxyl group at one end of the molecule. The hydrophobicity of cholesterol can be increased by esterification of the hydroxyl group to a fatty acid (Raven and Johnson, 1996). Compared to other hydrophobic membrane components, *e.g.* fatty acid tails, cholesterol is a bulky rigid structure, due to fused cyclohexane rings in the chair conformation (Raven and Johnson, 1996).

Early goals in liposome research were improving entrapment efficiency to obtain a good level of entrapment, once this was achieved the goals moved towards commercialisation shifting the emphasis on stability, vesicle size, reproducibility, sterility, scale-up, and process validation.

1.8.1 Types of Liposomes

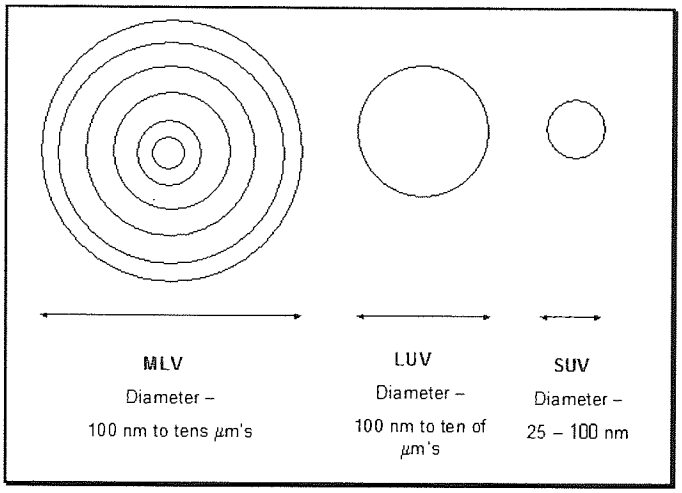

Figure 1.8 – Schematic representation of the different types of liposomes. The concentric circles represent lipid bilayers (adapted from Jones, 1995).

Different methods of liposome preparation exist; the chosen method determines the type of liposome formed and the number of bilayers (lamellae). Three basic morphological categories exist as described in Figure 1.8:

i. Multilamellar vesicles (MLVs) are composed of numerous concentric bilayers.

ii. Large unilamellar vesicles (LUVs) have one bilayer and are prepared from MLVs.

iii. Small unilamellar vesicles (SUVs) have one bilayer.

1.8.2 Size

SUVs have a diameter of approximately 25 – 100 nm, LUVs have a diameter of approximately 100 nm up to several microns and MLVs vary in size from approximately 100 nm to many microns (Gregoriadis, 2002 / 2003).

1.8.3 Entrapment

Liposome size, composition, charge and lamellarity can all be manipulated with ease, making them a good delivery system. Liposomes are currently exploited for the delivery of both hydrophilic and lipophilic drugs (Gregoriadis, 1985). Depending on the polar characteristics, these drugs are retained either within the hydrophilic aqueous core of the liposome or incorporated into the lipophilic region of the bilayer (Gregoriadis, 1976). Hydrophilic drugs can be entrapped in large quantities, in contrast to lipophilic drugs whose bilayer loading is influenced by the lipid bilayer composition and drug-lipid ratio. Drugs can express an intermediate degree of lipophilicity resulting in a partitioning effect between the two phases. The partition coefficient (LogP) of a drug will thus influence its location, incorporation and retention within the liposome carrier.

Drug molecules are classified into four groups – highly hydrophilic, highly lipophilic, amphiphilic drugs that exhibit good biphasic solubility and biphasic insolubility. The category of a drug will determine the location of entrapment, entrapment efficiency and release properties. Highly hydrophilic drugs with LogP values < -0.3 are exclusively located in the aqueous compartments of liposomes, *e.g.* cytosine arabinoside. Bilayer composition determines movement of highly hydrophilic drugs across the liposomal bilayer. Drugs with intermediate LogP values, 1.7 < LogP < 4, (*e.g.* mitomycin C or vinblastine) pose major problems because they partition between both phases, thus are easily lost from the liposomes. Stable liposomal formulations are produced when complexes are formed between the drug and membrane lipids. Lipophilic drugs have LogP values greater than five, and are entrapped almost exclusively in the lipid bilayer, *e.g.* cyclosporin. As lipophilic drugs are highly insoluble in water, loss of entrapped drug on storage is minimal. Biphasic insoluble drugs have a very low entrapment efficiency being insoluble in both the aqueous and lipid phase, *e.g.* 6-mercaptopurine or azathioprine. Lipophilic drugs have proved to be the most successful group in terms of cost, stability and utility (Gulati *et al.*, 1998). Anticancer, antiviral and steroids with varying solubility behaviour have been structurally altered to increase lipophilicity in order to achieve the optimal liposomal formulation (Gulati *et al.*, 1998).

Lipophilic drugs display a high level of entrapment and those with approximately 100% encapsulation efficiency can actually reduce the production costs of liposomes by reducing the amount of phospholipid(s) needed to prepare the formulation. Additionally, lipophilic drugs are located in the bilayer as stipulated by their aqueous solubility; consequently, the drug is rarely 'lost' to the surrounding aqueous environment. In contrast, the maximum entrapment efficiency for a water-soluble drug is approximately 70%, based on the theoretical maximum lipid concentrations. The remaining 30% volume is the void space due to the curvature of the liposomes (Gulati *et al.*, 1998).

With this in mind, hydrophilic drugs have been altered and new drugs synthesised with an increased lipophilicity in an attempt to overcome problems associated with hydrophilic drug entrapment that are not experienced with lipophilic drugs, including poor entrapment efficiency, physical and chemical instability (Gulati *et al.*, 1998).

Hydrophilic compounds to be incorporated are dissolved in the aqueous medium used to rehydrate the phospholipids; examples include anticancer and antimicrobial drugs, cytokines, nucleic acids, enzymes and proteins. Entrapment efficiency can be improved by using the appropriate method of preparation. The dehydration-rehydration (DRV) method can increase entrapment of water-soluble compounds (Kirby and Gregoriadis, 1984). The DRV method involves removal of the solvent(s) the phospholipid(s) are dissolved in, by rotary evaporation followed by rehydration with the aqueous solution containing the water-soluble compound. The solution is then sonicated and the solution is freeze-dried. The process of freeze-drying results in the phospholipid components and water-soluble solute being in very close proximity, the freeze-dried material is rehydrated in a controlled manner resulting in a higher entrapment of solute. Water-insoluble or lipid-soluble compounds are dissolved in solvent along with the phospholipids used for the preparation of the liposomes. The DRV method of preparation does not improve incorporation of water-insoluble compounds (Gregoriadis, 2002 / 2003).

1.8.4 Applications

Advances and improvements in cancer treatment are always being sought. The use of liposome technology has been included in these attempts. Low molecular weight cytotoxic drugs, peptides and proteins, have been administered using passively and ligand-targeted liposomes. Liposomes have been used to activate macrophages, driving them to become tumoricidal, by entrapping cytokines and immunomodulators in MLVs. Additionally, genetic material including DNA and antisense oligonucleotides have been delivered using liposomes to induce antitumor effects. A major area of liposome research is concentrated on improving vaccine delivery. DNA encoding for antigen to be vaccinated against has been inserted into plasmid DNA and the plasmid entrapped in liposomes; the liposome preparation is then administered, generally intramuscularly (i.m.). The antigen (protein) is then produced from the DNA, an immune response occurs and immunological memory is laid down.

Johnson and Johnson of the USA, have developed liposomal doxorubicin as a pharmaceutical product; the developed marketable product of doxorubicin is known as Doxil®. The licence for Doxil® was first obtained in 1995 making Doxil® the first liposomal preparation approved by the Food and Drugs Agency (FDA). Daunorubicin has also been entrapped in liposomes and a marketed product known, as DaunoXome™ (Gregoriadis, 2002 / 2003).

More recently, liposomes are being incorporated into moisturiser preparations for both hand and face, including anti-ageing products. The active ingredients are incorporated either in the aqueous phase or the lipid bilayer. The liposomes themselves do not penetrate the stratum corneum of the skin however; the active ingredients are entrapped and thus, can bring about their effects (Gregoriadis, 2002 / 2003).

1.8.5 Liposomes and the Oral Route

Although the oral route is the most convenient for drug delivery most peptide and protein drugs are not orally administered due to GI degradation by digestive enzymes (Chen and Langer,

1998). Peptide drugs are poorly absorbed because of high molecular weight and hydrophilicity (Takeuchi *et al.*, 2003). To overcome these problems the use of delivery vehicles including liposomes has been investigated.

1.8.5.1 Stability of Liposomes in Gastric Fluids

Liposomes must be stable in gastric fluids for them to be used as oral drug delivery vehicles. Instability of liposomes in the GI tract is a problem as they are easily degraded by bile salts in the GI tract because they interact with liposomes as surfactants (Iwanaga *et al.*, 1997) and they are prone to degradation by intestinal lipases (Chen and Langer, 1998; Keller, 2001). The stability of DSPC:Cholesterol liposomes was determined in the presence of bile salts (10 mM), pancreatic lipase and high and low pH. DSPC:Cholesterol liposomes were stable; therefore, they maybe suitable for oral administration of peptide including insulin (Rowland and Woodley, 1980). Glucose and carboxyfluorescein were incorporated into DSPC:Cholesterol and their stability was measured at an acidic pH, in the presence of bile salts and pancreatic lipase creating conditions similar to what would be expected in the GI tract. Substantial loss of glucose and complete loss of carboxyfluorescein was observed (Chiang and Weiner, 1987a). *In vivo* studies using DSPC:Cholesterol with hydrocortisone and salicylic acid entrapped re-iterated the *in vitro* findings; where excretion patterns from liposomes were not significantly different from encapsulated drugs (Chiang and Weiner, 1987b). Indicating that liposomes have little promise as a drug delivery system for oral administration (Chiang and Weiner, 1987b). However, research into the potential use of liposomes continued and liposomes composed of stearylamine, cholesterol and either DSPC or DPPC (dipalmitoylphosphatidylcholine) were compared. As an additional comparison ceramide was also included in the liposome formulation. The stability for oral administration was tested using an "Artificial Stomach-Duodenum" model empty liposomes were tested. This model simultaneously simulates gastric secretory flux, the gastric emptying rate, and bicarbonate secretory fluxes corresponding to duodenal alkaline secretion and to alkaline pancreatic secretion. DSPC was more resistant than DPPC to gastric attack, and that ceramides enhanced this effect. Thereafter, ceramides and DSPC were retained as preferable liposomes constituents, to protect superoxide dismutase from gastric attack in the course of oral administration. Maximal entrapment of superoxide

dismutase was obtained with ceramide-containing liposomes composed of DSPC, cholesterol, stearylamine and ceramides with a molar ratio of (14:7:4:1). Incorporation of ceramides into preparations increased the size of liposomes. Ceramide enhanced superoxide dismutase entrapment (Regnault *et al.*, 1996). To improve the stability of liposomes for oral delivery of peptide drugs (*e.g.* insulin) they were coated with the sugar portion of mucin or polyethylene glycol (PEG), neutral and positively charged liposomes were used as controls. Insulin release was measured from liposomes in an acidic solution (pH 2.0) and in the presence of bile salts (sodium glycocholate and sodium taurocholate). The liposomes were stable in the presence of the acidic solution and no significant release of insulin was measured. In contrast, insulin release from the uncoated liposomes was enhanced in the presence of bile salts, with 20-50 per cent being released; in comparison, only 2-10 per cent of insulin was released from the coated liposomes (Iwanaga *et al.*, 1997). Insulin degradation in the presence of intestinal fluid obtained from rats small intestine was measured using reverse phase HPLC. Insulin degradation was prevented using coated liposomes, and was reduced using neutral and positively charged liposomes compared to a free solution of insulin (Iwanaga *et al.*, 1997). Since the work presented within this thesis started, research into the use of liposomes has continued. Ovalbumin was incorporated into liposomes composed of DPSC and cholesterol (10:5) these were the unmodified liposomes. DSPC and cholesterol liposomes were modified by coating with PEG (DSPC, cholesterol and DSPC-PEG – 10:5:1). Release of ovalbumin was measured in artificial gastric and intestinal medium. No release was observed from any liposomal formulation into artificial gastric fluid (HCl solution containing 0.9 % NaCl at pH 2); however, partial release was measured in artificial intestinal medium. Coating the liposomes with PEG enhanced ovalbumin release. PEG increases phospholipase A action causing liposome degradation and thus release from liposomes increases (Minato *et al.*, 2003).

1.8.5.2 Insulin

Insulin has been used as a model peptide and liposomes are a potential drug carrier system to help to improve oral peptide delivery. Currently, insulin is administered by intramuscular (i.m.) injection; however, this is problematic for people who have difficulties injecting themselves. Due to the inherent problems of orally delivering peptides such as proteolytic degradation insulin has

been entrapped in liposomes to protect the insulin from degradation. Patel and Ryman (1976) entrapped insulin into liposomes composed of phosphatidylcholine, cholesterol and dicetyl phosphate (molar ratio 10:2:1). The same amount of insulin was orally administered to diabetic rats either as a free solution or as the liposome formulation. Blood-glucose levels were reduced to one-third of the initial value 3 hours after administration of the liposome formulation; however, the free insulin solution did not reduce the blood-glucose level, indicating that encapsulation of insulin within liposomes protects from proteolytic degradation in the GI tract (Patel and Ryman, 1976). Insulin was entrapped into dipalmitoylphosphatidylcholine (DPPC) and cholesterol (7:2) liposomes and induced hypoglycaemia in rats following administration in the buccal cavity (Weingarten *et al.*, 1981). Insulin was orally administered to rats as a free solution or entrapped in neutral or positively charged liposomes and liposomes coated with the sugar portion of mucin or PEG. Neutral liposomes and the free solution of insulin had no pharmacological effect. Blood glucose levels were significantly reduced following oral administration of insulin using positively charged and coated liposomes. Modification of the surface of liposomal surface through coating with PEG or mucin may enhance and sustain oral absorption of insulin (Iwanaga *et al.*, 1997). The lipid composition of liposomes influences the success of oral delivery. Insulin was entrapped into liposomes composed of dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidylethanol (1:1) and phosphatidylcholine and phosphatidylinositol (negatively charged) and given to diabetic rats. Blood-glucose levels were reduced following administration of dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidylethanol (1:1); conversely, blood glucose levels increased in the presence of phosphatidylcholine and phosphatidylinositol (negatively charged). At 37°C the liposomes composed of dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidylethanol (1:1) are solid compared to phosphatidylcholine and phosphatidylinositol liposomes that are fluid. The increase in blood glucose-levels in the presence of fluid liposomes may have been brought about because the fluid liposomes may be destroyed by proteolytic enzymes to a greater extent causing insulin to be more accessible to protease action with the result that it will lose activity (Kisel *et al.*, 2001). The use of absorption enhancers has increased in an attempt to improve drug absorption; sodium taurocholate is a known absorption enhancer that was used to improve insulin absorption. Liposomes were composed of DPPC, 15 mM, cholesterol 15 mM and methylcellulose, 50 mg. Insulin containing

liposome formulations with and without sodium taurocholate were orally given to mice and the penetration of insulin across Caco-2 cell monolayers was measured. Blood-glucose levels decreased to a greater extent following the oral administration of insulin-sodium taurocholate liposomes compared to mice that received insulin liposomes without sodium taurocholate. Insulin transport across Caco-2 cell monolayers was greatest in the presence in of insulin-sodium taurocholate liposomes and was least in the presence of a free insulin solution. A very good correlation was found between the *in vitro* (% penetrated fraction) and the *in vivo* (% absorbed fraction) experiments. A good *in vivo – in vitro* correlation was defined when R^2 value $\geq$ 0.9727 was calculated following the comparison of fraction absorbed (%; *in vivo*) versus penetrated fraction (%; *in vitro*) (Degim *et al.*, 2004). Sodium taurocholate was found to be an effective enhancer, acting by opening the tight junctions between barrier cells (Degim *et al.*, 2004).

1.8.5.3 Tetanus and Diphtheria Toxoid

Liposomes are a potentially promising delivery system for oral vaccines, they have been found to be stable in the GI tract and are taken up by Peyer's patches following their oral use. Uptake of liposomes by Peyer's patches is low; however, targeting liposomes to Peyer's patches using lectins for example, increases uptake (Hussain *et al.*, 2001; Clark *et al.*, 2001). Oral administration of radiolabeled liposomes showed that 6 per cent of the dose was absorbed in Peyer's patches (Hussain *et al.*, 2001). Tetanus toxoid was incorporated into liposomes (DSPC:Cholesterol) and orally administered to guinea pigs. The liposome formulation significantly increased the immune response (IgG) to tetanus toxoid (Alpar *et al.*, 1992). Diphtheria and tetanus toxoid were incorporated into PC:Cholesterol liposomes and orally given to rabbits. After the third administration of toxoid-laden liposomes 67 per cent of rabbits exhibited an immune response to diphtheria toxoid compared to 89 per cent for tetanus toxoid. The control rabbits did not respond to oral ingestion of conventional diphtheria and tetanus toxoid adsorbed onto aluminium hydroxide gel. Toxoid-laden liposomes were also orally administered to monkeys, after the third administration 62 per cent of monkeys presented a mild antibody response to both toxoids. The antibody responses were not considered protective. Toxoid-laden liposomes were adsorbed onto aluminium hydroxide and given to monkeys and

human volunteers. A weak antibody response in monkeys was observed following administration of toxoid-laden liposomes, the antibody response increased to a moderate to high antibody response following administration of toxoid-laden liposomes adsorbed onto aluminium hydroxide. The monkey and rabbit observations differ as rabbits had an antibody response to toxoid-laden liposomes in the absence of aluminium hydroxide, this difference may be attributed to the difference in physiological constitution of the GI tract of the rabbit and the monkey. There was a significant increase in diphtheria toxoid antibody titres in humans following the oral administration of toxoid-laden liposomes adsorbed onto aluminium hydroxide after the first and second ingestion. A significant increase in tetanus toxoid antibody titre was observed (Mirchamsy *et al.*, 1996).

1.8.5.4 Cholera

Cholera is caused by *Vibrio cholerae* and the possibility of an oral vaccine has been researched. Cholera toxin (CT) was covalently coupled to small unilamellar liposomes for targeting to Peyer's patch M cells, following their oral administration. The lipid composition of the liposomes was mainly saturated phospholipids since they form a more stable bilayer (low fluidity) compared to nonsaturated lipids. CT conjugated to liposomes induced a serum IgG anti-CT response in rats following oral administration (Harokopakis *et al.*, 1995). In another study liposome associated *Vibrio cholerae* lipopolysaccharide, fimbriae and procholeragenoid were orally administered to a group of Wister rats. Other groups of Wister rats were administered heat-killed fimbriated and non-fimbriated whole cell *Vibrio cholerae*. The liposome formulation with the associated *Vibrio cholerae* elicited the strongest immune response (IgM, IgG) (Kalambaheti *et al.*, 1998). Following this study a mixture of *Vibrio cholerae* antigens made up of crude fimbrial extract, lipopolysaccharide and procholeragenoid was administered to orally to Thai volunteers either as free antigen or associated with liposomes. Both vaccines elicited an immune response; however, the liposome vaccine induced a superior class-specific and antigen-specific antibody response compared to the free antigen vaccine (Chaicumpa *et al.*, 1998).

1.8.5.5 Cyclosporin A (CsA)

Cyclosporin A (CsA) is one of the most effective immunosuppressive drugs used today for the prevention of allograft rejection after organ transplantation. However, some currently available dosage forms suffer several disadvantages including slow and high variability of CsA absorption following oral administration of its oily solution. CsA was entrapped in liposomes, the most stable liposome formulation was CsA, dipalmitoylphosphatidylcholine (DPPC), and cholesterol in the molar ratio (1:0.2:1) respectively. The liposome formulation was compared to the commercially available oily solution – Sandimmune®; both were orally administered to New Zealand rabbits. A peak concentration was reached in less than 50 minutes for the liposome formulation compared to 225 minutes for Sandimmune®, indicating faster absorption of CsA from liposomes than from the commercially available oily solution. Although there was no significant ($p > 0.05$) difference in the maximum concentrations (C_{max}) between the two dosage forms, a statistically significant ($p < 0.05$) difference in the absorption rate was found. Higher individual variability was observed from Sandimmune® compared to the liposomes (Al-Meshal *et al.*, 1998).

1.8.5.6 Arteether

Arteether is a potent anti-malarial agent with limited water solubility (17 μg / ml) that is available as an oily solution intended for intramuscular injection. The oral route is an alternative for delivery of arteether; consequently, arteether was entrapped in liposomes composed of dipalmitoylphosphatidylcholine (DPPC), dibehynoyl-phosphatidylcholine (DBPC), cholesterol and arteether (molar ratio of 1:1:2:1). The liposomes and an oral aqueous suspension of micronized arteether were administered orally and intravenously to New Zealand rabbits. Higher bioavailability and a faster rate of absorption of arteether was observed for the liposome formulation compared to the aqueous suspension of micronized arteether. Almost complete arteether absorption was observed for oral liposomes where relative bioavailability was 97.91% compared to 31.83% for the oral suspension of micronized arteether, (where absolute bioavailability is defined as AUC_{oral} / AUC_{IV}). Intersubject variations were found to be relatively high in oral liposomes. These liposomal formulations can allow the use of the potent antimalarial arteether effectively in case of high-risk malarial patients (Bayomi *et al.*, 1998).

1.8.5.7 Additional Examples

DSPC and cholesterol (10:5) and DSPC, cholesterol and DSPE-PEG (10:5:1) liposomes containing ovalbumin were orally administered to mice at 20 mM and 50 mM; a free ovalbumin solution was used as a comparison. Following oral administration the levels of ovalbumin specific IgA antibody was measured. PEG-modification increased transit time in the intestine. Liposomes modified with PEG induced the systemic immune response to a lesser extent, possibly due to suppression of ovalbumin uptake by Peyer's patches (Minato *et al.*, 2003).

Recombinant human epidermal growth factor (rhEGF) was entrapped into polyethylene glycol (PEG)-coated liposomes (PC/DPPC, cholesterol and DOPE-PEG (10:5:1). Formulations were added to Caco-2 cell monolayer and orally administered to rats. There was no significant difference in growth stimulating activity between the rhEGF in solution and PC:DPPC liposomes, indicating encapsulated rhEGF in liposomes had bioactivity similar to free rhEGF. Encapsulation of rhEGF in liposomes significantly reduced degradation in Caco-2 cell homogenate. Flux of rhEGF was measured over Caco-2 cell monolayers. Flux of rhEGF was similar for rhEGF in solution and encapsulated in PC liposomes; however, flux was three times greater for DPPC liposomes, suggesting that DPPC liposomes could be used for the improvement of rhEGF transport. Liposomes composed of higher phase transition (T_m) lipids are internalised to a greater extent than liposomes consisting of lower T_m lipids, although high T_m lipids are extensively metabolised following intracellular absorption. Plasma concentration of rhEGF was measured after oral administration of rhEGF to rats. C_{max} was increased with liposome formulations compared to rhEGF solution, DPPC liposomes increased C_{max} to a greater extent compared to the PC formulation. Thus, the lipid composition affected oral bioavailability of rhEGF. Additionally, gastric ulcer healing was also measured, both PC and DPPC liposomes reduced the ulcer length faster than the control group, DPPC was faster than PC. Indicating that PC and DPPC liposomes were effective for acute gastric ulcer healing (Li *et al.*, 2003).

1.8.6 Advantages / Disadvantages of Liposomes as Drug Delivery Carriers

Liposomes have one major advantage as drug delivery carriers – they are capable of entrapping both hydrophilic and hydrophobic solutes, and they can be manipulated into a range of different sizes. Different starting lipids can be chosen to customise the rigidity if the bilayers and the charge of the liposomes. Advantages of liposomes are that they are biodegradable and non-toxic (Gregoriadis, 1976), allowing safe administration without serious side-effects. Through manipulation of the chosen lipids used to prepare a liposome suspension, the liposomes can have an overall neutral, positive or negative charge. Overall charge can be advantageous when entrapping charged solutes to enhance entrapment efficiency.

For commercial applications the disadvantages of liposomes include high cost, poor physical and chemical stability, leakage, and difficulty in sterilisation. These disadvantages can be improved through manipulation procedures mentioned previously (Gulati *et al.*, 1998). Liposomes with a size greater than 200 nm were rapidly cleared from the blood following i.v. administration, the fate of the liposomes was the fixed macrophages of the reticuloendothelial system (RES), mainly in the liver, spleen and the bone marrow (Gregoriadis, 1995). Coating liposomes, for example with PEG, has helped to improve the rapid clearance and prolong circulation time (Klibanov *et al.* 1991; Sudimack and Lee, 2000). PEG coated liposomes are also known as 'stealth' liposomes and in humans have a relatively long half life of approximately 1 day in blood circulation. The extended circulation time results from steric stabilisation of the liposome by the grafted polymer (Needham *et al.*, 1992), also the PEG hinders interaction between proteins and the liposome surface (Torchilin *et al.*, 1994; Levchenko *et al.*, 2002). An approach to improve delivery is ligand-targeted liposomes. Receptors for vitamins and growth factors have high expression levels on various forms of cancers therefore they have become targets for ligand-targeted liposomes (Drummond *et al.*, 2000). Immunoliposomes are a targeted therapy for human tumours (Ahmad *et al.*, 1993; Park *et al.*, 1997; Lopes de Menezes, *et al.*, 1998).

Efficient and site-specific delivery of drugs are important objectives that drug carriers have the potential to improve. Drug carriers are important because the free form of some drugs do not reach their final destination because of several factors including; rapid clearance (either from the site of introduction), from circulation, or biological barriers obstructing the drug, or the drug is toxic. Liposomes may alter delivery of drugs *via* site-directed delivery and offer protection by:

i. Protecting the drug from inactivation or destruction by the immune system (Farrell and Sirkar, 1997).

ii. Protecting the individual through decreasing toxicity of chemotherapeutic agents (Gregoriadis, 1995).

Liposomal membranes are similar to cellular membranes allowing liposomes to fuse / merge with cellular membranes, thus depositing their load. As the process is dependent on membranes fusing, delivery / release is controlled and prolonged. Delivery can be targeted to specific sites *via* cell surface receptors, *e.g.* cancer cells express different receptors, or they over-express receptors and this can be exploited for delivery of chemotherapeutic drugs.

Liposomes can be manipulated in several ways in order to control release:

- Bilayer composition – lipids have different transition temperatures; therefore, lipids are chosen dependent on the properties required. Leakage from liposomes can be reduced by using lipids with a high transition temperature *e.g.* DSPC (Simões *et al.*, 2004).
- pH-sensitive liposomes – are stable at physiological pH (pH 7.4); however, under acidic conditions they undergo destabilisation and have fusogenic properties due to the inclusion of lipids with fusogenic properties, resulting in the release of their aqueous contents (Straubinger, 1993; Simões *et al.*, 2004).
- Thermosensitive liposomes – are liposomes designed to release an entrapped drug preferentially at temperatures attainable mild local hyperthermia (Yatvin *et al*, 1978). Tumours (Chelvi *et al.*, 1995) and local infections are targets for thermosensitive liposomes (Yatvin *et al.*, 1978).

1.8.6.1 Oral Liposomes

Liposomes were first formulated in the 1970s for oral drug delivery because it is the preferred route for drug administration (Keller, 2001). Delivery vehicles are used to protect drugs from degradation by intestinal enzymes in the GI tract. To be effective as oral delivery vehicles they must satisfy certain criteria. To protect the encapsulated drugs the vehicle must be resistant to degradation in the GI tract and the drugs need to be absorbed with high efficiency in the GI tract (Chen and Langer, 1998). Liposomes have the advantage that they can be formed under mild conditions that can minimise denaturation of drugs during encapsulation. The stability of liposomes within the GI tract can be problematic (Section 1.8.5.1), disruption of the liposomal bilayers has serious consequence because the protective function of the bilayers is lost and the entrapped drugs are exposed to the GI tract (Chen and Langer, 1998). Although liposomes have the disadvantage of being unstable in the GI tract they also have the advantage that liposome formulations are easily manipulated and liposome behaviour *in vitro* and *in vivo* is dependent on size, bilayer rigidity, charge and morphology (*i.e.* unilamellar, multilamellar) (Storm and Crommelin, 1998). Consequently, progress has been made to stabilise liposomes for oral delivery. Polymerised liposomes have been developed and they have improved stability in the GI tract by creating a cross-linked network of liposomal membranes (Chen and Langer, 1998). Other methods used to improve stability involve the inclusion of cholesterol in the liposome bilayer, coating their surface with protecting polymers and putting them into gelatin capsules (Keller, 2001). Altering the liposome formulation has also had detrimental effects. Cationic liposomes were found to be highly toxic to mice inducing a profound and lethal hypothermia following oral administration (Filion and Phillips, 1998). Cationic liposomes down-regulate at least three of the immunomodulators, (nitric oxide, tumour necrosis factor-α and prostaglandin E_2) produced by activated macrophages which are important in immune and inflammatory responses (Filion and Phillips, 1998).

For the encapsulated drugs to reach their *in vivo* targets the encapsulated drug must be absorbed efficiently, this means that the drug delivery vehicle also needs to be absorbed at an efficient rate in the GI tract (Keller, 2001). Absorption of drug delivery vehicles in the GI tract is a major problem; it is estimated that less than 1 per cent of delivery vehicles can be absorbed

after oral administration. The extremely low absorption efficiency of delivery vehicles is the largest obstacle towards the potential application of using delivery vehicles to oral deliver complex molecules such as proteins and peptides. Modifying the surface with targeting molecules such as antibodies or lectins, or through slowing down intestinal transit of the particles can improve absorption of the delivery vehicles; however, the effectiveness of targeting in humans is unclear (Chen and Langer, 1998).

1.9 Excipients

The challenge for the pharmaceutical world is to deliver the required drug to the correct location and at the required amount *via* the most convenient administration route. As stated previously, the oral route is a favoured route for drug delivery; however, this route is hampered by the fact that many new drugs being developed by the pharmaceutical industry are lipophilic (Lipinski *et al.*, 2001; Leigh *et al.*, 2001). There are problems associated with the delivery of lipophilic drugs because for a drug to be successfully absorbed the drug must be in solution and lipophilic drugs have a low aqueous solubility; therefore, they have a poor oral bioavailability (Gershanik and Benita, 2000).

Excipients are defined as anything present in a drug formulation in addition to the active compound. Excipients are commonly used in oral drug formulations because they facilitate manufacturing of the product, to increase the stability of the formulation, for aesthetic reasons or for identification (Jackson *et al.*, 2000). Excipients have been used to increase solubility and thus absorption of poorly permeable drugs (Muranishi, 1990; Lee *et al.*, 1991; Junginger and Verhoef, 1998). Excipients used to improve solubility and dissolution are solubilising excipients and absorption enhancers (Saha and Kou, 2000). Chitosan, an amino polysaccharide, has been used as an excipient in numerous drug delivery systems (Bernkop-Schnürch and Kast, 2001). For example, chitosan has been used in direct tablet compression, for the production of controlled release dosage forms (Illum, 1998). An alternative approach to improve delivery and absorption of lipophilic drugs is by incorporation into surfactant-based delivery systems such as liposomes (Panchagnula and Thomas, 2000) soluble polymers, microparticles made of insoluble

or biodegradable natural and synthetic polymers, microcapsules, lipoproteins and micelles (Torchilin, 2001).

1.9.1 Excipients and Absorption

All components of a drug formulation will have an impact on the intended destination, *e.g.* the epithelium of the small intestine, and thus will influence how the active ingredient is absorbed. Excipients that have a toxic or detrimental effect on the active ingredient or the human body cannot be utilised for drug delivery. Prior to use, excipients need to be assessed for toxicity and any potential side effects; cell lines, including Caco-2 cells are commonly used for this purpose (Saha and Kou, 2000; Wagner *et al.*, 2001).

Excipients are used to increase solubility and dissolution of compounds resulting in increased absorption and thus improving bioavailability (Jackson *et al.*, 2000). Consequently, the absorption profile of the active ingredient is likely to be altered. Any alteration in absorption profile needs to be characterised.

Solubility and permeability are key determinants of a drug's oral bioavailability. Drugs with limited water solubility have always presented a formulation challenge for oral delivery; examples include griseofulvin, digoxin, phenytoin, sulphathiazole, and chloramphenicol (Leuner and Dressman, 2000). Polymers have been included into formulations in an attempt to control the drug release from the formulation (Khan and Zhu, 1999). Carbomers are oral pharmaceutical grade excipients; they influence drug release from formulations because they readily absorb water and swell. Carbomers have been investigated as excipients for hydrophilic matrix tablets containing atenolol (Pérez-Marcos, *et al.*, 1991). In addition to polymers, so-called filler excipients or co-excipients are used in drug formulations, these including lactose, microcrystalline cellulose and starch. The release rate of ibuprofen increased in the presence of lactose, microcrystalline cellulose and starch (Khan and Zhu, 1999). The solubility of prednisolone, griseofulvin and diazepam has been shown to be increased in the presence of gelatin (Kallinteri and Antimisiaris, 2001). In contrast, citric acid and succinic acid have been

shown to delay drug release (Nykänen *et al.*, 1999). Citric acid retards ibuprofen release *in vitro*; therefore, *in vivo* absorption of ibuprofen is delayed (Nykänen *et al.*, 2001).

The choice of excipient for a drug formulation is an important decision. Inclusion of sodium bicarbonate (630 mg) significantly increased the rate of paracetamol absorption compared to conventional and soluble formulations; however, calcium carbonate (375 mg) did not alter the absorption kinetics (Grattan *et al.*, 2000). The amount of excipient used is also very important. As noted above sodium bicarbonate (630 mg) significantly increased the rate of paracetamol absorption; in contrast, sodium bicarbonate (400 mg) did increase the rate of absorption compared to conventional tablets but not compared to soluble formulations (Grattan *et al.*, 2000).

1.9.2 Excipients and Efflux

In addition to improving solubility and dissolution, excipients are used to combat the effects of efflux – the active removal of compounds from cells. The goal is to deliver drugs to the small intestine and increase bioavailability by decreasing efflux. Inhibition of P-glycoprotein can be achieved by D-alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS) (Dintaman, 1999). Tween 80 (Yamazaki *et al.*, 2000), Pluronic block copolymers (Batrakova *et al.*, 1999a; Batrokova *et al.*, 1999b), Cremophor EL (Woodcock *et al.*, 1990; Watanabe *et al.*, 1996; Sparreboom *et al.*, 1998) have been tested *in vitro* and *in vivo* for their potential to modulate the multidrug resistance caused by the activity of P-glycoprotein and MRPs. The mechanism of action remains unclear (Rege *et al.*, 2002); however, changes in membrane fluidity resulting in transporter conformational changes have been reported; refer to Section 8.1.3 for more information.

Amprenavir is a potent HIV protease inhibitor and a P-gp substrate (Matheny *et al.*, 2001). The solubility of amprenavir was improved in the presence of vitamin E-TPGS (D-alpha-tocopheryl polyethylene glycol 1000 succinate); efflux of amprenavir was also decreased resulting in an overall increase in amprenavir absorption by increasing solubility and permeability (Yu *et al.*,

1999). Vitamin E-TPGS also increased oral bioavailability of cyclosporine in human volunteers (Chang *et al.*, 1996).

Digoxin is a lipophilic drug and a substrate of P-glycoprotein (Matheny *et al.*, 2001). Digoxin has a low oral bioavailability because of a low water solubility and active efflux. Surfactants are commonly used to improve solubilisation of poorly soluble drugs; surfactants used include Polysorbate 80 and Cremophor EL. In addition to improving solubility, Polysorbate 80 and Cremophor EL may also modulate the behaviour of P-glycoprotein thus having a positive effect on bioavailability. Polysorbate 80 and Cremophor EL have both been shown to improve uptake of digoxin in an *in vitro* model (Cornaire *et al.*, 2000). Therefore surfactants cannot only improve solubilisation but can also decrease efflux activity resulting in an improved absorption and bioavailability (refer to Section 8.1.3). Surfactants have been shown to modulate membrane fluidity; surfactants both increase and decrease fluidity. Changes in membrane fluidity can cause conformational changes in tight junctions, membrane transporters and receptors (Section 7.4) (Sinicrope *et al.*, 1992). Modulation of tight junctions can increase absorption of drugs *via* the paracellular route (Section 1.3.1).

1.9.3 Excipients and the Future

Excipients have been traditionally regarded as being inert; however, there are many excipients that have proved to have a positive effect on dissolution and bioavailability (Jackson *et al.*, 2000). Inclusion of excipients into drug formulations for enhancement of drug bioavailability is being actively researched as shown in the examples above. Research into surfactants as excipients is important because they appear to have a dual purpose, they improve solubility and they appear to have a positive effect on decreasing efflux; however, more research needs to be done to provide a more detailed picture and explanation. An alternative to surfactants are lipids – lipids form drug carriers known as liposomes.

Drug carriers offer several potential advantages – they offer the incorporated drug protection from the harsh GI environment. The components of the drug carrier, like surfactants, may aid

dissolution of the entrapped drug or may act as absorption enhancers by having an effect on the cellular membrane resulting in increased absorption and bioavailability. The effects the drug carrier components have on the epithelium of the small intestine and the absorption of the incorporated drug must be investigated and characterised.

Incorporating drugs into vehicles may also improve bioavailability by providing protection from CYP3A and either providing protection from or modulating P-glycoprotein; to be effective the vehicle must be taken up by cells. Liposome formulations increased the absorption of epirubicin across Caco-2 cell monolayers and reduced efflux *via* P-glycoprotein (Lo, 2000). Epirubicin is an anticancer drug that is actively pumped out of cells *via* P-glycoprotein; thus, inhibition of P-gp may enhance the oral bioavailability. The effect Tween 20, Tween 80 and acacia had on intracellular accumulation of epirubicin were investigated using the Caco-2 cell line. These surfactants all significantly increased the intracellular accumulation of epirubicin, indicating that the use of surfactants as excipients may increase the intestinal absorption of some drugs through P-gp inhibition and improve drug bioavailability for P-gp substrates (Lo *et al.*, 1998).

Sodium deoxycholate (a bile salt) and sodium caprate (a fatty acid) are potent absorption enhancers and their possible use as MDR reversing agents was examined. Epirubicin uptake and transport was measured using Caco-2 cells and everted gut sacs of the rat jejunum and ileum. Intracellular accumulation of epirubicin in Caco-2 cells was significantly increased in the presence of sodium deoxycholate and sodium caprate. These two enhancers significantly increased apical-to-basolateral absorption of epirubicin across Caco-2 monolayers and mucosal to serosal absorption of epirubicin in the rat jejunum and ileum. Furthermore, basolateral-to-apical efflux of epirubicin across Caco-2 monolayers was significantly reduced with the addition of sodium deoxycholate and sodium caprate. Low toxicity excipients may be used as absorption enhancers and MDR modulators in formulations to improve the therapeutic efficacy of epirubicin (Lo and Huang, 2000).

Epirubicin has shown reduced toxicity and enhanced therapeutic efficacy when encapsulated in liposomes. Liposomal encapsulation and empty liposomal pre-treatment both significantly increased intracellular accumulation of epirubicin in Caco-2 cells. Apical-to-basolateral absorption of epirubicin across Caco-2 monolayers was substantially increased with these two treatments. Likewise, these two treatments increased mucosal-to-serosal absorption of epirubicin in rat jejunum and ileum. Both liposome encapsulation and empty liposome pre-treatment reduced basolateral-to-apical efflux of epirubicin across Caco-2 monolayers. The reduction in epirubicin efflux may be partially due to inhibition of P-gp or other transporter proteins in the intestine. The therapeutic efficacy of epirubicin may be improved by using phospholipids as excipients and MDR modulators in the formulations (Lo, 2000).

Cyclosporin A (CsA) is a multidrug resistance (MDR) modulator; however, clinical applications have been hampered due to severe side effects *in vivo*. CsA was entrapped into liposomes in an attempt to reduce the toxicity. Uptake and transport of epirubicin in Caco-2 cells was measured. Intracellular accumulation of epirubicin was significantly enhanced in the presence of CsA entrapped in liposomes. Apical-to-basolateral transport of epirubicin was also enhanced and basolateral-to-apical transport was decreased in the presence of CsA entrapped in liposomes. Liposomal preparations of CsA may circumvent MDR and have the advantage of reducing side effects; thus, providing a useful alternative dosage form for intravenous administration of CsA to be combined with cytotoxic agents form the treatment of resistant tumours (Lo *et al.*, 2001).

Further research into how liposome formulations and their components influence absorption of drugs and consequently bioavailability is required. Toxicity of components needs to be established and how the components alter absorption mechanisms including both paracellular and transcellular mechanisms.

1.10 Aims and Objectives of Thesis

The aims of this Ph.D. project were to study the factors that influence drug incorporation and retention in liposome formulations and to evaluate the potential use of liposomes as a vehicle for oral drug delivery. As discussed previously; the oral route is favoured for drug delivery; however, there are problems associated with this route, especially for lipophilic drugs. Therefore, there is a need to improve oral delivery, especially for lipophilic drugs. In the work presented, liposomes were chosen as the model lipid vehicle and were used in a dual capacity; firstly, as a drug delivery vehicle for drugs with differing LogP values, and secondly, as excipients and the role of the excipients used in the formulation of liposomes in modulating the absorption profiles of mannitol, propranolol, glutamine and digoxin was assessed.

More specifically the objectives were to:

i. Evaluate the influence that LogP values have on drug incorporation into MLVs and retention within liposomal bilayers and to further examine the effect of bilayer composition on these parameters. The series of 'model' drugs (hydroxybenzoates) used were chosen because of intermediate LogP values (1.85 – 3.44).

ii. Determine the toxicity of different liposome formulations towards the Caco-2 cell model using the MTT assay and the crystal violet assay. The liposome formulations tested were PC, PC:Cholesterol (16 μM : 4 μM), DSPC, and DSPC:Cholesterol (16 μM : 4 μM). The total amount of lipid tested was in the range 100 – 1000 μg. The liposome formulations were then used to complete the following aims of the thesis.

iii. Assess the impact liposome formulations have on the paracellular route; mannitol was the chosen probe. Mannitol transport was determined in the absence and presence of the chosen liposome formulations to determine the impact the liposome formulations had on the paracellular route.

iv. Examine the influence liposome formulations have on the passive transcellular route; propranolol was the chosen probe. Propranolol uptake and transport was determined in

the absence and presence of the chosen liposome formulations to determine the impact the liposome formulations had on the passive transcellular route.

v. Investigate the effect liposome formulations have on the transcellular carrier-mediated route; glutamine was the chosen probe. Glutamine uptake and transport was determined in the absence and presence of the chosen liposome formulations to determine the impact the liposome formulations had on the transcellular carrier-mediated route.

vi. Evaluate the effect liposome formulations have on efflux; digoxin was the chosen probe. Digoxin uptake and transport was determined in the absence and presence of the chosen liposome formulations to determine the impact the liposome formulations had on efflux.

The work found in the first part of the thesis will help to determine the extent LogP values and liposome formulation effect drug incorporation and release. From the second part of the thesis it is anticipated that the results will determine whether liposome formulations can be used with the Caco-2 cell model and how they influence absorption profiles of mannitol, propranolol, glutamine and digoxin. Dependent on whether the effect on absorption profiles is positive or negative, will determine if liposomes have the potential to be used as an oral drug delivery vehicle and increase solubility, absorption and thus bioavailability.

Chapter 2

General Material and Methods

2. General Material and Methods

2.1 Summary

This chapter provides a summary of the general materials and methods frequently used. Specific experimental procedures are presented in the relevant sections of the subsequent chapters. In most cases, results are expressed as the mean ± the standard deviation, taken from at least three values. Statistical test performed include the one-way analysis of variance (ANOVA) and unpaired t-test using *Graphpad Instat 3.0*.

2.2 Materials

2.2.1 Cell-Culture Reagents and Materials

Caco-2 cells (passage 26) were kindly donated by Dr. Vanessa A. Moore of AstraZeneca, (Charnwood, Loughborough, UK); the original cells were Caco-2 cells (passage 20) purchased from the American Type Tissue Culture Collection (ATTC) (Rockville, MD, USA). Dulbecco's Modified Eagle's Medium (DMEM) (with sodium pyruvate and glutamine, Gibco Invitrogen catalogue number 41966-029), foetal bovine serum (FBS), penicillin / streptomycin, trypsin / EDTA, non-essential amino acids (NEAA), were purchased from Gibco Invitrogen, UK. Phosphate-buffered saline (PBS), trypan blue solution (0.4 %), dimethyl sulphoxide (DMSO), ethylene diamine tetraacetic acid (EDTA) and amino acids were purchased from Sigma, (Dorset, UK). Cell-culture vented flasks, plastic 24-well plates, 12 mm Transwell inserts (Figure 2.1), with a polycarbonate membrane (0.4 μM pore-size membrane) and cryovials were purchased from Costar, UK. Neubauer haemocytometer was purchased from Weber Scientific International Limited, UK. Cells were incubated in a Jencons Millennium incubator.

2.2.2 Liquid Scintillation Counting

Optiphase HiSafe III scintillation fluid was purchased from Fisher Scientific, UK. The scintillation counter was a Hewlett Packard Tricard 2000 CA scintillation analyser. 5 ml of scintillation fluid

was added to all samples to be counted; these included test solutions, samples taken at specific time points and all washings. A scintillation vial containing only 5 ml of scintillation fluid was used as a blank control and was counted before any samples were counted to ensure the scintillation counter was functioning properly and the counter was not contaminated. [^{3}H] and [^{14}C] samples were counted for 10 minutes.

2.2.3 Protein Determination

Aprotinin, benzydamine, dithiothreitol, EDTA, phenylmethylsulfonyl fluoride (PMSF), Tris-HCl, the bicinchoninic acid (BCA) protein assay containing BCA reagent solution and copper sulphate pentahydrate (4 % w/v), and bovine serum albumin were purchased from Sigma (Dorset, UK).

2.2.4 Radioactive Uptake and Transport Studies

Radiolabeled L-[U-^{14}C]-Glutamine (271 mCi/mmol), radiolabeled D-[^{14}C]-Mannitol (56 Ci/mmol) and radiolabeled [^{3}H]-Mannitol (17.00 Ci/mmol) were purchased from Amersham (Buckinghamshire, UK). Radiolabeled DL-Propranolol [4-^{3}H] Hydrochloride (33 mCi/mmol) was purchased from Sigma (Dorset). Radiolabeled [^{3}H(G)]-Digoxin (19 Ci/mmol) was purchased from Dupont New England Nuclear (NEN / Dupont) Research Products (Boston, USA). Hank's Balanced Salt Solution (HBSS) was purchased from Gibco Invitrogen (UK). 2-[*N*-morpholino]ethanesulphonic acid (MES) buffer, *N*-2-hydroxyethylpiperazine-*N'*-2-ethansulphonic acid (HEPES) buffer, sodium azide, sodium hydroxide, Triton X-100, testosterone, nifedipine and terfenadine were purchased from Sigma (Dorset, UK). (±) Verapamil and Cyclosporin A (CsA) were purchased from ICN, (Oxfordshire, UK). The 24-well plates were supplied by Costar, UK. Double-distilled water (ddH_2O) was used. The 12-well Transwell inserts were supplied by Costar, UK. The transepithelial electrical resistance (TEER) were measured using World Precision Instruments EVOM chop-stick electrodes.

2.2.5 MTT Toxicity Assay and Crystal Violet Assay

3[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), sodium dodecyl sulphate (SDS), PBS tablets and crystal violet were purchased from Sigma, (Dorset, UK). Glutaraldehyde (50 % in solution) was purchased from BDH, Poole, UK. Hydrochloric acid (HCl) and methanol were reagent analytical grade. Double-distilled sterile water was used. PBS was prepared with sterile, double-distilled water. A StudentCam camera was purchased from Fisher Scientific, UK.

2.2.6 Thin-Layer Chromatography (TLC)

Solvents (toluene and ethanol) were reagent analytical grade. Pre-coated plastic-backed sheets of silica gel $60F_{254}$ with a layer thickness of 250 μm were purchased from Merck Ltd. (Dorset, UK). Digoxin was purchased from Sigma, UK. Radiolabeled [^{3}H(G)]-Digoxin (19 Ci/mmol) purchased from NEN/Dupont (Boston, USA).

2.2.7 Liposome Studies

Grade 1 egg phosphatidylcholine (PC), distearoyl phosphatidylcholine (DSPC) dimyristoyl phosphatidylcholine (DMPC) were purchased from Lipid Products (Nuffield, UK). The purity of all lipids used was > 99 %.

Table 2.1 – Frequently used lipid components (Koynova and Caffrey, 1998; Walde and Ichikawa, 2001).

Cholesterol (> 99 %), methyl hydroxybenzoate (MP), ethyl hydroxybenzoate (EP), propyl hydroxybenzoate (PP), butyl hydroxybenzoate (BP), sodium chloride (NaCl), sodium taurocholate, calcium chloride ($CaCl_2$), phospholipase A_2 (PLA_2) and dialysis tubing, pore cut-off size 12 – 14 kDa were purchased from Sigma, (Dorset, UK). Double-distilled water (ddH_2O) was used. Hydrochloric acid (HCl) was analytical grade.

2.2.8 Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) was performed using a Pyris Series, Diamond DSC, Perkin Elmer Instruments, USA. Aluminium sample pans were purchased from Perkin Elmer. The DSC was calibrated using indium.

2.3 Cell-Culture Methods

All cell-culture procedures were performed under aseptic conditions in a Gelaire, biohazard level II, laminar flow cabinet supplied from ICN (Oxfordshire, UK).

2.3.1 Cell-Culture Media

Cell-culture medium was used to culture Caco-2 cells in both plastic vented flasks and plastic plates. Two cell-culture media were prepared to culture Caco-2 cells. A maintenance medium was used to maintain the cellular stocks in plastic vented flasks. A plate medium was used to cultivate Caco-2 cells on 24-well plates for uptake experiments and 12-well Transwell inserts for transport experiments (Figure 2.1).

Maintenance media comprised of Dulbecco's Modified Eagle's Medium (DMEM), with 20 % v/v foetal bovine serum (FBS), and 1 % non-essential amino acids (NEAA).

Plate media comprised maintenance medium supplemented with penicillin / streptomycin, 100 units (U)/ml and 100 μg/ml respectively. Both culture media were stored at 4°C and replaced after 14 days.

2.3.2 Cellular Stocks

2.3.2.1 Maintaining Stocks

Caco-2 cells were cultivated in plastic tissue-culture T-flasks, with non-wetting 0.22 μm hydrophobic microporous membrane vent with an area of 75 cm^2. Caco-2 cells were grown as monolayers in an atmosphere of 5 % carbon dioxide at 37°C and 90 % relative humidity. Cell cultures were maintained using the appropriate media – the media were aspirated and replaced every 48 hours.

Confluency was achieved 5 - 7 days post-seeding. These cells were then trypsinised and used to seed new flasks, plates or Transwell inserts. The cell-culture media were aspirated and the cells were washed 3 to 4 times with 20 ml pre-warmed (37°C) PBS; all washings were aspirated. 4 ml trypsin / EDTA was added to each flask and the flask was returned to the incubator for 2 - 3 minutes. The flask was gently tapped and fresh culture medium (6 ml) was added to deactivate the trypsin and give a total volume of 10 ml. Washing the cell-culture media over the cells several times generated a single-cell suspension. The single-cell suspension was centrifuged at 400 *g* for 5 minutes, brake 9; the cell culture-media containing trypsin was aspirated and the cell pellet re-suspended in 10 ml fresh culture media. The cell-culture media was drawn up and down a pipette several times to create a single-cell suspension. The single-cell suspension was used to seed new flasks. 3 ml cell suspension and 9 ml fresh cell-culture medium was added to each flask. The flasks were returned to the incubator allowing the cells to grow.

When seeding Caco-2 cells onto plates and inserts, cell viability was determined using the trypan-blue exclusion test – a ten-fold dilution of the Caco-2 cell suspension (0.5 ml suspension

plus 4.5 ml fresh medium) was used. Trypan blue (100 µl) was added to the suspension (400 µl, 1.25-fold dilution). This cell suspension was counted using a Neubauer Haemocytometer. Under a light microscope, viable cells have a clear cytoplasm, whereas non-viable cells have a blue cytoplasm because they absorb the dye. The number of cells present in 10 squares of a haemocytometer was counted. Cell suspensions were only used if cell viability was greater than 95 %.

Equation 2.1 can be used to calculate the number of Caco-2 cells per ml of cell suspension.

$$Cells\ ml^{-1} = \left(\frac{Number\ of\ Cells\ Counted}{10} \right) \times 1.25 \times 10^5$$

Equation 2.1 – Calculation of cell number (1.25 and 10^5 are dilution factors).

2.3.2.2 Freezing and Thawing Caco-2 Cells

Frozen stocks of Caco-2 cells were kept in a liquid nitrogen cell bank at -170°C. Cells were stored in a freezing medium that comprised 10 % v/v DMSO / FBS. Cells were trypsinised (Section 2.3.2.1) and counted. The cell suspension was centrifuged at 400 *g* for 5 minutes, brake 9; the cell-culture medium was aspirated and the cell pellet re-suspended in freezing medium at approximately 1 x 10^6 cells ml^{-1}. This cell suspension was transferred to cryovials. The cryovials were placed in a –20°C freezer for 12 hours, the vials were then transferred to a -80°C freezer for a further 12 - 24 hours, the vials were then stored in a liquid nitrogen cell bank at -170°C.

When required, cells were revived from the frozen state. The cryovial was warmed in a 37°C water bath, shaking gently until the freezing medium became liquid. Under aseptic conditions, the cells were transferred to a 25 cm^2 culture flask. Cell-culture medium (7 ml) was added to the flask drop-wise. Cell-culture medium was aspirated and replaced every 48 hours. Cells were split when confluent (Section 2.3.2.1) and seeded into 75 cm^2 culture flasks. Cells were split 5 times prior to experimental use.

2.3.3 Splitting and Seeding of Caco-2 Cells

Caco-2 cells were split and seeded onto 24-well plates or onto Transwell inserts for experimental work.

2.3.3.1 Seeding 24-Well Plates

Caco-2 cells were split and counted (Section 2.3.2.1). Caco-2 cells were seeded at a density of 8×10^5 cells ml^{-1}; 1 ml was added to each well. Cell-culture media were aspirated and replaced every 48 hours. The plates were used for experimental work seven days post-seeding.

2.3.3.2 Seeding Transwell Inserts

Transwell inserts were incubated at 37°C with pre-warmed (37°C) PBS (1.5 ml apical and basolateral) for at least 15 minutes prior to seeding. Caco-2 cells were split and counted (Section 2.3.2.1). Caco-2 cells were seeded at a density of 1.33×10^5 cells ml^{-1} (2×10^5 cells $insert^{-1}$); 1.5 ml was added to each insert (apical) and 1.5 ml of cell-culture media was added to the basolateral compartment. Cell-culture media was aspirated from both the apical and basolateral compartments and replaced every 48 hours. The Transwell inserts were used for experimental work 14 - 21 days post-seeding.

2.3.4 Solutions Used for Radioactive Uptake Experiments

A total of four different solutions were required for uptake experiments:

- Transport medium (pH 6)
- Radioactive-labelled probe solution
- Stop solution
- Detergent solution

Transport medium (pH 6) comprised 1 x Hank's Balanced Salt Solution (HBSS), 2-[*N*-morpholino]ethanesulphonic acid (MES) buffer (25 mM – 2.44 g per 500 ml), made up to volume

using ddH_2O, typically 500 ml. The pH was adjusted to pH 6 using 1 M NaOH. Transport medium was stored at 4°C.

The radioactive-labelled probe solution consisted of the radiolabel ([^{3}H] or [^{14}C]) and any compound being assessed *e.g.* inhibitors. Transport medium is the basis for the radioactive-labelled probe solution. A total volume of 2 ml (0.5 ml / well) was needed when assessing inhibitory effects or liposomal formulation effects. Allowing for excess volume, compounds were dissolved in 4 ml transport media (pH 6) containing the radioactive-labelled probe (glutamine, 2 μM; mannitol, 8 μM; propranolol, 14 nM; digoxin, 24 nM). All compounds *e.g.* inhibitors were tested at a final concentration of 1 mM (unless otherwise stated in the relevant chapters) or at a known total lipid content (100 μg, 500 μg, 1000 μg).

The stop solution contained 0.05 % w/v sodium azide / PBS (0.25 g sodium azide dissolved in 500 ml PBS). Sodium azide is a metabolic inhibitor (Bogucka and Wojczak, 1966) and poison. Sodium azide depletes ATP, thus all cellular functions cease and no further uptake occurs. The stop solution is stored at 4°C.

The detergent solution contained 1 % v/v Triton X-100 / ddH_2O, (5 ml Triton X-100 made up to 500 ml using ddH_2O). Triton X-100 was used to solubilise the cellular membrane. The detergent solution was stored at room temperature, approximately 20°C.

2.3.5 Solutions Used for Radioactive Transport Experiments

A total of four different solutions were required for transport experiments:

- Transport medium (pH 7.4)
- Donor solution
- Stop solution
- Detergent solution

Transport medium (pH 7.4) comprised 1 x Hank's Balanced Salt Solution (HBSS), *N*-2-hydroxyethylpiperazine-*N'*-2-ethansulphonic acid (HEPES) buffer (25 mM – 2.98 g per 500 ml), made up to volume using ddH_2O (Costa *et al.*, 2000; Epler *et al.*, 2003; Heckel *et al.*, 2003; Meng *et al.*, 2004). The pH was adjusted using 1 M NaOH. Transport medium was stored at 4°C.

The donor solution consisted of the radiolabel ([^{3}H] or [^{14}C]) and any compound being tested (glutamine, 2 μM; mannitol, 8 μM; propranolol, 14 nM; digoxin, 24 nM). The donor solution was transport media (pH 7.4) containing the [^{14}C]-D-Mannitol plus any compound or liposomal formulation being investigated. All compounds *e.g.* inhibitors were tested at a final concentration of 1 mM (unless otherwise stated in the relevant chapters) or at a known total lipid content (100 μg, 500 μg, 1000 μg). The stop solution and detergent solutions were the same as in Section 2.3.4.

2.3.6 Liquid Scintillation Counting

Liquid scintillation spectrophotometry was used to quantify beta-emitting radionucleotides [^{3}H] and [^{14}C]. Optiphase HiSafe III (5 ml) was added to each sample. All scintillation vials were vortexed to ensure adequate mixing and then counted on a Hewlett Packard Tricard 2000 CA liquid scintillation analyser. [^{3}H] samples and [^{14}C] samples were counted for 10 minutes. All samples were counted twice for continuity and accuracy of results. Counts per minute (CPM) were converted to disintegrations per minute (DPM) by comparison with standard quench-correction curves. DPM were used in calculations.

2.3.7 Protein Determination Assay (BCA Assay)

The protein content of cell monolayers was determined using a BCA protein assay kit. The protein reduces copper (Cu) from Cu^{2+} to Cu^{1+} in a concentration-dependent manner. BCA is a chromogenic reagent for Cu^{1+}, producing a purple complex with a maximum absorbance at 562 nm.

To prepare the cell homogenate the Caco-2 cells in 24-well plates were trypsinised and washed twice with PBS by centrifugation. Cell pellets were re-suspended in 0.5 ml of homogenising buffer (pH 74.) consisting of 100 mM Tris-HCl, 1 mM dithiothreitol, 1 mM PMSF, 1 mM benzydamine, 100 μg / ml aprotinin, 10 mM EDTA and sonicated for 20 seconds at 56W. Copper (II) sulphate pentahydrate (4 % w / v) (1 part) was combined with BCA reagent solution (50 parts) to produce the protein determination reagent. A calibration curve was produced using bovine serum albumin (0 – 100 μg). Samples to be tested (100 μl of protein standard or unknown) were mixed with the protein determination reagent 1900 μl), incubated at 37°C for one hour. Absorbance was measured at 562 nm. The amount of protein in the unknown samples was calculated using a calibration curve of absorbance versus protein concentration. The protein content of cell monolayers was determined in order to correct for differences in cell number in monolayers. Knowing the protein content of the Caco-2 cell monolayers allowed resulted to be expressed as mg protein^{-1}, where appropriate.

2.3.8 General Protocol for Uptake Studies

Caco-2 cells were seeded (8 x 10^5 cells well^{-1}) on plastic 24-well plates. All uptake experiments were performed using 24-well plates. Cell-culture medium (1 ml well^{-1}) was aspirated and replaced every 48 hours. The cells were used seven days post-seeding.

Transport media (pH 6) and all radioactive-labelled probe solutions were pre-warmed to 37°C. The stop (sodium azide / PBS) solution was put on ice. The cells were washed twice with pre-warmed transport media (500 μl well^{-1}) for a duration of five minutes. The test solutions containing the radioactive-labelled probe solution were then added (500 μl well^{-1}, four wells per condition) and incubated at 37°C with agitation on a rotating platform. After the required time, the solutions were collected in scintillation vials. Cell monolayers were washed twice with the stop solution (500 μl well^{-1}) for a further duration of five minutes. The washings were collected separately in scintillation vials. Detergent solution was added to each well (1 ml well^{-1}), and the plates incubated at 37°C overnight. The solubilsed cell solution was collected and placed into scintillation vials. 5 ml of scintillation fluid was added to each vial, all samples were left for at

least one hour and vortexed to ensure adequate mixing, prior to the amount of radioactivity measured using liquid scintillation counting.

2.3.9 General Protocol for Transport Studies

Caco-2 cells were seeded (2 x 10^5 cells $insert^{-1}$) onto 12 mm Transwell inserts (Figure 2.1), with a polycarbonate membrane. All transport experiments were performed using 12-well Transwell inserts. Cell-culture medium (1.5 ml) was added to the basolateral compartment. Cell-culture medium was aspirated and replaced every 48 hours. The cells were used 14 - 21 days post-seeding.

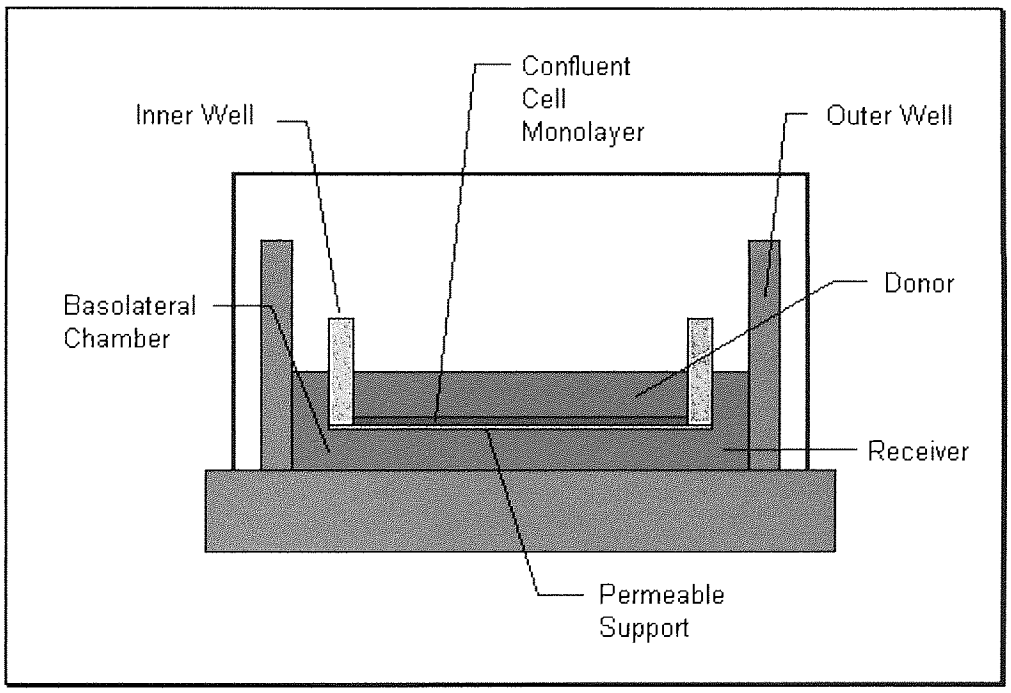

Figure 2.1 – Diagrammatic representation of a Transwell insert.

Transport media (pH 7.4) and all mannitol solutions were pre-warmed to 37°C. The stop (sodium azide / PBS) solution was put on ice. The cells were washed twice with pre-warmed transport medium for a duration of five minutes. For apical-to-basolateral (A – B) transport, the test solution containing the radioactive-labelled probe solution was added to the apical compartment (600 µl per insert) and 1.5 ml transport media (pH 7.4) was added to the basolateral compartment. For basolateral-to-apical (B – A) transport, the test solution (1.5 ml)

was added to the basolateral compartment and 600 µl transport media (pH 7.4) was added to the apical compartment. The cell monolayers were incubated at 37°C for the duration of the experiment with agitation on a rotating platform (Stuart Scientific Hybridisation Oven / Shaker SI 20H) to evenly distribute the radionucleotides in the receiving chamber.

At designated time points (0, 10, 20, 30, 40, 50 and 60 minutes), samples (100 µl) were collected and placed into scintillation vials; the transport medium was immediately replaced (100 µl). Following the last sampling time-point, both the apical and basolateral solutions were collected separately in scintillation vials.

Cell monolayers were washed twice with the stop solution (apical, 600 µl, and basolateral, 1.5 ml) for five minutes. The washings were collected separately in scintillation vials. Detergent solution was added to both compartments (apical, 600 µl, and basolateral, 1.5 ml) and the plate was incubated at 37°C overnight. The solubilised cell solution was collected and placed into scintillation vials. 5 ml of scintillation fluid was added to each vial, all samples were left for at least one hour and vortexed to ensure adequate mixing, prior to the amount of radioactivity measured using liquid scintillation counting.

Equation 2.2 was used to account for the sample removal at each time point and calculate cumulative amount transported.

$$M_t[n] = V_r . C[n] + V_s . \sum_{m=1}^{n-1} C[m]$$

Equation 2.2 – Equation used to calculate cumulative transport.

$M_t[n]$	–	Current cumulative mass (µg)
V_r	–	Volume in receiver (ml)
$C[n]$	–	Current concentration receiver (µg ml^{-1})

V_s – Sample volume remover (µl)

$\sum C[m]$ – Summed total of previous concentrations (µg ml^{-1})

2.3.10 MTT Toxicity Assay

The MTT toxicity assay was used to determine cell viability (refer to Section 4.2.2.1). Caco-2 cells were seeded in 24-well plates at a cell density of 8 x 10^5 cells ml^{-1}; 1 ml was added to each well and the cell-culture media was aspirated and replaced every 48 hours. Cells were used 7 days post-seeding.

Cells were washed with transport media (pH 7.4, methodology explained in Section 2.3.5) (500 µl per well). Samples to be tested were prepared in transport media. Transport media (pH 7.4), cell culture media and mannitol (0.1 % w/v) were used as negative controls, EDTA (0.5 % w/v) and sterile ddH_2O were used as the positive controls. A 5 mg / ml MTT solution was used and 10 % SDS / 0.01 M HCl was used to solubilise the MTT product overnight. Supernatants were read at 570 nm on an Anthos Reader 2001 plate reader (Anthos Labtech Instruments). Cell viability was calculated as a percentage of the negative control.

2.3.11 Crystal Violet Assay

The crystal violet assay was used in addition to the MTT assay to determine toxicity of liposomes towards Caco-2 cells. Caco-2 cells were seeded on Transwell inserts at a cell density of 1.33 x 10^5 cells ml^{-1} (2 x 10^5 cells insert^{-1}); 1.5 ml was added to each insert (apical) and 1.5 ml of cell-culture media was added to the basolateral compartment. Cells were used 14 – 21 days post seeding.

A 1 % v/v glutaraldehyde / PBS solution was prepared and stored at room temperature (2 ml glutaraldehyde made up to 100 ml using PBS). A 0.4 % w/v crystal violet / 30 % methanol solution was prepared and stored at room temperature.

Cell-culture medium was removed from both the apical and basolateral compartments. 1 % v/v glutaraldehyde / PBS solution was added with exceptional care to the apical (600 µl) and basolateral (1.5 ml) compartments. The 1 % v/v glutaraldehyde / PBS solution was left for one hour at room temperature, in order to fix the cells. After one hour, the 1 % v/v glutaraldehyde / PBS solution was then aspirated and the Caco-2 cells were washed for one minute using PBS, two washes were performed. With exceptional care, 0.4 % w/v crystal violet / 30 % methanol solution was added to the apical (600 µl) and basolateral (1.5 ml) compartments and left for 20 minutes at room temperature to stain the cells. After 20 minutes, the 0.4 % w/v crystal violet / 30 % methanol solution was aspirated and the cells were washed three times with double-distilled water at room temperature. The Transwell inserts were left to dry without the lid being replaced. Photographs of the Transwell inserts were taken using a StudentCam camera.

2.3.12 Thin-Layer Chromatography (TLC)

Digoxin is prone to degradation, therefore the purity of digoxin and [^{3}H(G)]-Digoxin was determined using thin-layer Chromatography (TLC). A mobile phase of toluene : ethanol (ratio 7 : 3) was used to identify digoxin; the mobile phase was left to stand for 45 minutes before use. The stationary phase was pre-coated plastic-backed sheets of silica gel 60 F_{254} with a layer of thickness of 250 µm. The TLC plates were heated at 120°C for 45 minutes to activate the plate's prior to use. The purity of digoxin and [^{3}H(G)]-Digoxin were determined on separate TLC sheets. Digoxin (1 mg / ml) was dissolved in chloroform : methanol (ratio 1 : 1), 1 µl was added to the TLC plate. The chromatogram was run and then dried rapidly. To visualise the cold digoxin, the plate was placed under ultraviolet (UV) light or sprayed with perchloric acid and heated in an oven to 100°C. For [^{3}H(G)]-Digoxin – 1 µl (32 kBq) was run on the TLC plate. The location of [^{3}H(G)]-Digoxin was determined by dividing the chromatogram into 5 mm horizontal sections and scraping off the silica into scintillation vials, 5 ml HiSafe III scintillation fluid was added to each vial and the [^{3}H(G)]-Digoxin content determined by liquid scintillation counting. The Rf value of digoxin is 62. Equation 2.3 was used to calculate the experimental Rf value for digoxin.

$$Rf = \frac{Distance\ the\ Substance\ Travels\ from\ the\ Origin}{Distance\ the\ Solvent\ Front\ Travels\ from\ the\ Origin}$$

Equation 2.3 – Calculation of Rf values.

2.4 Preparation of Liposomes

2.4.1 Hand-Shaking Method

The hand-shaking method was employed to prepare liposomes (Bangham *et al.*, 1965). Typically, the chosen lipids were dissolved at the required molar ratios in chloroform : methanol (9 : 1). These lipid solutions were added to a 100 ml round-bottomed spherical Quick-fit flask. The solvent was then removed *via* evaporation in a rotary evaporator above the liquid-crystalline temperature (T_c) (<0°C for PC, 37°C for DMPC, and 60°C for DSPC) resulting in a thin lipid film on the wall of the flask. The thin lipid film was flushed for about 60 seconds with oxygen-free nitrogen (N_2) to ensure complete solvent removal and to replace air. An appropriate amount of water was added into the flask to rehydrate the film and shaken vigorously until the lipid film transformed into a milky suspension – the suspension was left to stand above T_c for 30 minutes. Multilamellar liposomes of diverse sizes were formed during this period. This procedure was performed above the liquid-crystalline transition temperature (T_c) of the lipids and the suspension was left to stand above T_c for 20 – 30 minutes.

2.4.2 Incorporation of Hydrophobic Solutes

The hydrophobic drug(s) *e.g.* hydroxybenzoate series were dissolved in chloroform : methanol (9 : 1) at the required concentration (refer to Section 2.4.3 and Chapter 3) and added to the lipid mixture. The chosen lipids and hydrophobic drug were added into the round-bottomed spherical Quick-fit flask. The solvents (chloroform and methanol) were removed *via* evaporation in a rotary evaporator. The lipid film was flushed for 60 seconds with oxygen-free nitrogen followed by rehydration. The lipophilic drugs are incorporated during the rehydration process and located within the bilayer (Section 2.4.1).

2.4.2.1 Separation of Incorporated from Non-Incorporated Drug

The removal of non-incorporated drug was vital for experimental work. Incorporated and non-incorporated drug were separated by diluting the liposome suspension up to 35 ml with ddH_2O or PBS and centrifuging in a Beckman J2-21 centrifuge at 20,000 r.p.m. for 40 minutes at 4°C – this was utilised to remove non-incorporated drug from the liposome suspension. The pellet obtained was suspended a second time in 35 ml ddH_2O or PBS and centrifuged again under the same conditions. The supernatants were combined and the pellet was suspended in ddH_2O or PBS to the required volume. The supernatant was analysed to determine the amount of drug present and thus the amount of drug incorporated within the liposome formulation was calculated.

Surface adsorption of hydroxybenzoates was observed when the amount of hydroxybenzoate added to the liposome was too large for the liposome formulation to incorporate. In these instances the appearance of granular lipid films that did not rehydrate and re-crystallisation of the hydroxybenzoate was noted. These formulations were not used for any further experimental work. Surface adsorption was removed by reducing the amount of hydroxybenzoate.

2.4.3 Monitoring the Extent of Hydroxybenzoate Incorporation

A series of four hydroxybenzoates (methyl [MP], ethyl [EP], propyl [PP] and butyl hydroxybenzoate [BP]) were used due to their differing solubilities and LogP values. MP, EP, PP and BP were dissolved in chloroform : methanol (9 : 1) because of limited water solubility. Hydroxybenzoates were incorporated (1 mg, 2 mg, and 4 mg) in PC (16 μM) : cholesterol (0, 4, 8 and16 μM) liposomes. MP (1 mg) was incorporated in DSPC (16 μM) : cholesterol (0, 4, 8 and 16 μM) liposomes. The extent of hydroxybenzoate incorporation was estimated on the basis of UV absorbance. UV spectrophotometry was used to assay the hydroxybenzoates. MP and PP λ = 258 nm, EP and BP λ = 259 nm. Using samples taken from the combined supernatants and the re-suspended pellet obtained after centrifugation of the liposome preparations, the amount of drug was measured based on previously prepared calibration curves. The total amount of drug incorporated into liposomes was calculated by subtracting the amount of drug found in the

supernatant after centrifugation from the initial amount of drug added to the formulation, see Equation 2.4.

$$\%\,Incorporation = \left(\frac{(Total\ Amount\ of\ Drug\ Added - Drug\ in\ Supernatant)}{Total\ Amount\ of\ Drug\ Added} \right) \times 100$$

Equation 2.4 – Calculation of percentage incorporation.

2.4.4 Measuring Physical Attributes of Liposomes

2.4.4.1 Liposome Size Distribution

Liposome samples were sized by laser diffraction on a Malvern Mastersizer X (Malvern Instruments, UK). Liposome size was measured at 20°C in ddH_2O. The size of liposome samples were measured three times.

2.4.5 Liposome Release Studies

Release studies were performed as a method to compare liposome formulations. For the release studies, the liposomes were composed of PC:Cholesterol and DSPC:Cholesterol (16 μM : 4 μM) and 1 mg of the hydroxybenzoate. 'Empty' liposomes (containing no hydroxybenzoate) were used as control. After the liposome formulations were prepared they were centrifuged to remove non-incorporated hydroxybenzoate as described in Section 2.4.2.1. Following centrifugation the amount of hydroxybenzoates was determined and percentage incorporation calculated. The liposome pellet was resuspended in the appropriate receiver medium (10 ml) either water (see Section 2.4.5.1) or simulated gastric fluid (see Section 2.4.5.2). Refer to Section for 2.4.7 for complete experimental details.

2.4.5.1 Release into Water

Water was used as the receiver medium to monitor the extent of hydroxybenzoate release from liposomes (Section 2.4.5 and Section 2.4.7).

2.4.5.2 Release into Simulated Gastric Fluid

Liposome release into simulated gastric fluid (SGF) and simulated fasted intestinal fluid (FaSSIF) was measured (Section 2.4.5 and Section 2.4.7). Simulated gastric fluid comprised 0.04 M HCl (7 ml) made isotonic with NaCl (0.14 M), made up to a volume of 1000 ml. FaSSIF comprised of 5 mM sodium taurocholate, 3 mM $CaCl_2$, phospholipase A (PLA_2) (5 units ml^{-1}) made up to volume with PBS (Perrie *et al.*, 2002). PLA_2 was added immediately prior to the start of the experiment.

2.4.6 Liposome Transport Studies

Transport studies were used additionally to compare liposome formulations (Figure 2.2). Transport of hydroxybenzoates were measured across dialysis tubing; the dialysis tubing was soaked in water for approximately 72 hours prior to the beginning of the transport experiment, the water was changed several times during the 72 hour time period. For the transport studies, the liposomes were composed of PC:Cholesterol (16 μM : 4 μM) and 1 mg of the hydroxybenzoate was incorporated. 'Empty' liposomes (containing no hydroxybenzoate) and free hydroxybenzoate were used as controls. After the liposome formulations were prepared they were centrifuged to remove non-incorporated hydroxybenzoate as described in Section 2.4.2.1. Following centrifugation the amount of hydroxybenzoates was determined and percentage incorporation calculated – the amount of hydroxybenzoates incorporated in the liposome formulation was then used for the corresponding free hydroxybenzoate. The liposome pellet was resuspended in 1 ml of water. Refer to Section for 2.4.8 for complete experimental details.

2.4.6.1 Transport into Water

Water was used as the receiver medium to monitor the extent of hydroxybenzoate transport from liposomes (Section 2.4.6).

2.4.7 General Protocol for Liposome Release Studies

A modified version of the USP Apparatus 2 was used. Constant shaking agitation was employed in replacement of paddles. All samples were placed in a shaking water bath at 37°C. Hydroxybenzoate release at 37°C was monitored over 48 hours (receiver medium – 10 ml); samples (1 ml) were taken at 0, 0.5, 1, 2, 4, 24 and 48 hours. Parafilm was used to prevent evaporation of the samples. Samples were centrifuged at 14,000 r.p.m. for 30 minutes at 6°C to remove any lipid components present. The supernatant was decanted and analysed by UV and the amount of hydroxybenzoate present was calculated (see Section 2.4.3). To ensure that the centrifugation process removed all the lipid components present in the aliquots, the turbidity of the supernatant was measured at 350 nm.

2.4.8 General Protocol for Liposome Transport Studies

Transport from the liposomal formulations was examined using a model transport study based on Tabandeh and Ghasemi (1998), and Yoon and Burgess (1997). Dialysis tubing (12 – 14 kDa cut off) was used as an artificial membrane, transport of incorporated hydroxybenzoate and free hydroxybenzoate across the membrane was monitored over 24-hours at 37°C (Figure 2.2). The liposome solution or hydroxybenzoate solution (1 ml) was placed in the dialysis bag (Figure 2.2); the dialysis bag was placed into the receiver medium (100 ml). A water bath was used to maintain the temperature at 37°C and a magnetic stirrer was used to provide agitation. Sample times were 0, 0.5, 1, 2, 4, and 24 hours. Parafilm was used to prevent evaporation of the samples. Aliquots (1 ml) removed during sampling were centrifuged at 14,000 r.p.m. for 30 minutes at 6°C in order to remove any lipid components present. The supernatant was decanted and analysed by UV and the amount of hydroxybenzoate present was calculated – turbidity was measured at 350 nm.

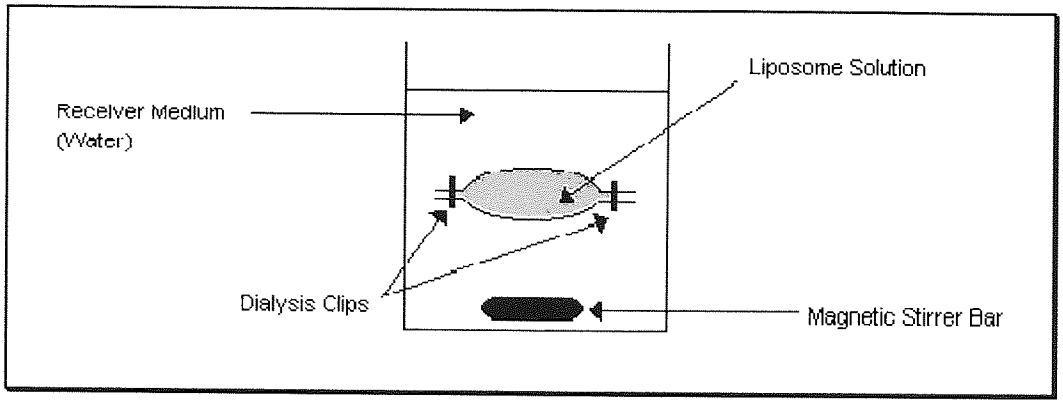

Figure 2.2 – Schematic of liposome transport.

2.4.9 Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) was performed on liposome samples. Aluminium pans for volatile samples were used. Approximately, 15 μl (15 mg) of liposome formulation was used with a scanning rate of 5°C per minute was employed over the required temperature range. Three DSC scans were completed for each sample.

Chapter 3

General Characterisation of Liposomes

3. General Characterisation of Liposomes

3.1 Introduction

Due to the increasing number of lipophilic drugs being produced by the pharmaceutical industry alternative formulations need to be investigated due to the problems associated with delivery of lipophilic drugs (discussed in Section 1.9). Consequently, new formulations and delivery strategies are needed. One option open for investigation is the use of liposomes. Liposomes are composed of insoluble phospholipids (Gregoriadis, 1976). Following hydration a highly ordered arrangement of concentric closed phospholipid bilayers form; the bilayers formed are continuous and unbroken (Figure 3.1).

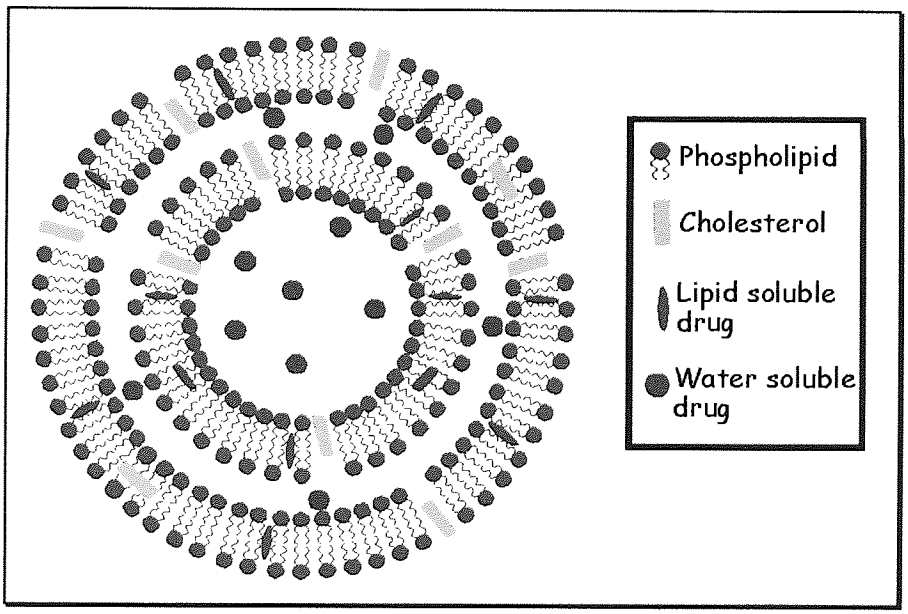

Figure 3.1 – Schematic representation of a liposome showing the orientation of the phospholipid constituents and the location of lipid-soluble and water-soluble drugs (adapted from Jones, 1995).

3.1.1 Bilayer Stability and Rigidity

Bilayer stability and rigidity of the liposome formulation can be modified to produce liposomes with differing degrees of rigidity thus allowing control over release from the liposomes. Phospholipids display a characteristic known as the liquid-crystalline transition temperature (T_c). T_c is defined as the temperature when the phospholipids undergo the phase transition between

the two phases. Below the T_c, the phospholipids are in the gel phase and enter the liquid crystal phase above the T_c (Figure 3.5).

Liposome bilayers are easily manipulated primarily through the selection of the phospholipid components chosen for the liposome formulation. Phospholipids may be chosen because of their liquid-transition temperature or because of their alkyl chain length. Transition temperature was the characteristic that influenced the chosen lipids used here. The two chosen lipids – PC and DSPC have very different transition temperatures, < 0°C and 57°C respectively (Table 2.1). Therefore at room temperature (20°C) and at physiological temperature (37°C) the two lipids will be in different states. PC will be in the liquid crystal phase whereas DSPC will be in the gel phase; consequently the liposomes containing DSPC will have more rigid bilayers (Figure 3.5).

3.1.2 Partition Coefficient (LogP)

Partition coefficient (LogP) is often used as a measure of lipophilicity. LogP is defined as the logarithm of the equilibrium constant of a solute's distribution between a non-polar solvent (1-octanol) and a polar solvent (water). LogP is an important factor that needs consideration when choosing a drug delivery system because it can influence uptake, bioavailability and toxicity (Section 1.2, Section 1.3.2.1 and Section 6.1).

$$\text{LogP} = \frac{C_o}{C_w}$$

Equation 3.1 – Partition Coefficient (LogP).

C_o – Is the solutes distribution in the non-polar solvent (1-octanol)

C_w – Is the solutes distribution in the polar solvent (water)

3.1.3 Hydroxybenzoates

3.1.3.1 Applications of Hydroxybenzoates

Hydroxybenzoates are commonly known as 'parabens' and are esters of *p*-hydroxybenzoic acids. The parabens are a homologous series including methyl, ethyl, propyl, butyl, parabens. Parabens are a class of antimicrobial agents with a broad spectrum of activity over a wide pH range. Parabens were first used as preservatives in the pharmaceutical industry in the mid-1920s (Soni *et al.*, 2001); however, they became widely used in cosmetic, food and pharmaceutical industry approximately 50 years ago (Rauha *et al.*, 1996; Wang and Chang, 1998; Giordano *et al.*, 1999; Soni *et al.*, 2001).

3.1.3.2 Hydroxybenzoates as a Model for Incorporation into Liposomes

Models are frequently used in pharmaceutical research to provide fundamental data and information that can then be applied to specific drugs. From the homologous series, methyl, ethyl, propyl and butyl hydroxybenzoate were chosen because they have differing solubilites and increasing intermediate LogP values whist having similar molecular weights (Table 3.1).

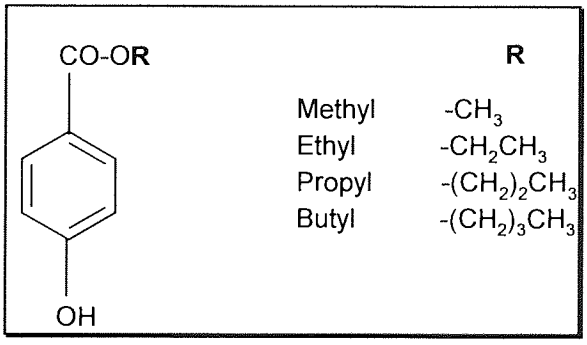

Figure 3.2 – General structure of hydroxybenzoates.

The length of the side group (R-group) increases from methyl hydroxybenzoate through to butyl hydroxybenzoate (Figure 3.2). The length of the side group (R-group) correlates with solubility and the LogP value. Increasing R-group length associated with an increasing LogP value and hence, increasing lipophilicity and consequently decreasing solubility.

Table 3.1 – Physical properties of hydroxybenzoates (Giordano *et al.*, 1999; British Pharmacopoeia 1988).

3.1.4 Objective

The objective of this chapter was to investigate the influence of LogP values on drug incorporation into MLVs and retention within liposomal bilayers and to further investigate the effect of bilayer composition on these parameters. A series of hydroxybenzoates were chosen as a model because of their range of intermediate LogP values (1.85 - 3.44).

Different liposomal formulations were prepared using phosphatidylcholine (PC), distearoyl phosphatidylcholine (DSPC) and cholesterol. The aims were two-fold, firstly to assess the influence liposome formulation has on drug incorporation and release and secondly the influence LogP values have on drug incorporation and release. It is hoped that the results of this chapter will be provide an optimum liposome formulation for the incorporation of hydroxybenzoates. The optimum formulation will provide the highest incorporation and a steady release rate. The optimum liposome formulation will be used in Chapter 4 – 8.

3.2 Material and Methods

The methods described in Chapter 2 were used; refer to section 2.4 for a detailed explanation of methodology used.

Liposomes composed of PC:Cholesterol (16:0, 16:4, 16:8 and 16:16 μmoles) and DSPC:Cholesterol (16:0 μmoles) were prepared using the hand-shaking method (Bangham *et*

al., 1965) (Section 2.4.1). Methyl hydroxybenzoate (MP), ethyl hydroxybenzoate (EP), propyl hydroxybenzoate (PP) and butyl hydroxybenzoate (BP) were incorporated into liposomes at 1 mg, 2 mg and 4 mg. Non-incorporated hydroxybenzoate was separated by centrifuging the liposome formulation. The liposome suspension was made up to a volume of 35 ml with ddH_2O or PBS and centrifuged at 20,000 r.p.m. for 40 minutes at 4°C. To ensure complete removal of non-incorporated hydroxybenzoate the pellet obtained was suspended a second time in 35 ml ddH_2O or PBS and centrifuged again under the same conditions. The supernatants were combined and analysed to determine the amount of drug present and thus the amount of drug incorporated within the liposome formulation was calculated. The pellet was suspended in ddH_2O or PBS to the required volume.

For incorporation studies, liposome size studies, release studies and transport studies, liposome formulations were prepared in at least triplicate. Due to the poor solubility of the hydroxybenzoates (Table 3.1) methanol was used as a co-solvent for MP and BP in the transport studies for the free MP solution and the free BP solution (Section 3.3.5). For the release and transport studies the amount of hydroxybenzoate incorporated was calculated, from the initial amount of hydroxybenzoate incorporated, percentage release and percentage transport were calculated. The data presented is an average and standard deviation of at least three formulations.

3.3 Results and Discussion

3.3.1 Incorporation into the Bilayer

The incorporation of MP, EP, PP and BP (1, 2 and 4 mg) into liposomes composed of PC:Cholesterol (16:0, 16:4, 16:8 and 16:16 µmoles) was estimated (refer to Section 2.4.3). As an additional comparison, 1 mg MP was incorporated into similar liposomes where DSPC replaced PC within the formulation (Section 3.3.1.2).

From this range of formulations, hydroxybenzoate incorporation was influenced by both the liposome composition and the drug characteristics, indeed with some formulation combinations liposomes were not formed when hydration of the lipid/drug film was attempted. In these instances the appearance of granular lipid films that did not rehydrate properly were noted. Such formulations are denoted as 'unsuccessful' and represented by '-' in Table 3.2.

3.3.1.1 Hydroxybenzoate Incorporation into Liposomes Composed of PC and Cholesterol

MP, EP, PP and BP were firstly incorporated at 1 mg; results obtained are presented in Table 3.2. All liposome formulations successfully incorporated MP, EP, PP and BP at a quantity of 1 mg, except for BP into PC:Chol, 16:16 (Table 3.2). Incorporation was observed at varying extents with the highest percentage incorporation being 74.6 per cent for PP in PC-only liposomes, compared to MP in PC:Chol, 16:16 liposomes with lowest incorporation of 33.4 per cent (Table 3.2).

Following the 1 mg hydroxybenzoate incorporation studies, the quantity of hydroxybenzoate was increased to 2 mg (Table 3.2). 2 mg of MP, EP, PP and BP were not as successfully incorporated into liposomes compared to 1 mg of the hydroxybenzoates (Table 3.2). All liposome formulations failed to incorporate 2 mg of BP; in contrast, MP, 2 mg was successfully incorporated into all liposome formulations. The highest incorporation was for PP into PC:Chol, 16:4 liposomes (66.4 per cent) and the lowest incorporation was 33.1 per cent for MP incorporation into PC:Chol, 16:16 liposomes (Table 3.2).

To further investigate the incorporation of hydroxybenzoates the quantity to be incorporated was increased to 4 mg (Table 3.2). 4 mg of MP, EP, PP and BP proved to be the least successful amount incorporated. MP, 4 mg, was the only hydroxybenzoate successfully incorporated; however, 4 mg MP was not successfully incorporated into PC:Chol, 16:16 liposomes (Table 3.2). Triamcinolone was incorporated into liposomes at 10, 2.5, 5.0 mg/ml and percentage incorporation remained approximately the same 90.6, 93.0 and 96.4% respectively (Lopes de

Menezes and Vargha-Butler, 1996). In contrast, entrapment of diclofenac in soya phosphatidylcholine liposomes was dependent on the concentration added. Increasing the concentration of diclofenac added resulted in a higher amount being incorporated until a plateau was reached at high concentrations (Lopes *et al.*, 2004).

It was predicted that PC:Cholesterol, 16:0 formulation would have the highest incorporation of hydroxybenzoates because there is no cholesterol present in the bilayer; therefore, will have the largest bilayer volume available to accommodate the hydroxybenzoates. MP, EP, PP and BP were all successfully incorporated into PC-only liposomes at 1 mg (Table 3.2) however, increasing the quantity of hydroxybenzoate resulted in a decreased success rate. MP and EP were the only hydroxybenzoates successfully incorporated into PC-only liposomes at 2 mg (Table 3.2). Further increasing the quantity to 4 mg resulted in only MP being successfully incorporated (Table 3.2). Introducing cholesterol to the formulation – PC:Cholesterol, (16:4) produced a very similar pattern of hydroxybenzoate incorporation to PC-only liposomes; however, there was one improvement – PP, was successfully incorporated at the increased quantity of 2 mg (Table 3.2). Increasing the cholesterol content to PC:Cholesterol, 16:8 resulted in decreased incorporation of hydroxybenzoates. All hydroxybenzoates were successfully incorporated at 1 mg; however, only MP was successfully incorporated at increased quantities of 2 mg and 4 mg (Table 3.2). Further increasing the cholesterol content to PC:Cholesterol, 16:16 had a negative effect on hydroxybenzoate incorporation. MP, EP and PP were incorporated at 1 mg and MP was also incorporated at 2 mg; however, BP, 1, 2 and 4 mg were not successfully incorporated into PC:Cholesterol, 16:16 liposomes and all hydroxybenzoates were unsuccessfully incorporated at the increased quantity of 4 mg (Table 3.2).

Liposome Cholesterol Content	Drug-Loading (Percentage of Amount Used)															
	MP			EP			PP			BP						
	1	2	4	1	2	4	1	2	4	1	2	4				
0	47.6 ± 10.4	60.1 ± 0.3	67.6 ± 4.3	64.1 ± 2.9	63.8 ± 3.7	-	74.6 ± 2.1	-	-	45.1 ± 9.5	-	-				
4	65.4 ± 3.4	58.0 ± 7.9	61.0 ± 6.0	61.7 ± 3.1	63.3 ± 0.4	-	64.3 ± 1.5	66.4 ± 0.3	-	49.2 ± 5.1	-	-				
8	46.5 ± 6.8	48.5 ± 5.3	48.6 ± 8.2	62.0 ± 0.6	-	-	61.5 ± 5.6	-	-	70.1 ± 6.5	-	-				
16	33.4 ± 16.2	33.1 ± 1.9	-	62.0 ± 1.2	-	-	60.0 ± 1.6	-	-	-	-	-				

Table 3.2 – Effect of bilayer composition on hydroxybenzoate incorporation.

MLV vesicles composed of phosphatidylcholine (PC; 16 µmoles) and cholesterol (0 –16 µmoles) were prepared as described in Section 2.4. Vesicles were prepared incorporating methyl (MP), ethyl (EP), propyl (PP) and butyl hydroxybenzoate (BP); 1 – 4 mg. '-' represents unsuccessful formulations. Results represent mean ± SD, n = at least 3.

From Table 3.2 two factors appear to dictate hydroxybenzoate incorporation: cholesterol content and drug molecular weight and / or volume. Cholesterol is an important bilayer component that has major effects on the physical properties of membranes and is used as a membrane stabiliser primarily effecting membrane fluidity (Devaraj *et al.*, 2002). For example, Essa *et al.*, (2004) used cholesterol to compare non-rigid liposomes (pure PC) and membrane stabilised liposomes (PC:Cholesterol). Cholesterol is a steroid and unlike phospholipids is only weakly amphipathic. The major structural entity of cholesterol is steroidal and flat allowing the bulky molecule to easily fit between adjacent alkyl chains (Kyrikou *et al.*, 2004). The interaction between cholesterol and phospholipids varies and is dependent on the individual phospholipid. The length of the alkyl groups of the phospholipids has a huge impact on the miscibility of cholesterol; for example cholesterol's miscibility is limited in the presence of negatively charged phospholipids (Bach and Wachtel, 1989; Borochov *et al.*, 1995; Bondar and Rowe, 1998). Cholesterol miscibility decreases as alkyl chain length increases (Bach *et al.*, 1992; Borochov *et al.*, 1995; Bondar and Rowe, 1998). Low concentrations of cholesterol causes positional disordering due to mismatch of the steroidal part with the alkyl chains of phospholipids (Kyrikou, *et al.*, 2004).

Incorporation values were also influenced by the presence of cholesterol in the liposome bilayer; increasing the cholesterol content from 20 (16:4) to 50 (16:16) per cent (total lipid), was seen to reduce incorporation values of all of the hydroxybenzoates tested (Table 3.2). Increasing the cholesterol content of the lipid bilayer may result in smaller amounts of the lipophilic drug being incorporated as there is finite volume in the bilayer (Gregoriadis, 1976). However, from the incorporation studies, it can be ascertained that a small quantity of cholesterol can enhance bilayer incorporation of the drug. For example, MP (1 mg) incorporation was significantly increased ($p < 0.05$) from 47.6 per cent for PC-only liposomes to 65.4 per cent for PC:Cholesterol, 16:4 liposomes (Table 3.2). This small amount of cholesterol may be required for bilayer integrity (Vemuri and Rhodes, 1995), as removal of cholesterol from the bilayer composition results in an increase in liposome size suggesting inefficient lipid packaging. A higher cholesterol content of 16:8 and 16:16 dramatically reduced incorporation; for example, PP (1 mg) incorporation significantly decreased ($p < 0.001$) from 74.6 per cent in PC-only

liposomes to 60.0 per cent in PC:Cholesterol, 16:16 liposomes (Table 3.2). This reduction in incorporation efficiency maybe due to cholesterol and the hydroxybenzoates having to compete for space within the lipid bilayer. Incorporation efficiency of ibuprofen into MLV's composed of PC:Cholesterol (16:0-16 μM) has been shown to be influenced by cholesterol content (Mohammed *et al.*, 2004). Ibuprofen was most successfully incorporated into liposomes composed of PC:Cholesterol, 16:4 μM and further increases in cholesterol content (8 and 16 μM) saw a decrease in ibuprofen incorporation (Mohammed *et al.*, 2004). Addition of cholesterol into liposome formulations improved incorporation of the anti-malarial agent Arteether (Al-Angary *et al.*, 1996).

The presence of cholesterol alters the formation of liposomes from dried lipid films (Barriocanal *et al.*, 2001). PC-only liposomes form as a result of a net breaking of bonds (endothermic), followed by a net formation of bonds (exothermic). The overall enthalpy of formation for PC-only liposomes is exothermic; however, this ceases upon inclusion of cholesterol, suggesting that cholesterol modifies phospholipid arrangement within the film (Barriocanal *et al.*, 2001); and this alteration in energetics may explain why liposome collapse was observed upon incorporation of hydroxybenzoates into PC-only liposomes, and was not observed in the presence of cholesterol.

There are two potential positions for incorporated drugs (and cholesterol) to be located, the first being alongside the phospholipids acting as an 'anchor' in the membrane and the second being in-between the phospholipid tails, *i.e.* in the middle of the membrane (Figure 3.1) (Vemuri, 1995). The molecular weights of all hydroxybenzoates are considerably less than that of cholesterol, less than 195 and 386 respectively. The conformation of cholesterol is more rigid than the hydroxybenzoates because of greater steric hindrance. This maybe one of the reasons why none of the hydroxybenzoates appear to stabilise the membrane and reduce the vesicle size to the same extent as cholesterol, resulting in the need for a small amount of cholesterol to be present for a stable conformation (Table 3.4).

The two most stable conformations for the hydroxybenzoates are with the R-group in the plane of the ring, and perpendicular to the plane of the ring (Figure 3.3). When the R-group is in the plane, the electron charge is spread over the ring; therefore, dipole values are significantly smaller (Appendix 2).

MP has the smallest R-group side chain out of the four hydroxybenzoates and has the most similar conformation to that cholesterol – hence, this may enable MP to 'sit' in the bilayer without straining the bilayer to the same extent as EP, PP and BP. With increasing R-group size the phospholipid arrangement may be disrupted leading to a decreased tolerance for hydroxybenzoate incorporation in the bilayer and resulting in liposome collapse or the inability for bilayers to form. Although MP is the smallest hydroxybenzoate, successful incorporation of MP, EP, PP and BP into the phospholipid bilayer resulted in a decrease in size similar to the reduction observed following the inclusion of cholesterol in the liposome formulation (Section 3.3.3; Table 3.4).

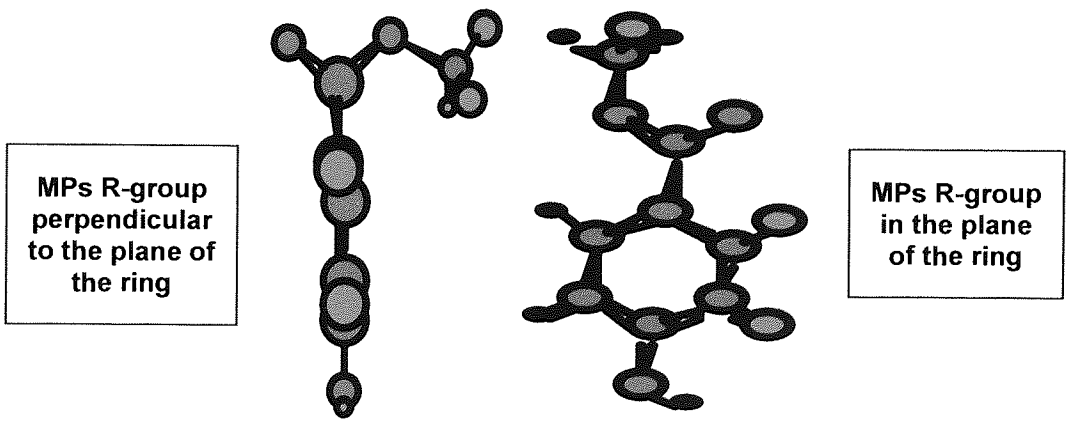

Figure 3.3 – Conformation of hydroxybenzoates using MP as an example.

Overall incorporation of the four hydroxybenzoates was shown to be influenced by both the model drug structures and the liposome composition. MP incorporated into the bilayer most effectively, despite having the lowest LogP and hence lipophilicity, with percentage incorporation of 65.4 per cent compared to 49.2 per cent in the case of BP in liposomes composed of PC:Chol (16:4 μM) (Table 3.2). The observed difference in MP and BP

incorporation into 16:4 liposomes is significantly ($p < 0.001$) different. Despite increased lipophilicity MP – BP, this was attributed to the increased structural size and hence the decreased packing ability of BP within the hydrocarbon core of the lipid bilayer compared to MP. The influence of hydroxybenzoate structure on bilayer loading was also demonstrated by employing increasing quantities of solutes. Up to 4 mg of MP could be incorporated into the lipid bilayer (16:4 μM) at a similar loading efficiency to that of 1 mg where as a maximum of 2 mg of both EP and PP could be incorporated into MLV at the same efficiency (61.7 – 66.4 %) (Table 3.2). Increasing the quantity of EP and PP above 2 mg resulted in an inability to form vesicles. At a reduced initial solute loading of 1 mg, all four hydroxybenzoates were successfully incorporated into PC:Chol (16:4 μM); suggesting the structure of the drug intended for incorporation was more influential than its lipophilicity on drug loading efficiency. The amount of lipophilic drugs incorporated into liposomes can be limited due to drugs interfering with bilayer formation and stability (McCormack and Gregoriadis, 1998).

Investigating the drug loading efficiency of the four hydroxybenzoates with increasing LogP values revealed that the influence of the drug's LogP is not the dominant factor in predicting lipid bilayer loading. As was shown in Table 3.2, despite the increased lipophilicity of BP over MP (suggesting that BP would preferentially enter the lipophilic core of the bilayer), drug loading efficiencies did not correlate with this hypothesis. MP has the lowest LogP value and thus the lowest lipophilicity was the most successfully incorporated hydroxybenzoate; high incorporation of MP was expected because liposomes are ideal for incorporating hydrophilic drugs. MP was unsuccessfully incorporated into only one formulation, 16:16 at 4 mg (Table 3.2). In contrast, BP has the highest LogP and thus is the most lipophilic was incorporated the least successfully. BP was successfully incorporated into only three formulations – PC:Chol, 16:0, 16:4, 16:8 at 1 mg (Table 3.2). EP and PP have LogP values that fall between MP and BP and incorporation success rate was better than BP and worse than MP. These results show that the optimum loading efficiency of bilayer-incorporated drugs are influenced by the drug structure and the presence of cholesterol in the lipid bilayer. In addition, several physical parameters, other than LogP, including molecular mass, conformation, size of R-group, dipole, and critical volume may influence the incorporation of the hydroxybenzoates (Table 3.1; Appendix 2). Moreover,

incorporation of MP was more successful than the incorporation of BP (Table 3.2), the critical volume of BP is greater than MP is 585.5 cm mol^{-1} and 417.5 cm mol^{-1} respectively. The Connolly area and Connolly volume are both greater for BP compared to MP providing further evidence that the presence of BP in the bilayer causes more strain and thus the collapse of the bilayer (Table 3.2; Appendix 2). Previous findings have shown that lipophilicity is not the primary factor in determining the extent of incorporation into liposomes (Stevens and Green, 1972). Incorporation of testosterone esters into liposomes was dependent on length of the side chain and structure opposed to lipophilicity. Incorporation was reduced by approximately half when straight side chain esters were replaced with branched chain esters with the same lipophilicity (Stevens and Green, 1972). In contrast, incorporation of corticosteroids into liposomes was dependent on lipophilicity; triamcinolone, the most lipophilic, was incorporated to a greater extent compared to hydrocortisone, the least lipophilic (Lopes de Menezes and Vargha-Butler, 1996).

The least successful formulation was PC:Chol, (16:16) because only MP, EP and PP were successfully incorporated (Table 3.2). The second least successful formulation was PC:Chol, (16:8), although all four hydroxybenzoates were successfully incorporated, MP, EP and PP were incorporated less efficiently compared to 16:0 and 16:4 (Table 3.2). The two remaining formulations PC:Chol, (16:0; Table 3.2) and PC:Chol, (16:4; Table 3.2) were very similar, with there being no significant difference in incorporation for MP, EP and BP; however, 16:4 has a significantly ($p<0.01$) higher incorporation (%) than 16:0 (%); therefore, making PC:Chol (16:4) the most successful formulation.

3.3.1.2 Incorporation of MP – PC Versus DSPC

To investigate the effect of lipid alkyl chain length liposomes were prepared where PC (C_{14} alkyl chain length) was replaced with DSPC, which contains two 18-carbon long alkyl chains. As previously noted (Section 3.1.1), DSPC has a higher transition temperature compared to PC (<0°C versus 57°C respectively); therefore DSPC liposomes form more rigid bilayers.

MP (1 mg) was successfully incorporated into all DSPC formulations, within the range 60.0 – 69.5 per cent (Figure 3.4). The longer alkyl chain length of DSPC and the subsequently increased hydrophobic volume of the liposomes resulted in a significantly higher incorporation of MP (1 mg) compared to the relative PC formulation, except for the 16:4 μM formulation (Figure 3.4). MP (1 mg) was incorporated into both PC:Cholesterol and DSPC:Cholesterol (16:4) with a very similar percentage incorporation, 65.4 per cent compared to 65.8 per cent respectively; therefore, it is possible for these two formulations to be used in further investigations to compare the behaviour of hydroxybenzoates incorporated into liposomes.

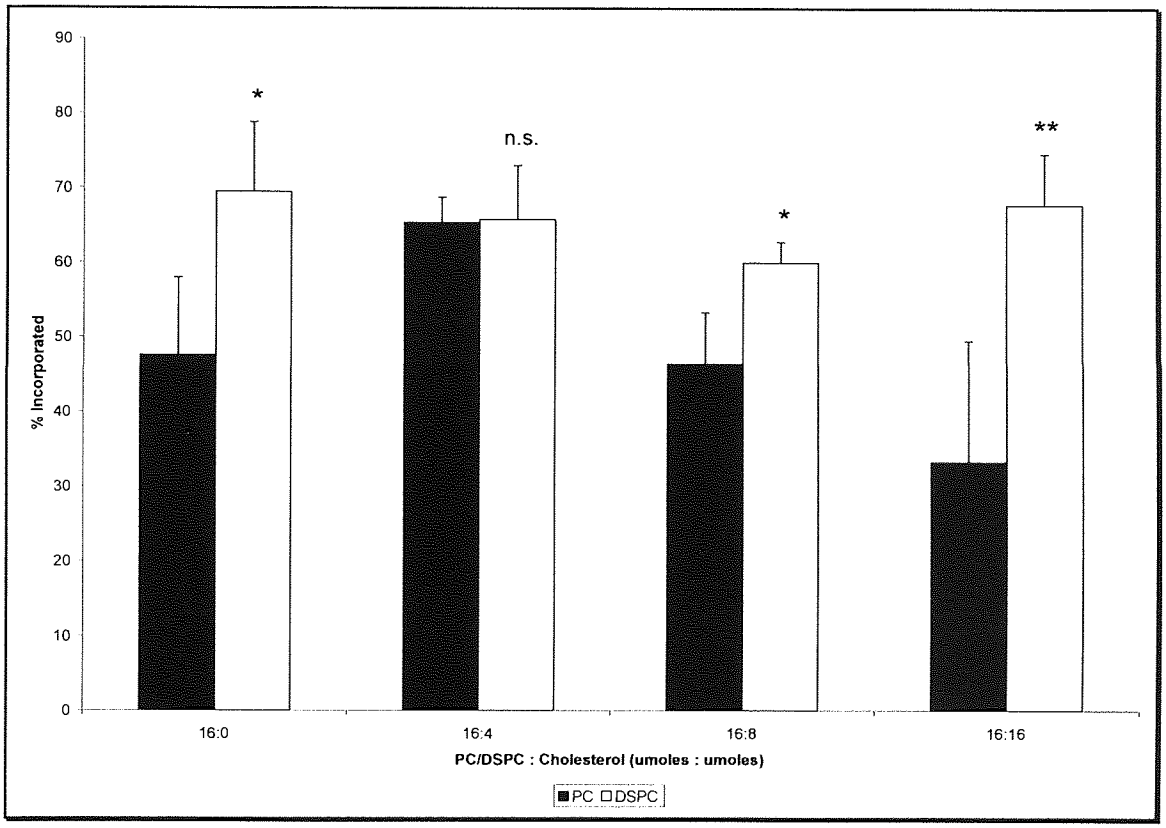

Figure 3.4 – Incorporation of 1 mg MP into PC:Cholesterol and DSPC:Cholesterol (16:0, 16:4, 16:8 and 16:16) liposomes.

* Denotes a significant ($p < 0.05$) difference. ** Denotes a very significant ($p < 0.01$) difference. n.s. denotes no significant difference ($p > 0.05$).

Increased incorporation in the presence of longer alkyl chain lipids can be attributed to an increased hydrophobic volume within liposome bilayers (Egerdie and Singer, 1982). Higher rigidity of longer alkyl chain lipids *e.g.* DSPC (C_{18}) may also increase incorporation (Bedu-Addo and Huang, 1995; Gløgård *et al.*, 2002).

Alkyl chain length of phospholipids has previously been shown to influence incorporation of solutes into liposome formulations. Increasing alkyl chain length has been shown to increase the incorporation of penicillin, Actinomycin D (Gregoriadis, 1973), arteether (Al-Angary *et al.*, 1996), gadolinium chelate (Gløgård *et al.*, 2002), antigenic proteins from *Pasteurella multocida* incorporation (Daghastanli *et al.*, 2004), ibuprofen (Mohammed *et al.*, 2004) and Na,K^+ATP-ase (de Lima Santos *et al.*, 2005).

Based on these results, further investigations employed liposomes composed of PC:Cholesterol or DSPC:Cholesterol (16:4 μmoles). This formulation was chosen in preference of 16:0, 16:8 and 16:16 μmoles because 16:16 μmoles failed to successfully incorporate all hydroxybenzoates (Table 3.2). Although hydroxybenzoate incorporation in 16:0, 16:4 and 16: 8 μmoles was similar, 16:4 μmoles successfully incorporated a higher number of hydroxybenzoates compared to 16:0 and 16:8 μmoles. Moreover, the percentage incorporation for MP was higher in 16:4 μmoles liposomes compared to 16:0 and 16: 8; for example MP, 1 mg 65.4 ± 3.4, 47.6 ± 10.4 and 46.5 ± 3.8 per cent respectively (Table 3.2).

3.3.2 Differential Scanning Calorimetry (DSC)

A very useful tool for studying the effects different membrane components have on a lipid bilayer is differential scanning calorimetry (DSC). DSC allows the changes and alterations in the bilayer to be observed through shifts in transition temperature and differences in enthalpy values. Phase transition is measured using DSC and is an important characteristic of phospholipids because it effects how the phospholipids will behave at particular temperatures (refer to Section 3.1.1). The phospholipids are capable of being in two states (Figure 3.5):

i. Gel Phase – the alkyl chains of the phospholipids are tightly packed and ordered, in a solid-like phase with limited movement.

ii. Liquid Crystal Phase – the alkyl chains of the phospholipids are disordered, in a liquid-like phase with 'free' movement.

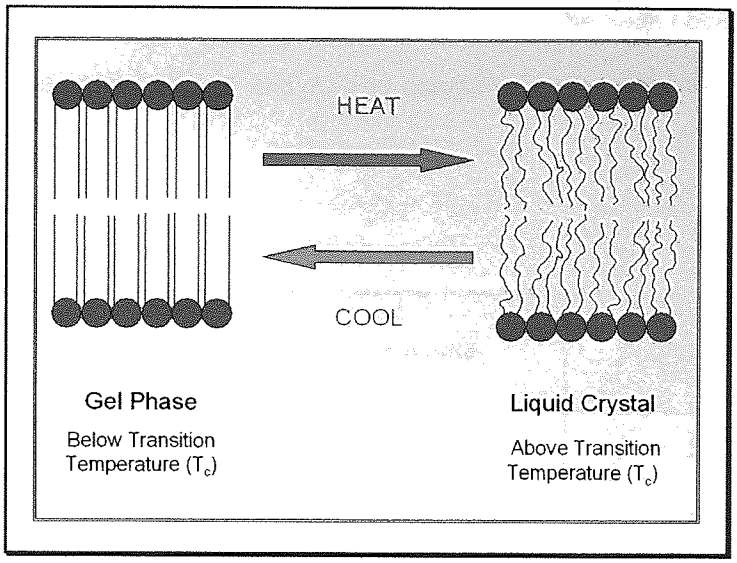

Figure 3.5 – Schematic representation of phase transition of phospholipids.

DSC scans were performed as described in Section 2.4.9; briefly, 15 µl (15 µg) of liposome solution was heated at a scan rate of 5°C / minute. DSC scans were performed on the following liposome formulations in order to determine the effect of increasing cholesterol content on liposome bilayers and the effect of incorporating the four hydroxybenzoates (1mg) in DSPC liposome formulations containing cholesterol (0-16 µmoles). From the DSC scans the phase-transition temperature was measured (Table 3.3).

The phase-transition temperature (T_c) of DSPC is 57°C (Table 2.1); from the DSC scan of DSPC-only liposomes, (Table 3.3) the experimental phase-transition temperature obtained was 57.29°C ± 2.27, with a narrow smooth peak. The addition of cholesterol to the bilayer reduced the phase transition temperature (Table 3.3). Cholesterol has previously been shown to alter to the phase transition temperature of phospholipid bilayers and broaden the peak of DPPC (dipalmitoyl phosphatidylcholine) (McMullen *et al.*, 1993; McMullen *et al.*, 1995; Rosser *et al.*, 1999; Kyrikou *et al.*, 2005). Here, the experimental phase transition temperature was reduced by 10.31°C following the addition of 4 µmoles of cholesterol to the liposome formulation. Increasing the amount of cholesterol to 8 µmoles resulted in a 15.18°C reduction in phase transition temperature; similarly, following the addition of 16 µmoles of cholesterol the phase transition temperature was reduced by 16.24°C. In addition to the reduction in phase transition

temperature following the inclusion of 16 μmoles of cholesterol the peak obtained from the DSC scan (Figure 3.6) was not as smooth and defined compared to liposome formulations with less cholesterol in the bilayer.

Liposome Composition (DSPC (μM) : Chol (μM)	Hydroxybenzoate	Peak Temperature (°C)
16:0	None	57.29 ± 2.27
16:4	None	46.98 ± 2.26
16:8	None	44.59 ± 4.48
16:16	None	41.05 ± 3.54
16:4	MP	41.23 ± 1.24
16:4	EP	37.05 ± 0.96
16:4	PP	35.07 ± 2.29
16:4	BP	41.19 ± 4.49

Table 3.3 – Summary of DSC scan data obtained from DSPC:Chol liposomes (16:0, 16:4, 16:8 and 16:16) and the effect incorporation of MP, EP, PP and BP (1 mg) had on DSPC:Chol, 16:4 liposomes.

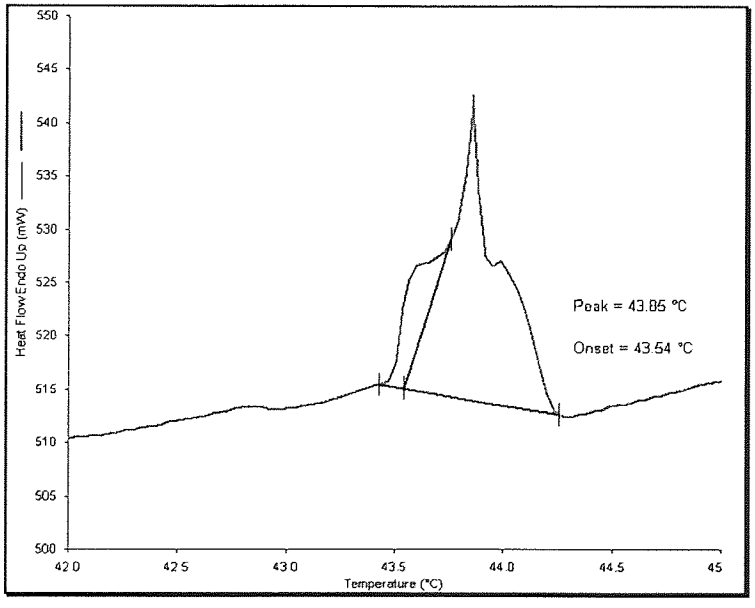

Figure 3.6 – DSC scan for DSPC:Chol, (16:16) liposomes.

In addition to the effects of cholesterol on PC bilayers, the effect alcohols have on PC bilayers has also previously been investigated – cholesterol is known to order cell membranes whereas ethanol is known to disorder cell membranes (Rosser *et al.*, 1999). The presence of ethanol shifts the phase transition temperature of phosphatidylcholine bilayers to a lower temperature (Rowe, 1981; Rowe, 1982; Rosser *et al.*, 1999). Methanol, ethanol and propanol induce biphasic effects in PCs (Rowe, 1985). Here, the effects MP, EP, PP and BP have on DSPC bilayers were investigated using DSC. Additionally, Kyrikou, *et al.*, (2004), used DSC to show that cholesterol is not only a buffer of fluidity of membrane bilayers but also a biomolecule that affects thermal properties of pharmaceutical molecules in membrane bilayers.

The presence of hydroxybenzoates further reduced the phase-transition temperature compared to DSPC:Chol, (16:4) liposome formulation (Table 3.3). Incorporation of 1 mg MP further reduced the phase transition temperature by 7.34°C; incorporation of 1 mg EP further reduced the phase transition temperature by 11.52°C; incorporation of 1 mg PP further reduced the phase transition temperature by 13.50°C; and incorporation of 1 mg BP further reduced the phase transition temperature by 7.38°C (Table 3.3).

The reduction in phase transition temperature observed following the incorporation of both EP and PP is more pronounced than MP (Table 3.3). The reduction in phase-transition temperature results from a less rigid bilayer. The reduction in bilayer rigidity results in a faster rate of release as observed in the release studies comparing PC and DSPC (see Section 3.3.4); PC has a lower phase-transition temperature and hydroxybenzoate release was faster from PC than DSPC; which has a higher phase transition temperature and thus increased bilayer rigidity. MP had a slower release rate compared to EP, PP and BP; this observation is probably due to increased bilayer rigidity. In contrast, the presence of both BP and MP reduced the phase-transition temperature approximately the same extent (Table 3.3); therefore, these two liposome formulations should have approximately the same bilayer rigidity; and thus, display the same rates of release. This was not the case; BP was released at a faster rate than MP. A closer look at the DSC scans offers a possible explanation. The MP DSC scan (Appendix 2) shows a

smooth peak; whereas the BP DSC scan (Figure 3.7) shows two overlapping peaks. This will influence and skew the calculated phase-transition temperature. The conclusion is that the BP liposome bilayer is less rigid compared to MP liposome thus showing a faster rate of release. Using a slower heating rate may separate the two peaks observed on the BP scan. Incorporation of sclareol into DPPC liposomes at high concentrations resulted in multiple transition peaks (Kyrikou *et al.*, 2005). Previous studies have also shown that the presence of solutes in the lipid bilayer affects transition temperature, for example inclusion of lutein in DMPC liposomes reduced the transition temperature, increasing the amount of lutein in the bilayer caused further reductions in transition temperature (Castelli *et al.*, 1999). The presence of chlorpromazine hydrochloride in DPPC:DMPC liposomes broadens the phase transition peak (Nerdal *et al.*, 2000) as did arteether (Al-Angary *et al.*, 1996). Therefore, the reduction in transition temperature in the presence of MP, EP, PP and BP suggests the hydroxybenzoates are interacting with the lipid bilayer; the hydroxybenzoates are positioned adjacent to the hydrocarbon chains of the phospholipid components in a similar manner to cholesterol (Figure 3.1).

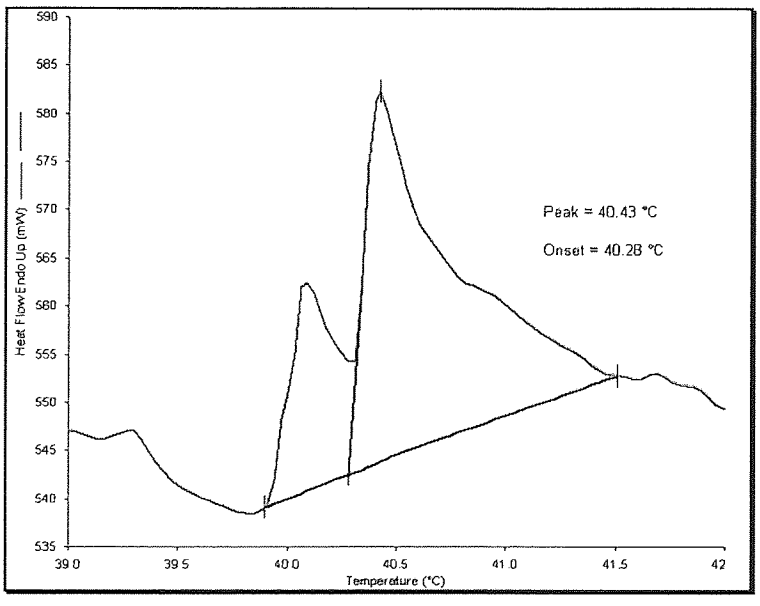

Figure 3.7 – DSC scan for DSPC:Chol, (16:4) liposomes with 1 mg BP incorporated.

The EP, PP and BP scans (Appendix 2 and Figure 3.7), do not display smooth peaks compared to the MP scan (Appendix 2), this may portray decreased bilayer stability resulting in faster

release rates. The presence of the hydroxybenzoates in the bilayer broadened the transition peak (Appendix 2). Inclusion of sclareol in DPPC bilayers also broadens the transition peak (Kyrikou *et al.*, 2005). Insulin incorporated into liposomes created a shoulder on the main endothermic transition temperature peak, the transition peak was also at a lower temperature indicating that a portion of insulin interacts with the lipid bilayer through both hydrophobic and electrostatic mechanisms (Kisel *et al.*, 2001).

3.3.3 Liposome Size

3.3.3.1 Effect of Incorporating Hydroxybenzoates on Liposome Size

The liposome size of vesicles with and without incorporated hydroxybenzoates (Section 3.3.1) was also investigated (Table 3.4). The mean diameter of the liposomes and the average span of the data were calculated from 3 liposome samples *per* formulation (Table 3.4). Span is a measurement of the width of the distribution. Span is a dimensionless number which illustrates whether the or not the spread of the distribution is narrow or wide. MLV size range is 100 nm to tens μms (Figure 1.8); therefore, due to the large size range of MLVs span is stated in Table 3.4 as an indicator of the spread of the size data. The spread of the size data was span: 1.260 ± 0.05 – 2.467 ± 0.28 (Table 3.4).

Liposome Cholesterol Content	Empty Vesicles Size (μm)	Drug-Loaded Vesicles (μm)											
		MP			EP			PP			BP		
		1	2	4	1	2	4	1	2	4	1	2	4
0	14.39 ± 0.20 (1.371 ± 0.01)	11.49 ± 0.72 (1.662 ± 0.12)	10.08 ± 0.26 (1.508 ± 0.05)	9.00 ± 0.93 (1.653 ± 0.17)	8.05 ± 0.10 (1.487 ± 0.05)	7.96 ± 0.48 (1.390 ± 0.07)	-	9.05 ± 0.01 (1.363 ± 0.18)	-	-	7.27 ± 0.94 (1.469 ± 0.10)	-	-
4	7.73 ± 0.16 (1.299 ± 0.04)	7.59 ± 0.58 (1.393 ± 0.08)	9.90 ± 0.68 (1.777 ± 0.27)	8.64 ± 0.03 (1.435 ± 0.26)	6.62 ± 0.38 (1.517 ± 0.08)	7.69 ± 0.22 (1.573 ± 0.09)	-	5.99 ± 0.24 (1.375 ± 0.07)	8.19 ± 0.27 (1.320 ± 0.11)	-	9.92 ± 3.98 (1.930 ± 0.23)	-	-
8	7.31 ± 0.07 (1.260 ± 0.05)	8.42 ± 1.14 (1.547 ± 0.18)	7.15 ± 0.96 (1.570 ± 0.15)	7.91 ± 1.49 (1.602 ± 0.11)	7.42 ± 1.42 (1.857 ± 0.15)	-	-	7.92 ± 0.24 (1.776 ± 0.42)	-	-	6.29 ± 0.63 (1.462 ± 0.32)	-	-
16	7.53 ± 0.47 (1.529 ± 0.05)	7.27 ± 0.91 (1.967 ± 0.27)	7.89 ± 1.54 (2.268 ± 0.43)	-	10.19 ± 1.17 (1.555 ± 0.30)	-	-	8.91 ± 1.30 (2.467 ± 0.28)	-	-	-	-	-

Table 3.4 – Effect of bilayer composition and hydroxybenzoate incorporation on liposome size.

MLV vesicles composed of phosphatidylcholine (PC; 16 μmoles) and cholesterol (0 –16 μmoles) were prepared as described in Section 2.4. Vesicles were also prepared incorporating methyl (MP), ethyl (EP), propyl (PP) and butyl hydroxybenzoate (BP). Vesicles were prepared with 1 to 4 mg initial drug concentration. Mean liposome size and

average span of size data are from at least 3 liposome samples *per* formulations. Span of the data is expressed in parentheses.

Lipid bilayer composition of the MLV had a marked effect on the vesicle size – liposomes composed of only PC (16 μM) were significantly larger than PC:Cholesterol liposomes ($p < 0.05$; all ratios). For example, liposome size decreases from 14.39 ± 0.20 μm for 16:0 liposomes to 7.73 μm ± 0.16 for 16:4 liposomes (Table 3.4). However, the data also suggests that after the initial decrease in vesicles size after addition of 4 μmoles of cholesterol no further significant change in vesicle size was noted with additional increases in bilayer cholesterol content (Table 3.4). Cholesterol is known to aid packing in bilayers; therefore, the observed reduction in size in the presence of cholesterol is probably due to improved packing and higher cohesion in the bilayer (Valenti *et al.*, 2001; Sinico *et al.*, 2005). Liposome size can both increase and decrease in following the inclusion of cholesterol in the lipid bilayer for example, DPPC liposome size increased following inclusion of cholesterol in the lipid bilayer (Kyrikou *et al.*, 2005). Although liposome size increased with cholesterol in the lipid bilayer, DPPC:Cholesterol liposomes proved to be stable (Kyrikou *et al.*, 2005).

The reduction in liposome size trend was followed with MP (1 mg) present in the MLV bilayer with PC liposomes being significantly larger ($p < 0.05$) than PC:Chol (16:4 μmol) (11.49 ± 0.72 μm versus 7.59 ± 0.58 μm respectively) (Table 3.4). Again, there was no significant difference between vesicles containing 4 to 16 μmoles cholesterol respectively. This trend, whilst not significant, was also noted for 2 and 4 mg of MP. Liposome size also decreased when the amount of cholesterol increased in the presence of EP, PP and BP; however, discrepancies were apparent (Table 3.4). These included, liposome size increasing after incorporation of EP and PP into PC:Cholesterol, 16:16 (Table 3.4).

PC-only liposomes are the only liposome formulation where liposome size significantly ($p < 0.01$) decreases following the incorporation of all the hydroxybenzoates, (MP, EP, PP and BP; all amounts) (Table 3.4). The observed reduction in PC-only liposome size following the incorporation of the hydroxybenzoates occurred because the hydroxybenzoates were

interacting with the lipid bilayer in a similar manner to cholesterol; thus, stabilising and improving bilayer packing resulting in a smaller liposome size. Inclusion of MP, the least lipophilic hydroxybenzoate reduced liposome size the least. In contrast BP, the most lipophilic hydroxybenzoate reduced liposome size to the greatest extent, indicating that BP interacted more effectively with the lipid bilayer which was expected as BP is the most lipophilic. The presence of BP in the bilayer reduced liposome size to approximately the same extent as the inclusion of 4 μm cholesterol – 7.27 ± 0.94 μm compared to 7.73 ± 0.16 μm. Incorporation of hydroxybenzoates into PC:Cholesterol 16:4 μM; 16:8 μM and 16:16 μM (Table 3.4) did not reduce liposome size any further. Thus, indicating that the presence of both cholesterol and hydroxybenzoates in the bilayer did not further stabilise or improve bilayer packing.

In contrast, to the hydroxybenzoate size data, incorporation of 3,3',4',5-tetrachlorosalicylanilide into egg phosphatidylcholine liposomes had no effect on liposome size (Barratt and Weaver, 1979). However, incorporation of essential oils (Valenti *et al.*, 2001; Sinico *et al.*, 2005), sodium diclofenac (Lopes *et al.*, 2004) and sclareol (Kyrikou *et al.*, 2005) have all significantly decreased liposome size. The reduction in liposome size is as result of high packing and cohesion in the bilayer (Valenti *et al.*, 2001; Sinico *et al.*, 2005).

3.3.4 Drug Release from the Liposome System

3.3.4.1 Hydroxybenzoate Release into Water

To further investigate these systems, the release and transport (Section 3.3.5) profiles of the four hydroxybenzoates from the liposome systems were determined (Section 2.4.5). PC:Chol and DSPC:Chol (16:4 μM) were the chosen formulations as they offered the highest drug loading efficiency (%) for all four of the hydroxybenzoates tested (Table 3.2 and Figure 3.4). These systems were employed in both release and transport studies (Section 3.3.5).

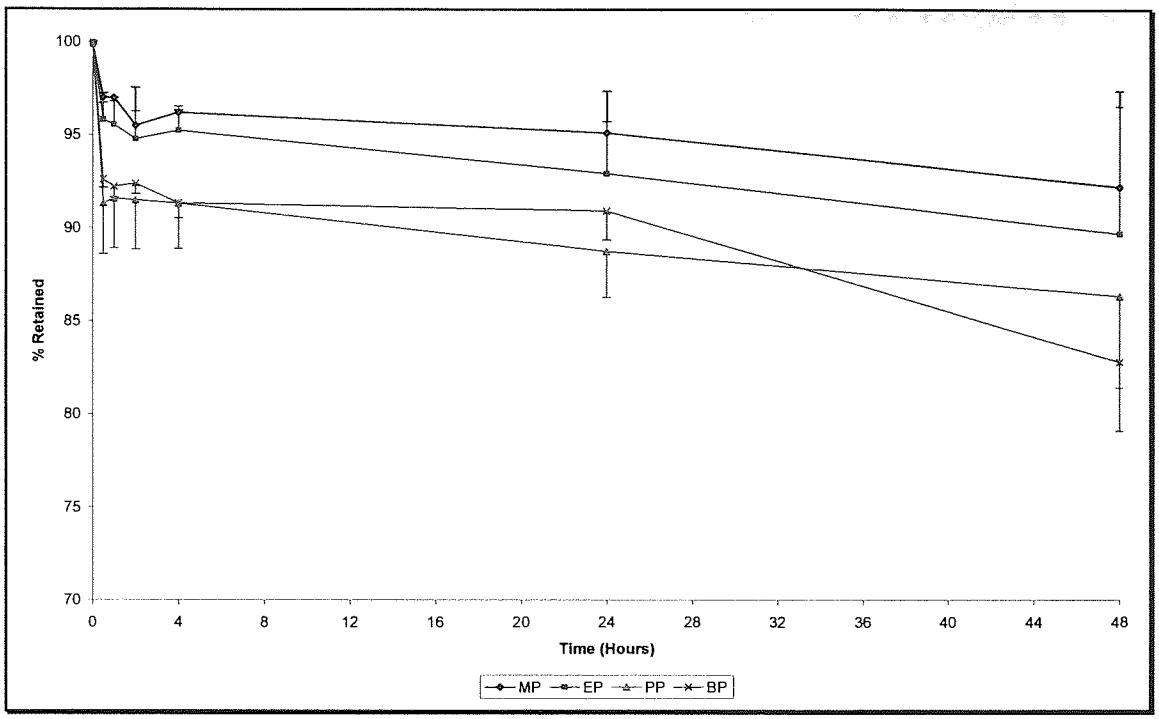

Figure 3.8 – Percentage retainment of incorporated hydroxybenzoates from PC:Chol (16 μM : 4 μM) liposomes (n = 3).

For PC:Chol (16:4 μM) a similar release profile was observed for all hydroxybenzoates; there was a burst effect with hydroxybenzoate being released (1 – 2 hours); (Figure 3.8), followed by slow controlled release; however, the extent of drug release differed between the four solutes tested. Following the initial release of hydroxybenzoates release was limited. Of the four hydroxybenzoates, MP was released from the liposomes the least and thus the highest amount was retained, 92% after 48 hours (Figure 3.8). In contrast, BP was released the most and therefore the least amount was retained, 83% after 48 hours. Overall hydroxybenzoate release were contrary to what would be expected based on LogP values with, generally, release being in the order of BP > PP > EP > MP. This was highlighted even after 1-hour incubation of the liposome systems (Figure 3.8). After the 48-hour period, high levels of the incorporated hydroxybenzoates remained incorporated and have not been released (Figure 3.8). The initial faster release of the hydroxybenzoates might be due to the burst effect *i.e.* release from the outer bilayers of the MLV structure, followed by a slower release due to restricted movement of the hydroxybenzoate from the inner lamellae across a series of bilayers (Lopes de Menezes and Vargha-Butler, 1996). Repeating the release experiment with LUVs or SUVs in replacement of MLVs would confirm whether the burst effect happened because LUVs and SUVs have a

single bilayer; therefore, all the incorporated hydroxybenzoates would be released if the burst effect occurred.

Although the release profiles for all the hydroxybenzoates from PC:Chol, (16:4) liposomes were similar (Figure 3.8); statistical analysis showed that the release of hydroxybenzoates from PC:Chol, (16:4) were significantly different at a number of different time points (Table 3.5). Significant difference in hydroxybenzoate release predominately occurred in the early stages of the experiment. MP with the lowest LogP and BP with the highest LogP have significantly different release rates at 0.5, 1, and 4 hours (Table 3.5).

In agreement with incorporation studies, these release studies also suggest that the smaller MP is more stably incorporated within the lipid bilayer with only 7.8 % of the incorporated solute being released after 48-hours compared to 17.2 % in the case of BP. Again, these results are in contrast to the LogP values for MP and BP (1.85 and 3.44 respectively), which would predict that BP would favour the lipophilic bilayer of liposomes compared to the aqueous medium. It was predicted that LogP values would influence the release of MP, EP, PP and BP and thus the release rate would be in the order of MP > EP > PP > BP; however, the order of release was generally BP > PP > EP > MP. Therefore, another factor of the hydroxybenzoates series must have influenced the release pattern. In addition to increasing LogP values from MP – BP the size of the hydroxybenzoate increases. Increasing the size of a solute in the bilayer results in an increased strain on the bilayer structure leading to an unstable bilayer. The increased strain on the bilayer caused by the increase in hydroxybenzoate size may have caused the release profiles obtained *i.e.* MP being the smallest (R group = CH_3) will cause the least disturbance to the bilayer resulting in a low release and BP being the largest hydroxybenzoate (R group = $(CH_2)_3CH_3$) will cause the greatest strain on the bilayer resulting in the highest release rate (Figure 3.8 and Figure 3.9).

Time (Hours)	MP vs EP	MP vs PP	MP vs BP	EP vs PP	EP vs BP	PP vs BP
0.5	n.s.	**	*	*	n.s.	n.s.
1	n.s.	**	*	*	n.s.	n.s.
2	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
4	n.s.	**	**	**	**	n.s.
24	n.s.	*	n.s.	n.s.	n.s.	n.s.
48	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Table 3.5 – Statistical comparison of release from PC:Chol (16:4) liposomes.

* Denotes a significant ($p < 0.05$) difference; ** Denotes a very significant ($p < 0.01$) difference; n.s Denotes not significant ($p > 0.05$).

Triamcinolone and hydrocortisone have different lipophilicities; their release from liposomes was measured. After 24-hours the less lipophilic hydrocortisone was retained at greater amounts than the more lipophilic triamcinolone. Hydrocortisone may have been retained at higher amounts because it is able to be encapsulated in both the hydrophobic regions and in the aqueous environment of the MLVs due to its higher aqueous solubility (Lopes de Menezes and Vargha-Butler, 1996). Release of vincristine, vinblastine and vinorelbine from liposomes was measured because they have similar chemical structures; however, their LogP values differ (Zhigaltsev *et al.*, 2005). In agreement with the release data presented here, vinblastine has the highest LogP value and thus lipophilicity and was released more rapidly than both vincristine and vinorelbine. Vincristine with the lowest lipophilicity was retained at the greatest amount (Zhigaltsev *et al.*, 2005). In contrast to the release data presented here, release of *p*-hydroxybenzoic acid esters from Pluronic F-127 gels was the opposite with release rates decreasing as lipophilicity increased (Gilbert *et al.*, 1986).

3.3.4.1.1 The Effect of Alkyl Chain Length on Hydroxybenzoate Release

The same release profile was observed for the DSPC:Chol, (16:4) liposome formulation (Figure 3.9), as for PC:Chol, (16:4) (Figure 3.8); an equilibrium was established after 1 – 2 hours, with approximately 98% of MP being retained, compared to 96, 93 and 93% retention of EP, PP and

BP respectively. Even after the 48-hour time period the incorporation remained high (96, 87, 89 and 84% respectively; Figure 3.9).

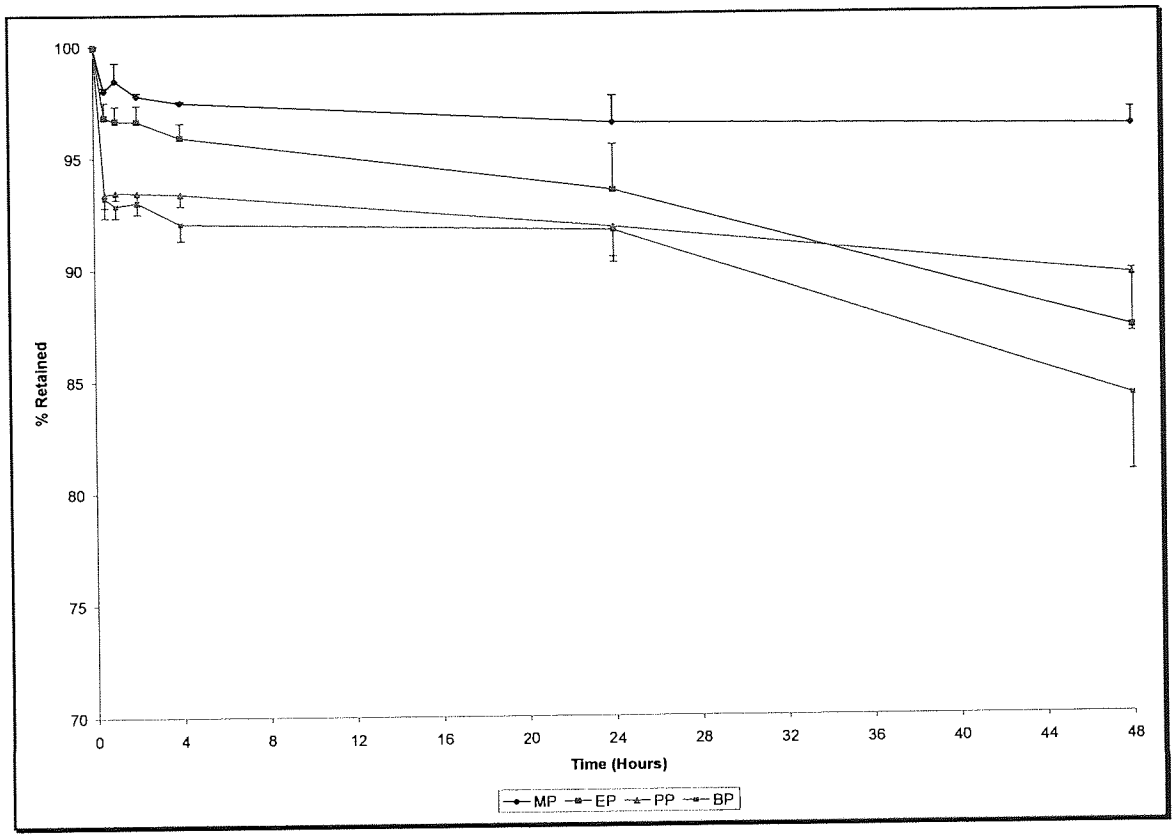

Figure 3.9 – Percentage retainment of incorporated hydroxybenzoates (1 mg) from DSPC:Chol (16 μM : 4 μM) liposomes (n = 3).

Hydroxybenzoate release from DSPC:Chol (16:4) liposomes followed a similar profile to the release from PC:Chol (16:4) liposomes; however, statistical analysis showed that hydroxybenzoate release from DSPC:Chol (16:4) liposomes were significantly different at many more time points (Table 3.6). Unlike PC:Chol (16:4) liposomes, there were significantly different release rates from DSPC:Chol (16:4) liposomes throughout the experiment and not just at the beginning. For example, MP and BP have significantly different release rates at all time points.

Hydroxybenzoates with close LogP values *e.g.* MP and EP, or PP and BP were expected to have similar release profiles, this was observed from PC:Chol, (16:4) where no significant difference in release profiles for MP and EP or PP and BP were observed (Table 3.5). This was

also observed with release from DSPC:Chol, (16:4); however, some significant differences were observed (Table 3.6).

Time (Hours)	MP vs EP	MP vs PP	MP vs BP	EP vs PP	EP vs BP	PP vs BP
0.5	n.s.	**	**	**	**	n.s.
1	*	**	**	**	**	n.s.
2	n.s.	**	**	**	**	n.s.
4	*	**	**	**	**	*
24	n.s.	*	*	n.s.	n.s.	n.s.
48	**	*	**	n.s.	n.s.	n.s.

Table 3.6 – Statistical comparison of release from DSPC:Chol (16:4) liposomes.

* Denotes a significant ($p < 0.05$) difference; ** Denotes a very significant ($p < 0.01$) difference; n.s Denotes not significant ($p > 0.05$).

PC has a lower transition temperature and shorter alkyl chain length than DSPC therefore DSPC liposomes form bilayers with increased rigidity; thus it was expected that different release profiles for the hydroxybenzoates would be observed. The differences in transition temperature did not produce different release profiles from the two formulations (Figure 3.8 and Table 3.7). A significant difference in the amount of MP released from the two formulations was observed at 0.5, 1 and 4 hours; however, no significant difference was observed for EP, PP or BP (Table 3.7). Alkyl chain length and phase transition temperature have previously been shown to influence release from liposomes, typically the shorter the alkyl chain length the greater the release (Senior and Gregoriadis, 1982; Lopes de Menezes and Vargha-Butler, 1996). Increasing the alkyl chain length, increases the van der Waals forces between the lipid chains; therefore more energy is needed to disrupt the ordered packing. At 37°C DSPC liposomes will be in the ordered gel phase and release is reduced. Release of arteether (Al-Angary *et al.*, 1996), budesonide, hydrocortisone (Saarinen-Savolainen *et al.*, 1997), ibuprofen (Mohammed *et al.*, 2004), doxorubicin (Charrois and Allen, 2004) from liposomes decreases as alkyl chain length increases.

Time (Hours)	MP	EP	PP	BP
0.5	Very Significant (p = 0.0033)	n.s. (p = 0.2042)	n.s.. (p = 0.2899)	n.s. (p = 0.1629)
1	Significant (p = 0.0375)	n.s. (p = 0.2485)	n.s. (p = 0.2968)	n.s. (p = 0.2371)
2	n.s. (p = 0.1299)	n.s. (p = 0.1269)	n.s. (p = 0.2835)	n.s. (p = 0.2432)
4	Extremely Significant (p = 0.0001)	n.s. (p = 0.4763)	n.s. (p = 0.2283)	n.s. (p = 0.3334)
24	n.s. (p =0.4073)	n.s. (p = 0.8031)	n.s. (p = 0.1328)	n.s. (p = 0.5837)
48	n.s. (p =0.2502)	n.s. (p = 0.5906)	n.s. (p = 0.3713)	n.s. (p = 0.6566)

Table 3.7 – Statistical comparison of release of hydroxybenzoates from PC:Chol (16:4) and DSPC:Chol (16:4).

3.3.4.2 Hydroxybenzoate Release into Simulated Fluids

The release experiments were repeated using either SGF or FaSSIF as the receiver medium. The lipids PC and DSPC were compared in further investigations to assess the stability of these systems after oral delivery. Experiments were performed using PC and DSPC liposomes as above (Figure 3.10).

Results from these studies show a marked difference between the two formulations (Figure 3.10) – even after 1-hour in the presence of SGF, DSPC liposomes were able to retain significantly more ($p < 0.05$) of the incorporated drug compared to their egg PC counterparts (Figure 3.10). Similarly, in the presence of FaSSIF, DSPC liposomes were able to retain significantly more ($p < 0.01$) after 1-hour compared with PC liposomes (Figure 3.10). After 24-hours the pattern remained the same (Figure 3.10). Release into SGF media was very slow for both formulations; in contrast, release into FaSSIF was much faster for both formulations (Figure 3.10). The release profile of hydroxybenzoates into simulated fluids was similar to release into water (Figure 3.8 and Figure 3.9). To confirm that there was a burst effect resulting

in hydroxybenzoate release followed by slow hydroxybenzoate release, the release experiment into simulated fluids needs to be repeated using LUVs or SUVs because they have a single bilayer, therefore all incorporated hydroxybenzoate will be released if there is a burst effect. To determine if PLA_2 is the rate limiting step in the release of hydroxybenzoates the release experiment needs to be repeated with varying amounts of PLA_2.

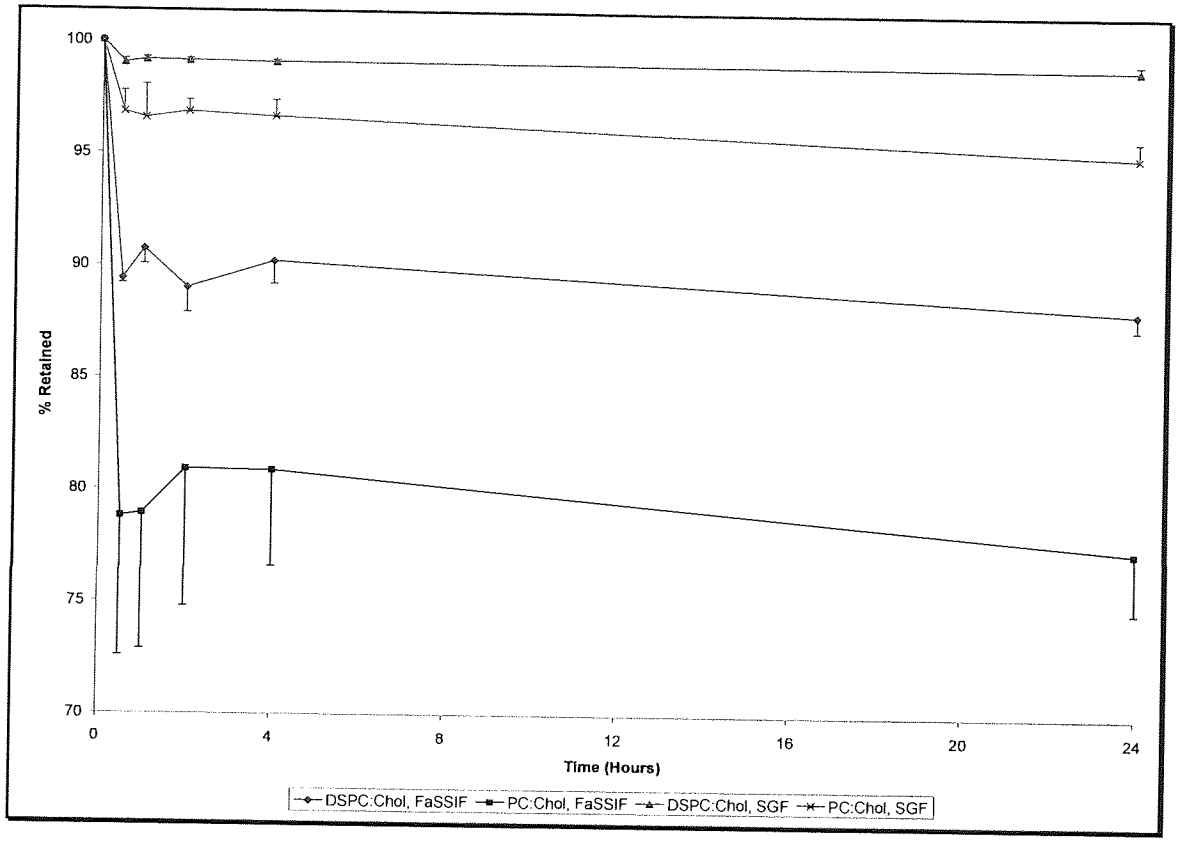

Figure 3.10 – Percentage of MP retained versus time from different liposome formulations into simulated gastro-intestinal fluids (n = 3).

Previous studies have shown that release of insulin (Iwanaga *et al.*, 1997), ovalbumin (Minato *et al.*, 2003; Alonso-Romanowski *et al.*, 2003) and glucose-6-phosphate (Alonso-Romanowski *et al.*, 2003) is limited in simulated gastric solution (pH 2.0); however, release is enhanced in simulated intestinal fluid. Indicating that liposome formulations are stable in the gastric lumen and that bile salts (including sodium taurocholate) interact with the liposomal membrane and burst them.

Time (Hours)	PC vs DSPC FaSSIF	PC vs DSPC SGF
0.5	Significant (p = 0.0414)	Significant (p = 0.0166)
1	Significant (p = 0.0285)	Significant (p = 0.0391)
2	n.s. (p = 0.0876)	Very Significant (p = 0.0017)
4	Significant (p = 0.0214)	Very Significant (p = 0.0045)
24	Very Significant (p = 0.0026)	Very Significant (p = 0.0010)

Table 3.8 – Statistical analysis of MP release into simulated gastric fluids – comparing PC:Chol (16:4) and DSPC:Chol (16:4).

These results may be due to the presence of DSPC in the liposome bilayer – DPSC is known to form more rigid bilayers (Senior and Gregoriadis, 1982a) compared to PC at physiological temperature due to DSPC's higher transition temperature (57°C compared to approximately 0°C for DSPC and PC respectively). The slow release of MP from DSPC:Cholesterol liposomes into SGF, even after 24-hours (1.0% compared to 4.9% from PC:Cholesterol (Figure 3.10); suggests DSPC:Cholesterol liposomes are more likely to retain a significantly higher proportion of incorporated drug on entering the intestine than PC:Cholesterol liposomes. The enhanced drug release for both formulations in the FaSSIF may be due to the presence of the phospholipase enzyme PLA_2 that is known to degrade phospholipids. However, it can be seen even under these conditions, that significant amounts of the MP are retained by the DSPC liposomes (88.1% of MP is retained after 24-hours). The results above show that the rigid bilayers produced by DSPC may be advantageous in the harsh environment of the GI tract fluids, allowing significantly more drug retention compared to liposomes containing egg PC that has a transition temperature of less than 37°C.

In contrast, to release studies using water as the receiver medium there is a significant difference in MP release from PC:Chol (16:4) and DSPC:Chol (16:4) liposomes. There was a significant difference in MP release into FaSSIF at all time points except for 2 hours (Table 3.8). DSPC:Chol (16:4) liposomes retained a significantly higher amount of MP compared to PC:Chol (16:4) (Figure 3.10 and Table 3.8). These observations were repeated with SGF as the receiver medium; however, DSPC:Chol (16:4) liposomes retained significantly more MP at all time points (Table 3.8). The higher transition temperature of DSPC producing liposomes with an increased bilayer rigidity had a significant effect on MP release into both FaSSIF and SGF.

Time (Hours)	FaSSIF vs SGF PC	FaSSIF vs SGF DSPC
0.5	Very Significant ($p = 0.0076$)	Extremely Significant ($p < 0.0001$)
1	Very Significant ($p = 0.0080$)	Extremely Significant ($p < 0.0001$)
2	Significant ($p = 0.0109$)	Extremely Significant ($p < 0.0001$)
4	Very Significant ($p = 0.0033$)	Extremely Significant ($p = 0.00010$
24	Extremely Significant ($p = 0.0004$)	Extremely Significant ($p < 0.0001$)

Table 3.9 – Statistical analysis of MP release into simulated gastric fluids from PC:Chol (16:4) and DSPC:Chol (16:4) – comparing the two simulated gastric fluids.

Not only did the lipid in the liposome formulation have an impact on MP release (Table 3.8), the receiver medium also had a significant effect (Table 3.9). MP release from PC:Chol (16:4) into SGF and FaSSIF was significantly different at all time points (Table 3.9). PC:Chol (16:4) retained a significantly higher amount of MP in SGF compared to FaSSIF (Figure 3.10). The same observations were observed for the DSPC:Chol (16:4) liposome formulation (Table 3.9) where MP was retained at a significantly higher amount when SGF was the receiver medium opposed to FaSSIF (Figure 3.10 and Table 3.9).

Release studies also suggest that the MP, with its small critical volume (Table 3.1) is more strongly incorporated within the lipid bilayer. Once again, we see that these results are in contrast to the LogP values for MP and BP (1.85 and 3.44 respectively); (Table 3.1) – this suggests that BP would favour the lipophilic bilayer of liposomes compared to the aqueous medium.

3.3.5 Model Drug Transport – The Influence of Liposome Incorporation

As an additional comparison, transport of MP and BP (1 mg) across an artificial membrane was measured. MP and BP were chosen because MP has the lowest LogP value and BP the highest LogP value. MP and BP (1 mg) were incorporated into PC:Cholesterol (16:4) liposomes. The percentage incorporation was measured and a solution containing the same amount of MP and BP was made – this was known as free MP or free BP and the liposome formulations referred to as incorporated MP and incorporated BP. Refer to Section 2.4.8 and Section 3.2 for details of the methodology used; briefly 1 ml of the liposome or free MP / BP solution was placed in a dialysis bag and then placed into 100 ml of water (pre-warmed to 37°C), at time intervals aliquots (1 ml) were taken and the amount of hydroxybenzoate determined. The data obtained is presented in Figure 3.11.

Model transport studies were seen to reflect drug release profiles from the liposome bilayers (Figure 3.8 – Figure 3.10). An equilibrium was established after 1 – 2 hours, followed by a steady rate of transport (Figure 3.11). It was predicted that MP and BP incorporated into PC:Chol, (16:4) liposomes would be transported at much slower rate compared to free MP and free BP because incorporated MP and BP would have to be released from the liposome formulation and then diffuse across the dialysis membrane; whereas free MP and free BP only have to diffuse across the dialysis membrane to be transported. As predicted free MP and free BP were transported at a faster rate compared to incorporated MP and BP (Figure 3.11).

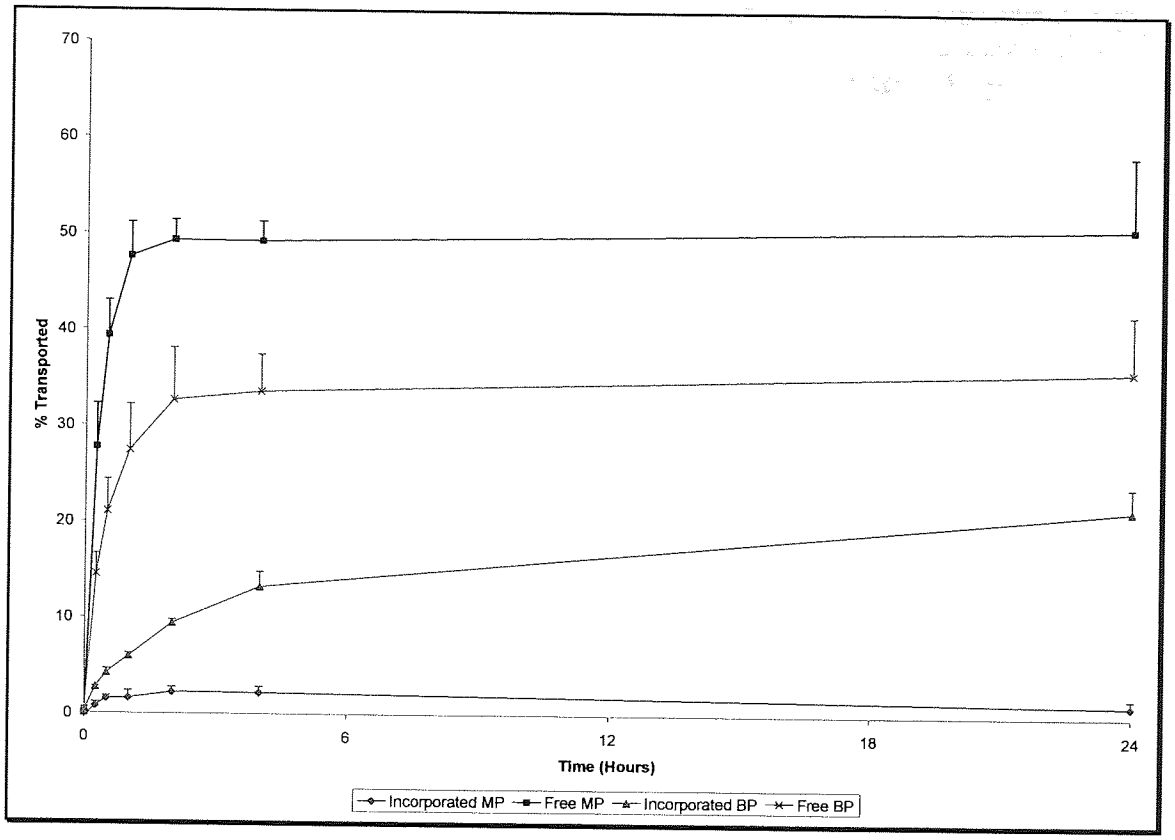

Figure 3.11 – Model transport studies of hydroxybenzoates (n = 3).

Free MP and free BP transported at a significantly faster rate at all time points compared to the equivalent liposome formulation (Table 3.10). It was expected that free MP would be transported at a faster rate than free BP because MP has a much higher aqueous solubility (Table 3.1). The increased transportation rate of free MP compared to free BP, continued throughout the 24-hour time period. Free MP was transported at a significantly higher rate compared to free BP at all time points, except for 24 hours (Figure 3.11 and Table 3.11). In contrast, incorporated MP was transported at a significantly slower rate compared to incorporated BP throughout the duration of the experiment, even though MP has a higher aqueous solubility (Figure 3.11 and Table 3.11). A slower MP transport was expected because MP was retained at higher amounts than BP in the release studies (Figure 3.8 and Figure 3.9); therefore if MP is released from liposomes at a slower rate compared to BP, then transport across the dialysis membrane will also be less. After 4-hours 2.2 per cent of MP had been transported compared to 13.2 percent for BP.

Time (Hours)	Free MP vs Incorporated MP	Free BP vs Incorporated BP
0.25	Extremely Significant ($p = 0.0005$)	Extremely Significant ($p = 0.0007$)
0.5	Extremely Significant ($p < 0.0001$)	Extremely Significant ($p = 0.0005$)
1	Extremely Significant ($p < 0.0001$)	Very Significant ($p = 0.0015$)
2	Extremely Significant ($p < 0.0001$)	Very Significant ($p = 0.0018$)
4	Extremely Significant ($p < 0.0001$)	Very Significant ($p = 0.0011$)
24	Extremely Significant ($p = 0.0004$)	Significant ($p = 0.0178$)

Table 3.10 – Statistical comparison of incorporated MP / BP and free MP / BP transport.

Time (Hours)	Free MP vs Free BP	Incorporated MP vs Incorporated BP
0.25	Significant ($p = 0.0102$)	Very Significant ($p = 0.0013$)
0.5	Very Significant ($p = 0.0026$)	Extremely Significant ($p = 0.0009$)
1	Very Significant ($p = 0.0041$)	Extremely Significant ($p = 0.0009$)
2	Very Significant ($p = 0.0076$)	Extremely Significant ($p < 0.0001$)
4	Very Significant ($p = 0.0035$)	Extremely Significant ($p = 0.0004$)
24	Not Significant ($p = 0.0561$)	Extremely Significant ($p = 0.0002$)

Table 3.11 – Statistical comparison of incorporated MP and incorporated BP; and free MP and free BP.

In parallel to the release studies, incorporated MP displayed a slower rate of transport compared to BP (Figure 3.11). Due to the intermediate LogP value 1.85, it would be expected that MP would have equilibrated between the bilayer, the dialysis membrane, the aqueous compartments of the liposomes, and the aqueous receiver phase.

However, in both cases, transport of incorporated hydroxybenzoate was reduced substantially compared to that of the free form, suggesting incorporation of the hydroxybenzoate in liposomes decreases hydroxybenzoate transport. These results highlight the dramatic effect that formulation and excipients can have on a drug's physico-chemical and biological activities.

Hydroxybenzoate transport was measured from MLVs; however, to verify if the burst effect happened causing the high hydroxybenzoate transport (1 – 2 hours; Figure 3.11) the transport study needs to be repeated using LUVs or SUVs. If the burst effect occurs all of the incorporated hydroxybenzoate will be transported from LUVs or SUVs because they only have a single bilayer.

3.3.6 Interdigitation

Incorporation of hydroxybenzoates into the PC:Cholesterol (16:0, 16:4, 16:8 and 16:16 μM) and DSPC:Cholesterol (16:4 μM) may have induced interdigitation in the liposomal bilayers. The presence of compounds in the lipid bilayer can alter the structure of the bilayer and induce the formation of an interdigitated bilayer. An interdigitated bilayer forms when the terminal methyl groups of the alkyl chains extend beyond the bilayer mid-plane, effectively interpenetrating into the opposing monolayer (Komatsu and Okada, 1995a). When small amphiphilic molecules are located in the interfacial region of the gel state liposomes, they anchor to the interface by virtue of their polar moiety, with the non-polar part of the molecule intercalating between the rigid alkyl chains. In the case of short amphiphilic molecules whose non-polar moieties are not as long as the hydrocarbon chains, this would potentially cause voids between chains in the bilayer interior. Since the energy of formation of holes in hydrocarbons is extremely large, the chains must

eliminate the voids. This can be achieved by the bilayer interdigitating. The lowest energy phase is the interdigitated phase (Komatsu and Okada, 1995b; Hata *et al.*, 2000a). The interdigitated gel phase has been demonstrated for saturated symmetrical and asymmetrical phosphatidylcholines (PCs) (Bondar *et al.*, 1998). It was originally thought that interdigitation only occurs above the transition temperature of phospholipids *i.e.* in the gel phase; however, interdigitation can occur above and below the phase transition temperature although, interdigitation of lipids exists mostly in the gel phase (Wang *et al.*, 1993; Hao *et al.*, 2000). Interdigitation is lipid specific, vinblastine induced interdigitation with DPPC but did not with shorter (DMPC) or longer (DSPC) alkyl chain lengths (Kyrikou *et al.*, 2004).

Many compounds have the ability to induce the interdigitated phase of PCs, these include glycerol (Boggs and Rangaraj 1985), ethylene glycol (Li and Kam, 1997), benzyl alcohol, chlorpromazine (Nerdal *et al.*, 2000), tetracaine (Maruyama *et al.*, 1997a; Hata *et al.*, 2000a), and thiocyanate ion (Komatsu and Okada, 1997; Bondar *et al.*, 1998; Tran *et al.*, 2004).

Experimental techniques used to verify interdigitated membranes include high sensitivity DSC, X-ray diffraction, neutron diffraction, electron spin resonance, atomic force microscopy, fluorescence studies (DPH fluorescence quenching method and the pyrene-PC fluorescence method) (McIntosh *et al.*, 1983; Bondar *et al.*, 1998; Bondar and Rowe, 1998; Nerdal *et al.*, 2000). DSC has been used extensively in studies of the interdigitated phase, DSC is a powerful tool for investigating the thermotropic phase transitions of a phospholipid bilayer. Indicators of interdigitation include the disappearance of the pre-transition and the biphasic effect (Tran *et al.*, 2004). Hysteresis, *i.e.* a difference in the main phase transition temperature between the heating and cooling scans, is a reliable indicator of the presence of interdigitation in the gel phase (Bondar and Rowe, 1998). The main transition temperature of lipids decreased with increasing concentrations of chlorpromazine (Nerdal *et al.*, 2000) and tetracaine hydrochloride (Maruyama *et al.*, 1997b; Hata *et al.*, 2000a; Hata *et al.*, 2000b), a broader transition peak was also noted.

In agreement with these findings the presence of hydroxybenzoates in DSPC:Cholesterol (16:4 μM) reduced the transition temperature (Table 3.3). Further DSC scans need to be performed, these include using different amounts of cholesterol and hydroxybenzoates in the formulation. All of the DSC scans need to be repeated using PC as the phospholipid in replacement of DSPC. In addition, to measuring heating scans, cooling scans need to be measured to determine if there is hysteresis, as it good indicator of interdigitation. The additional scans will provide more insight as to whether hydroxybenzoates induce interdigitation.

In the first step of the phase change process to the interdigitated state, dynamic changes in liposomal structure would be induced, for example, in the phase transition from normal bilayer to interdigitated structures changes in liposomal size and surface area (and as a result, inner volume) become apparent (Komatsu and Okada, 1997). The interdigitated membrane is characterised by a thinner structure and more rigid hydrocarbon regions in the layer than its non-interdigitated counter part (Komatsu and Okada, 1995a). Bilayer thickness of DPPC bilayers was measured in the presence and absence of chlorpromazine. In the presence of chlorpromazine the bilayer thickness measured 30 Å, which is about 20 Å smaller than two fully-extended molecules (McIntosh *et al.*, 1983). The size of DPPC liposomes was measured in the presence of ethanol. DPPC vesicle size was approximately 100 nm, vesicle size increased to a mean diameter of 1300-2300 nm in the fully interdigitated gel state. Thus, it appears that the ethanol-induced lipid interdigitation results in an increase in vesicle size. However, the increase in vesicle size at high ethanol concentrations (1.6 M) is due to vesicle aggregation, rather than vesicle fusion because the mean diameter of the vesicle returned to its original size (100 nm) following sonication (Zeng *et al.*, 1993).

Incorporation of hydroxybenzoates reduced liposome size for PC:Cholesterol, 16:0 μM (Table 3.4). Moreover, in agreement with interdigitation not changing liposome size, incorporation of hydroxybenzoates into PC:Cholesterol, 16:4, 16:8 and 16:16 μM did not change liposome size to the same extent as 16:0 μM (Table 3.4). Liposome size was measured at 20°C; therefore, PC was above the transition temperature (Table 2.1) in the liquid crystal phase (Figure 3.5). As

stated previously interdigitation can occur in both the gel phase and liquid crystal phase, however, interdigitation mostly occurs in the gel phase. Consequently, the size study needs to be repeated using a phospholipid with a higher transition temperature, *e.g.* DSPC, that will be in the gel phase at 20°C to determine if incorporation of hydroxybenzoates has the same affect on liposome size.

Interdigitated membranes have an increased permeability; carboxyfluorescein molecules leak through interdigitated bilayers approximately 100-times more rapidly compared to standard DPPC bilayers (Löbbeceke and Cevc, 1995). Determining if hydroxybenzoates induce interdigitation and if MP, EP, PP and BP have different abilities to induce interdigitation was beyond the scope of the work; however, if it was ascertained that the hydroxybenzoates were able to induce interdigitation under certain conditions, the influence interdigitation has on release of hydroxybenzoates from liposome formulations could be compared.

The presence of compounds in the bilayer cannot only induce interdigitation but they can also prevent interdigitation. Cholesterol can prevent the ethanol-induced interdigitated structure formation in DPPC liposomal membranes. Ethanol induces the interdigitated structure in DPPC membranes below 20 mol% cholesterol, whereas above 20 mol% cholesterol the interdigitated structure is prevented from forming (Komatsu and Okada, 1997). Cholesterol was used at 0, 4, 8, and 16 μM with PC for the incorporation and size studies (Section 3.3.1 and Section 3.3.3); therefore, the affect cholesterol has on the ability of hydroxybenzoates to induce interdigitation could be determined. Size and incorporation studies need to be repeated with DSPC in replacement of PC because it has a higher transition temperature; therefore, will be in the gel phase and the hydrocarbon chain of DSPC is longer than PC.

In order to verify if hydroxybenzoates induce interdigitation further experimental work must be performed, including direct assessment of interdigitation by X-ray diffraction and neutron diffraction studies and indirect assessment of interdigitation using DSC and electron spin resonance (Bondar *et al.*, 1998).

3.4 Conclusions

The research undertaken was primarily to investigate the effect of LogP values of methyl, ethyl, propyl and butyl hydroxybenzoates on incorporation into liposomes (PC:Cholesterol and DSPC:Cholesterol) and further analyse release and transport profiles to determine retention within liposomal bilayers, this initiated investigations into the effect of lipidic drug carrier systems on the transport of lipophilic drugs.

As stated previously the model drugs (methyl, ethyl, propyl and butyl hydroxybenzoates) used for this study were chosen because they have different octanol-water partition coefficients (Log P) whilst having similar molecular weights. Since this work began different drug delivery methods have been explored and hydroxybenzoates have been used as model drugs (Zhang and Kirsch 2003; Akomeah *et al.*, 2004).

Incorporation of hydroxybenzoates into liposomes produced a system with sustained release and transport profiles. This system has further potential to be modified to incorporate targeting groups to increase desired cell uptake (Hashida *et al.*, 2001; Meers, 2001; Clark *et al.*, 2002). The limitations of the system appear to be the structure and conformation (Figure 3.3) of the solute and the amount incorporated is probably dictated by how much the solutes presence within the bilayer disrupts the bilayer structure and hence integrity. MP is smaller than BP (Figure 3.1; Table 3.1) therefore; the presence of MP in the bilayer will cause less strain and disruption to the bilayer compared to the larger BP; consequently larger amounts of MP could be incorporated into the bilayer compared to BP (Table 3.2).

From the incorporation studies, it can be seen that MP can be incorporated into DSPC liposomes as effectively, if not more so, compared to PC-containing liposomes (Figure 3.4). Further, from the release studies, it can be determined that DSPC forms more stable liposomes compared to PC, resulting in increased incorporation and slower release into both water (Figure 3.8) and simulated fluids (Figure 3.10) at physiological temperatures. Consequently, it is proposed, that in future studies, DSPC be used in preference of PC.

From the series of experiments performed, it can be ascertained that several factors, including liposome composition and the solute size and structure are influential in the incorporation of solutes into liposomes. The size and structure of the solute plays an integral part in determining incorporation efficiency. If the structure of the solute disrupts the bilayer too much, the liposome integrity will be comprised and the liposome will collapse. LogP values will determine where the solute resides (Gulati *et al.*, 1998); however, from this study, the importance of solute size and structure has been observed. Size and structure affected all aspects investigated. The amount of hydroxybenzoate incorporated decreased with increasing R-group size. Decreased incorporation paralleled faster release and transport re-iterating the strain induced by the presence of hydroxybenzoates in the bilayer (see Section 3.3.4 and 3.3.5). It is predicted that MP 'fits' better into the liposomal bilayer than the EP, PP and BP; therefore, release and consequently transport will be slower from liposomes with incorporated MP in their bilayers. This is because MP is situated in the bilayer; therefore if MP was released too rapidly, this would cause destabilisation of the liposomes leading to collapse.

Bilayer cholesterol content, drug conformation and LogP values can all influence the incorporation, release and transport of hydroxybenzoates loaded into the bilayer of liposomes; thus suggesting, that each factor must be considered when attempting to use these systems for lipophilic drug delivery.

Chapter 4

Characterisation of the Caco-2 Cell Line and Toxicity Studies

4. Characterisation of the Caco-2 Cell Line and Toxicity Studies

4.1 Introduction

The Caco-2 cell line was utilised for the studies presented here because they spontaneously differentiate like mature intestinal enterocytes (see Sections 1.6 and 1.7 for more detail). For the Caco-2 cell line to be used in subsequent studies presented in Chapters 5, 6, 7 and 8; it must first be determined if the liposome formulations are toxic towards the cell line. If the formulations are toxic the Caco-2 cell line cannot be used.

4.1.1 Drug Absorption and Excipients

In the pharmaceutical field, the drug for administration is never administered as a single entity – additives known as excipients are mixed with the drug and then administered. Excipients were previously discussed in Section 1.9. Excipients can be used to enhance the solubility of drugs because a drug must be dissolved before absorption is achievable. An increased solubility can be achieved by mixing the drug with excipients that will aid the dissolution of the drug or a delivery vehicle can be used to enhance solubility. Additionally, excipients are commonly used as absorption enhancers and to improve wetting properties, thus increasing drug delivery (Refer to Section 1.9). Therefore, if the absorption of a drug is being investigated, the effects that the excipients may have on the drug and the intended destination must also be investigated.

With an ever increasing number of new drugs displaying limited water-solubility the goal is to develop excipients that aid delivery of drugs, aid drug dissolution and enhance absorption of the drugs. A number of excipients that have been assessed using the Caco-2 cell model to try and improve drug transport of poorly soluble compounds. These include povidone, pluronic F68, gelucir, propylene glycol / Tween 80 and PEG 300 (polyethylene glycol) (Saha and Kou, 2000).

In the work presented here the potential use of liposomes as excipients and drug carriers was investigated.

4.1.1.1 Liposomes as Drug Delivery Vehicles Used to Improve Solubility

One technique employed to improve solubility and thus get the desired drug to its intended destination is *via* encapsulation of the drug in a delivery vehicle. For example, camptothecin, a potent cytotoxic alkaloid showing anticancer activity, and its analogues have limited water-solubility and display a number of toxic effects (Slichenmyer *et al.*, 1993; Dancey and Eisenhauer, 1996; Cortesi *et al.*, 1997). To increase camptothecin solubility and reduce toxicity problems Cortesi *et al.*, (1997) formulated the drug within liposomes, micellar solutions and microemulsion. Solubility was increased between 5-fold and 23-fold and these drug delivery systems containing camptothecin displayed a similar or slightly enhanced effect compared to the free drug, thus proposing a promising starting point for future experimental therapy (Cortesi *et al.*, 1997). Additionally, the phospholipids in the liposome composition may act as absorption enhancers increasing drug absorption (refer to Section 1.9).

Since the work presented within this thesis began, interest in this area has increased and numerous compounds have been investigated as potential excipients to improve solubility and increase absorption. These include non-ionic surfactants that have been shown to inhibit P-gp using Caco-2 cell monolayers (Rege *et al.*, 2002). Nonionic surfactants shown to inhibit efflux transporters include Tweens, Spans, Cremphors, Pluronic block copolymers and vitamin E TPGS (D-alpha-tocopheryl polyethylene glycol 1000 succinate) (Rege *el al.*, 2002). Takahashi *et al.*, (2003), compared the effects of excipients on 3-day cultured Caco-2 cells compared to the more traditional 21-day cultured Caco-2 cells. They concluded that, even at low concentrations, cell viability was reduced in the presence of propylene glycol, hydroxypropyl-β-cyclodextrin, PEG 400 and Tween 80; and concluded that the 3-day cultured Caco-2 cells could not be used to determine the effects of the chosen excipients – cell viability was maintained at 21-day cultured Caco-2 cells. Takahashi *et al.*, (2003), chose propranolol, nadolol and FITC-dextran as their test drugs, they concluded that transport did not differ from the control in the

presence of propylene glycol, Tween 80, PEG 400, hydroxypropyl-β-cyclodextrin and Tween 80 + PEG 400; therefore, these excipients can be utilised along with the 21-day cultured Caco-2 cell monolayers to estimate human intestinal absorption – excipients must be chosen dependent on the physicochemical properties of chosen compounds to be tested. Intracellular accumulation and transport kinetics of epirubicin were investigated using Caco-2 cells and everted gut sacs of rat jejunum and ileum in the presence of Span 80, Brij 30, Tween 20, Tween 80, Myrj 52 and sodium lauryl sulphate (Lo, 2003). All excipients tested increased epirubicin uptake and transepithelial flux in Caco-2 cells and increased epirubicin absorption in rats. Epirubicin efflux was reduced in the presence of Tween 20, Tween 80, Myrj 52 and Brij 30 indicating that in addition to changes in membrane permeability P-gp was inhibited to some degree in the presence of the excipients (Lo, 2003).

4.1.2 Objective

The objective of this chapter was to validate the use of the Caco-2 cell line for study with liposome formulations to be tested in later Chapters. Confluent Caco-2 cell monolayer formation was verified using TEER measurements and mannitol flux. Toxicity assessments of the liposome formulations towards the Caco-2 cell line were determined using the MTT assay and crystal violet assay to verify that the Caco-2 cell line and chosen liposome formulations can be used for subsequent investigations.

4.2 Material and Methods

The general material and methods were previously described in Chapter 2.

4.2.1 Monolayer Formation

The formation of confluent cell monolayers is vital for experimental work. The progression of monolayer development is easily monitored when using Transwell inserts; transepithelial electrical resistance (TEERs) measurements are taken. TEERs were measured using chopstick electrodes to determine and monitor monolayer integrity. The TEER for the blank inserts was

115 ± 6 Ω, and this value was subtracted from all measured TEER values before they were multiplied by the surface area of the inserts (1 cm^2). TEERs were calculated using Equation 4.1.

$$TEER = [(Average\ Measured\ TEER) - (115 \pm 6)] x\ 1\ \Omega.cm^2$$

Equation 4.1 – TEER calculation (area of Transwell = 1 cm^2).

In addition, the flux of a paracellular marker, for example mannitol or fluorescein, can be measured. Visual assessment using a microscope (x 100 magnification) and a cell count is used for 24-well plates.

4.2.2 Toxicity Assays

Excipients used in the liposome formulations must be assessed for toxicity if they are to be used in further studies with the Caco-2 cell line. Two methods were used the MTT assay and crystal violet assay (see Section 2.3.10 and 2.3.11 for complete details).

MTT toxicity assay and the crystal violet assay were chosen to determine toxicity towards Caco-2 cells. The primary assay used was the MTT assay, which has previously been used with Caco-2 cells. Ekmekcioglu *et al.*, (1999), successfully employed this assay to determine toxic effects of beverages including fresh orange juice, a cola drink and several different types of tea towards Caco-2 cells. The crystal violet assay was used here as a visual guide to toxicity; however, the crystal violet assay has another step in the procedure – following the drying of the crystal violet, the crystal violet is solubilised, lysing the cells and the absorbance measured at 570 nm; from this cell number can be calculated (Waldenmaier *et al.*, 2003). The crystal violet assay has been employed with the Caco-2 cell model. Wolter *et al.*, (2001), used the crystal violet assay to determine the effects of resveratrol on cell growth. This assay has also been used with a number of different cell lines including Caco-2 cells (Wolter *et al.*, 2002) human Tenon fibroblasts (Hueber *et al.*, 2002) and macrophages (Harhaji *et al.*, 2004).

4.2.2.1 MTT Assay

The MTT (3[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay is a measurement of viable cells using a colorimetric assay. MTT is a pale yellow tetrazolium salt and when cleaved forms a dark blue coloured formazan. MTT is cleaved by the mitochondrial enzyme succinate-dehydrogenase to produce the formazan. Only viable cells have active mitochondrial enzymes; therefore, the amount of formazan produced is directly proportional to the number of living, metabolically active cells (Mosmann, 1983). Denizot and Lang (1986) proposed modifications to the original protocol of Mosmann (1983); modifications included the absence of serum and phenol red due to their interference in protein precipitation and high background absorbance readings.

The MTT assay was used to measure toxicity of liposomal formulations – PC:Cholesterol, DMPC:Cholesterol and DSPC:Cholesterol (16:0, 16:4, 16:8 and 16:16), 1 – 1000 μg; refer to Section 4.3.2. Caco-2 cells were grown on 24-well plates and the liposomal formulations were applied to the Caco-2 cells 7-days post seeding. Liposomal formulations were in contact with the Caco-2 monolayers for 1 hour. The time period of 1 hour was chosen because uptake and transport experiments do not exceed 1 hour.

4.2.2.2 Crystal Violet Assay

The crystal violet assay was used as a visual identification of toxicity, crystal violet is a basic dye that binds to any negatively charged molecules in the cell *i.e.* DNA and proteins, and thus stains the cells purple. Therefore, cells killed through toxicity will be washed away and only the viable cells will remain adhered to the Transwell inserts, and thus stain. The crystal violet assay was used to further confirm and verify that the liposomal formulations are not toxic to Caco-2 cells. Caco-2 cells were seeded on to Transwell inserts; liposomal formulations (PC; PC:Cholesterol, 16:4; DSPC; DSPC:Cholesterol, 16:4; 500 μg) were applied 14 - 21 days post seeding. The formulations were in contact with the Caco-2 monolayers for 1 hour. Sodium azide (40 mM) and Triton-X (0.05 % v/v) were used as positive controls. Sodium azide is used as a stop solution for uptake and transport experiments at 15 mM (Section 2.3.4 and 2.3.5); however,

to ensure sodium azide was toxic towards the Caco-2 cell monolayers, 40 mM sodium azide was used.

4.3 Results and Discussion

4.3.1 Monolayer Development and Integrity

Monolayer development was measured using TEER measurement. The flux of mannitol was measured during the experiment to determine if monolayer integrity was maintained throughout the experiment duration. Additionally, the flux of mannitol was measured through empty Transwell inserts in order to determine the background resistance.

Transepithelial electrical resistance (TEERs) were used to monitor cell monolayer development and ensure Caco-2 cell monolayers were ready for experimental use. For experimental use Caco-2 cell monolayers were only used if the measured TEER was greater than 250 Ω cm^2.

4.3.1.1 Transepithelial Resistance (TEERs)

As stated previously, TEERs are a measure of monolayer integrity, thus TEER values increases with increasing age and development of a monolayer. TEER values greater than 250 Ω cm^2 and within in the range 250 – 600 Ω cm^2 were accepted for experimental use in agreement with values found in the literature (Walter and Kissel, 1995; Artursson *et al.*, 1993). Caco-2 cells were seeded onto Transwell inserts on day 0 and TEER values were measured approximately every 48-hours when the Caco-2 cells were fed. Progression and development of Caco-2 cell monolayer formation was measured using TEER values are depicted in Figure 4.1.

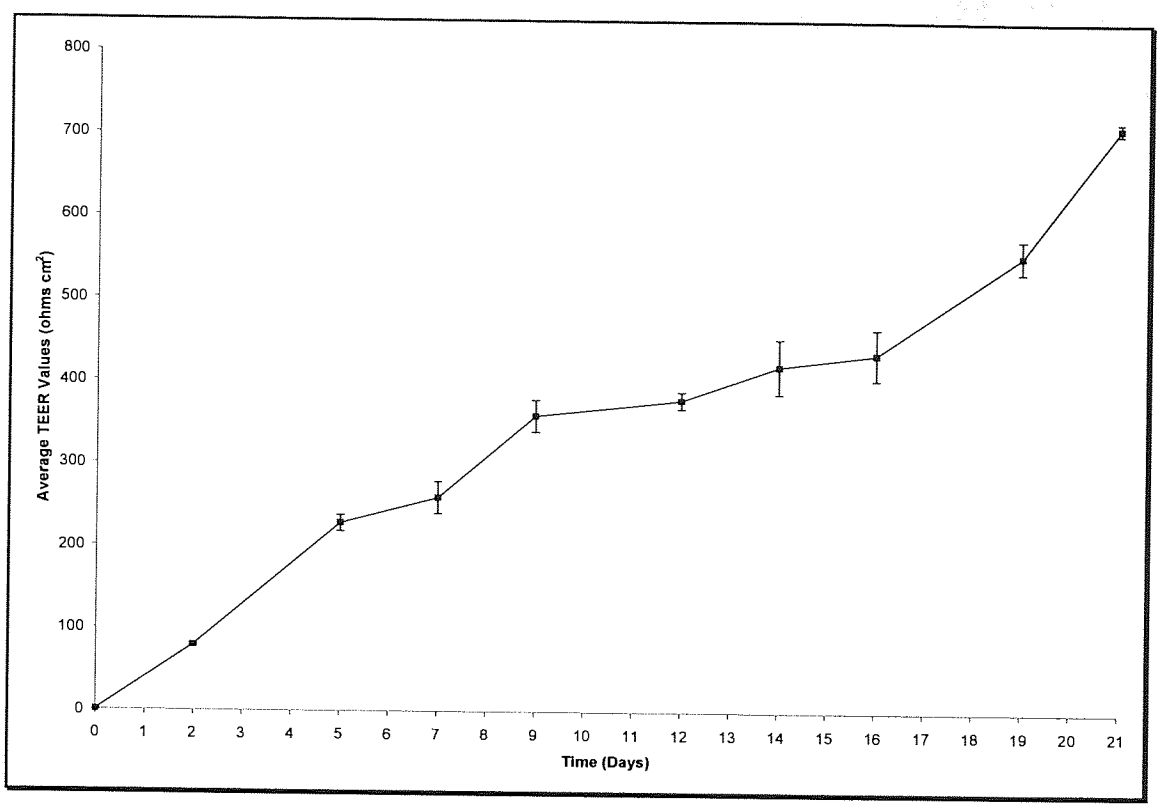

Figure 4.1 – Caco-2 cell monolayer formation.

Caco-2 Cell, passage 26-40, were seeded onto Transwell inserts. TEER measurements were taken using chopstick electrodes approximately every 48-hours to determine monolayer development. Data are mean values and standard deviation of results from four wells.

Caco-2 cell monolayers were used for experimental use between day 14 – 21, and from Figure 4.1 it can be seen that TEER values exceed 250 Ω cm^2 – at day 14 the TEER value is 374.1 Ω cm^2 and fits within the experimental range 250 – 600 Ω cm^2 (Walter and Kissel, 1995; Artursson *et al.*, 1993).

4.3.1.2 Mannitol Flux

Mannitol has been used extensively as a marker for paracellular transport (Artursson, 1990; Artursson, 1991; Boulenc *et al.*, 1993; Caro *et al.*, 1995). As stated previously, mannitol flux is measured as a determinant of monolayer integrity and a flux of < 1 % is accepted over a 60-minute period; therefore, mannitol flux through Transwell membranes was determined.

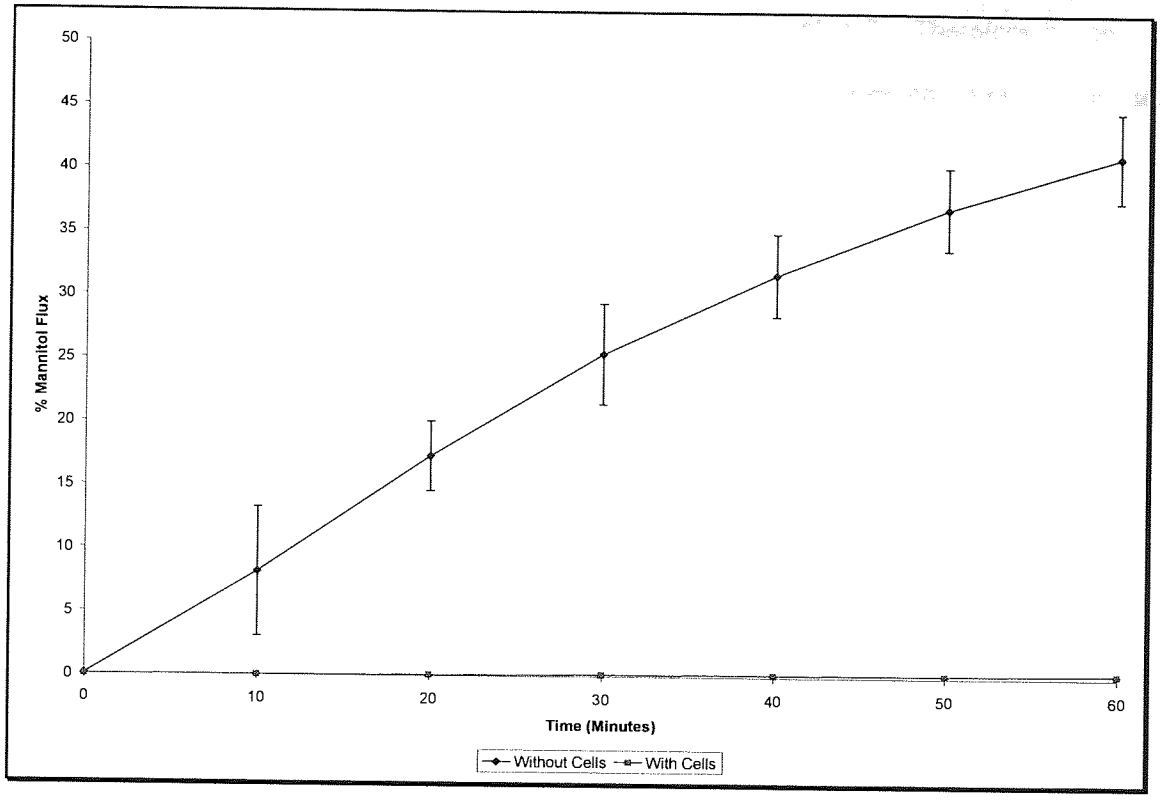

Figure 4.2 – Apical-to-basolateral flux of D-[^{14}C]-Mannitol (8 μM) through Transwell inserts (n = 4) in the absence and presence of a Caco-2 cell monolayer (passage 26-40, used 14-21 days post seeding).

Prior to experiments commencing with test compounds mannitol transport was measured in the presence and absence of a Caco-2 cell monolayer to verify that the Transwell membrane did not impede transport mannitol transport (apical to basolateral) (Figure 4.2). The presence of the Caco-2 cell monolayer significantly ($p < 0.0001$; unpaired t-test) increased TEER measurements from 115 ± 6 (without cells) to 619 ± 37 (with cells). The presence of cells significantly (two-tailed unpaired t-test) reduced mannitol flux at all time points (Figure 4.2). The average apparent permeability for mannitol in the absence of a Caco-2 cell monolayer was P_{app} 7.91 x 10^{-4} ± 9.94 x 10^{-5} cm s^{-1} compared to P_{app} 9.88 x 10^{-7}± 7.48 x 10^{-8} cm s^{-1} in the presence of a Caco-2 cell monolayer; the difference in apparent permeabilities is significantly different, $p < 0.0001$ (two-tailed unpaired t-test).

A mannitol flux less than 1 % over a 60-minute time period is experimentally acceptable because this indicates a well-developed Caco-2 cell monolayer with intact tight junctions. At 60 minutes percentage mannitol flux was only 0.28 ± 0.03 per cent in the presence of a Caco-2 cell

monolayer compared to 41.2 ± 3.5 per cent in the absence of cells. Therefore it can be concluded that the Transwell membrane, as would be expected, does not impede mannitol transport and this type of Transwell can be used for further studies.

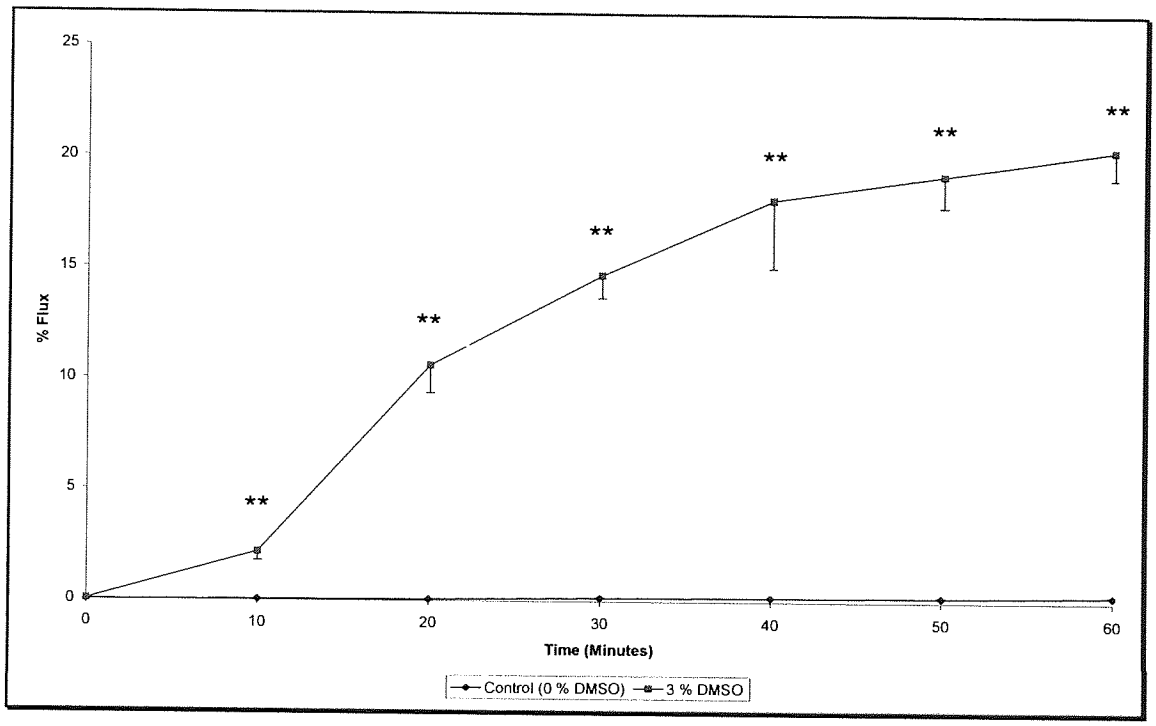

Figure 4.3 – The effect of 3% (v/v) DMSO on apical-to-basolateral flux of D-[^{14}C]-Mannitol (8 μM) across Caco-2 cells. Caco-2 cells passage 26-40, were used 14-21 days post seeding, flux was measured at 37°C. Data are mean values and standard deviation of results from four inserts. ** denotes an extremely significant difference compared to the control, two-tailed unpaired t-test.

Mannitol flux (*via* the paracellular route) is commonly measured to determine monolayer integrity throughout the duration of experiments and therefore to determine if compounds have a detrimental effect on Caco-2 cell monolayers (Meaney and O'Driscoll, 2000). Compounds that are toxic to Caco-2 cells disrupt the Caco-2 cell monolayer causing an increase in mannitol flux. DMSO is an organic solvent used to aid dissolution of numerous compounds. Being an organic solvent means that DMSO may be toxic to cells and therefore, is commonly used at < 1% v/v to aid dissolution (Yamashita *et al.*, 2000; Ingels *et al.*, 2002). DMSO is toxic towards cells if used at greater than 1% v/v consequently transport media (pH 7.4) containing 3 % v/v DMSO was used to confirm that toxic compounds increase mannitol flux; the control used was 0 % DMSO *i.e.* only transport media (pH 7.4) (Figure 4.3). The presence of 3 % DMSO significantly ($p < 0.0001$) increased P_{app} compared to the control (0 % DMSO).

It can be concluded from Figure 4.3, 3 % v/v DMSO is toxic to cells resulting in a significant increase in mannitol flux compared to 0 % DMSO. At 30 minutes mannitol flux increased to 14.7 ± 1.0 per cent compared to 0.16 ± 0.02 per cent for the control (0 % DMSO). There is a slight lag during the first ten minutes of experiment for 3 % v/v, followed by a faster rate of mannitol flux. The lag occurred because DMSO toxicity is not instant and during the first ten minutes of the experiment cell damage is in the early stages and after ten minutes cell damage has occurred and continues to progress throughout the remainder of the experiment, resulting in a faster rate of mannitol flux. This is in agreement with previous findings that DMSO decreases TEER values; thus, compromising Caco-2 cell monolayer integrity (Yamashita *et al.*, 2000). Consequently, DMSO is commonly used at ≤1 % (Yamashita *et al.*, 2000; Ingels *et al.*, 2002).

4.3.2 MTT Assay

The MTT assay was used to verify toxicity of liposomal formulations towards Caco-2 cell monolayers. Controls used included cell culture media, transport media (pH 7.4) and mannitol (0.01 % w/v) as negative controls because they are known not cause any detrimental effects towards Caco-2 cells. Conversely, the positive controls chosen are known to cause damage to Caco-2 cells, positive controls used were EDTA (0.5 % w/v) and sterile ddH_2O. Mannitol and EDTA were dissolved in transport media (pH 7.4), and the sterile water was a total replacement of transport media (pH 7.4).

Referring to Table 4.1 it can be seen that negative controls – cell-culture medium and transport medium, pH 7.4 do not significantly reduce cell viability; however, the positive controls do significantly reduce cell viability. Mannitol (0.01 % w/v) was used as an additional negative control, mannitol did not significantly reduce cell viability, which was maintained at 98.5 ± 1.1 %. The positive controls, EDTA and water significantly ($p < 0.01$) reduced cell viability compared to the control using ANOVA (Dunnett's multiple comparison test) analysis. It can be concluded that the MTT assay controls are satisfactory; therefore, all compounds tested should fall in between the two sets of controls.

Condition	% Cell Viability ± SD	Statistical Significance (ANOVA)
Cell Culture Media	100 .0 ± 1.0	-
Transport Media (pH 7.4)	100.1 ± 4.3	n.s.
Mannitol	98.5 ± 1.1	n.s.
EDTA	20.6 ± 6.0	**
Water	20.0 ± 0.9	**

Table 4.1 – MTT assay controls – cell viability.

The MTT assay was used to determine the toxicity of cell culture media, transport media (pH 7.4), mannitol (0.01 % w/v), EDTA (0.5% w/v) and sterile water towards Caco-2 cells. Caco-2 cells were seeded onto plastic 24-well plates, passage 26-40 and used 7-days post seeding, n = 4. Cell culture media, transport media (pH 7.4), mannitol (0.01 % w/v), EDTA (0.5% w/v) and sterile water were incubated with Caco-2 cells for 1 hour at 37°C. Cell viability was calculated using the MTT assay. ** Denotes a significant ($p < 0.01$) difference compared to cell culture media; n.s. denotes not significant ($p > 0.05$) (ANOVA).

Condition	% Cell Viability ± SD	Statistical Significance (ANOVA)
Cell Culture Media	100 .0 ± 1.0	-
PC, 1 μg	94.1 ± 5.8	n.s.
PC, 10 μg	98.4 ± 7.1	n.s.
PC, 100 μg	94.3 ± 2.1	n.s.
PC, 1000 μg	90.2 ± 2.8	n.s.
PC:Chol (16:4) 1 μg	92.9 ± 0.7	n.s.
PC:Chol (16:4) 10 μg	95.4 ± 5.7	n.s.
PC:Chol (16:4) 100 μg	90.2 ± 5.8	n.s.
PC:Chol (16:4) 1000 μg	93.0 ± 3.8	n.s.
PC:Chol (16:8) 100 μg	97.9 ± 4.3	n.s.
PC:Chol (16:8) 1000 μg	94.8 ± 3.5	n.s.
PC:Chol (16:16) 100 μg	98.6 ± 1.8	n.s.
PC:Chol (16:16) 1000 μg	96.6 ± 1.9	n.s.

Table 4.2 – Cell viability of Caco-2 cells measured using the MTT assay following treatment with PC:Cholesterol (16:0, 16:4, 16:8, 16:16) liposomes.

n.s. Denotes not significant ($p > 0.05$).

The toxicity of PC:Cholesterol (16:0 – 16:16) was determined at a range of total lipid amounts, 1 – 1000 μg; refer to Table 4.2. Cell viability was maintained above 90.2 per cent in the presence of PC:Cholesterol formulations tested (Table 4.2).

Increasing the total amount of lipid used from 1 μg to 1000 μg did not have a detrimental effect on cell viability nor did increasing the cholesterol content from 0 to 16 μM. ANOVA (Dunnett's multiple comparison test) analysis confirmed that there was not a statistically significant decrease in cell viability compared to the cell culture media control. Therefore concluding that PC and PC:Cholesterol are suitable lipids and formulations to be used with the Caco-2 cell model.

Different lipids are used in liposome formulations because of different properties; one important property is the phase transition temperature of lipids, PC has a low phase transition temperature (< 0°C) whereas DMPC and DSPC have higher phase transition temperature (23°C and 57°C respectively). The toxicity of DMPC and DSPC were determined (Table 4.3).

Condition	% Cell Viability ± SD	Statistical Significance (ANOVA)
Cell Culture Media	100 .0 ± 1.0	-
DMPC, 1 μg	93.0 ± 5.4	n.s.
DMPC, 10 μg	94.3 ± 2.2	n.s.
DMPC, 100 μg	95.1 ± 5.0	n.s.
DMPC, 1000 μg	94.9 ± 4.5	n.s.
DMPC:Chol (16:4) 1 μg	98.3 ± 5.3	n.s.
DMPC:Chol (16:4) 10 μg	97.2 ± 3.4	n.s.
DMPC:Chol (16:4) 100 μg	93.0 ± 0.8	n.s.
DMPC:Chol (16:4) 1000 μg	98.8 ± 1.0	n.s.

Condition	% Cell Viability ± SD	Statistical Significance (ANOVA)
DMPC:Chol (16:8) 100 μg	97.1 ± 3.8	n.s.
DMPC:Chol (16:8) 1000 μg	95.9 ± 6.9	n.s.
DMPC:Chol (16:16) 100 μg	95.2 ± 4.5	n.s.
DMPC:Chol (16:16) 1000 μg	93.2 ± 5.5	n.s.
DSPC, 1 μg	96.7 ± 6.8	n.s.
DSPC, 10 μg	95.3 ± 2.3	n.s.
DSPC, 100 μg	95.9 ± 2.4	n.s.
DSPC, 1000 μg	93.4 ± 3.2	n.s.
DSPC:Chol (16:4) 1 μg	97.6 ± 5.3	n.s.
DSPC:Chol (16:4) 10 μg	94.1 ± 4.7	n.s.
DSPC:Chol (16:4) 100 μg	86.0 ± 1.6	**
DSPC:Chol (16:4) 1000 μg	93.5 ± 3.1	n.s.
DSPC:Chol (16:8) 100 μg	96.5 ± 1.8	n.s.
DSPC:Chol (16:8) 1000 μg	99.5 ± 6.1	n.s.
DSPC:Chol (16:16) 100 μg	96.2 ± 0.4	n.s.
DSPC:Chol (16:16) 1000 μg	95.7 ± 3.6	n.s.

Table 4.3 – Cell viability of Caco-2 cells measured using the MTT assay following treatment with DMPC:Cholesterol and DSPC:Cholesterol (16:0, 16:4, 16:8, 16:16) liposomes.

** Denotes a significant ($p < 0.01$) difference; n.s. Denotes not significant ($p > 0.05$).

Cell viability was maintained above 92.0 per cent in the presence of DMPC:Cholesterol formulations (Table 4.3). As observed with PC, increasing the total lipid content or increasing cholesterol content did not have an increased detrimental effect on cell viability (Table 4.3). Cell viability was not significantly decreased compared to cell culture media (ANOVA Dunnett's multiple comparison test analysis), thus DMPC and DMPC:Cholesterol are suitable formulations to be used with the Caco-2 cell model.

DSPC:Cholesterol (16:0, 16:4, 16:8 and 16:16) liposome formulations were not detrimental to cell viability. Cell viability was maintained above 93.4 per cent, excluding DSPC:Chol (16:4), 100 μg, this formulation reduced cell viability to 86.0 per cent. Cell viability was maintained when total lipid content and cholesterol content was increased (Table 4.3). ANOVA (Dunnett's multiple comparison test) analysis confirmed that there was not a statistically significant decrease in cell viability compared to the cell culture media control, except for DSPC:Chol (16:4), 100 μg, ($p < 0.01$). Although DSPC:Chol (16:4), 100 μg, significantly ($p < 0.01$) reduced cell viability compared to cell culture media, a cell viability of 86.0 per cent is not as low as known toxic compounds *e.g.* EDTA (20.6 per cent) (Table 4.1 and Table 4.3). The MTT assay is widely used with many different cell lines and because cell lines vary there are no published levels of accepted reductions in cell viability, consequently, positive controls (EDTA, sterile water) were used. The positive controls reduced Caco-2 cell viability to approximately 20 per cent (Table 4.1); this is in agreement with Ingels *et al.*, (2002) who determined that FeSSIF (fed state simulated intestinal fluid; pH 5) was toxic to Caco-2 cells – cell viability was reduced to 23.1 ± 1.6 %. All DSPC and DSPC:Cholesterol liposome formulations tested maintained Caco-2 cell viability (> 86.0 %) considerably higher than the positive controls; therefore, concluding that DSPC and DSPC:Cholesterol are suitable lipids and formulations to be used with the Caco-2 cell model.

The toxicity of the liposomal formulations measured using the MTT and crystal violet assay was expected to be limited because liposomes are generally considered to be essentially non-toxic, biodegradable and non-immunogenic and remarkably safe for pharmacuetical use (Parnham and Wetzig, 1993; Allen and Moase, 1996; Jovcic *et al.*, 1999; Kozubek *et al.*, 2000; Oku *et al.*, 2000). PC, DPPC and DMPC have been shown not be toxic towards cell lines (Jett and Alving, 1983; Mayhew *et al.*, 1987; Berrocal *et al.*, 2000). However, DMPC liposomes were better tolerated by human skin fibroblasts compared to PC liposomes (Berrocal *et al.*, 2000). Although liposomes are considered to be non-toxic a decline in the number of mature granulocytes, monocytes, and lymphocytes has been reported following administration of pure egg yolk phosphatidylcholine or soybean phosphatidylcholine to mice (Jovcic *et al.*, 1999). The addition of constituents to liposomes can alter toxicity; for example, positively-charged liposomes

displayed greater toxicity both *in vitro* and *in vivo* compared to neutral and negatively-charged liposomes (Olson *et al.*, 1982; Parnham and Wetzig, 1993). Generally, small liposomes were more toxic than large liposomes (Mayhew *et al.*, 1987).

Limited liposomal toxicity towards the Caco-2 cell line was also expected because there are commercially available liposome formulations. Available liposome formulations include Caelyx® (Doxil® in the US) and AmBisome®. Doxorubicin hydrochloride and amphotericin B are the active components of the liposome formulations, in both cases the entrapment in liposomes has reduced the toxic profile of the active drug without reducing activity (Baas *et al.*, 2000; Judson *et al.*, 2001; Hann and Prentice, 2001). Although these formulations are for i.v. administration and not oral delivery their use shows that liposomes are tolerated by the human body.

Caelyx® liposomes are composed of fully hydrogenated soybean phosphatidylcholine (HSPC), cholesterol, and N-(carbonyl-methoxy polyethylene glycol 2000)-1,2-distearoyl-*sn*-glycerol-3-phospho-ethanolamine sodium salt (MPEG-DSPE) (Amantea *et al.*, 1997). Greater than 90% of the drug is encapsulated in the liposomes which have a mean diameter of 100 nm (Amantea *et al.*, 1997). To improve circulation time and to protect the drug from the immune system, Caelyx® liposomes are pegylated (STEALTH®) (Caponigro *et al.*, 2000). Common side effects reported with Caelyx® therapy include reduced red blood cell count (anaemia), reduced white blood count (neutropenia), nausea and fatigue (Coukell and Spencer, 1997; Keller *et al.*, 2004).

The toxicity of PC, DMPC and DSPC was determined as individual lipids and with cholesterol in the formulation, thus the toxicity of cholesterol was determined as an individual component (Table 4.4; Figure 4.4).

Condition	% Cell Viability ± SD	Statistical Significance (ANOVA)
Cell Culture Media	100 .0 ± 1.0	-
Cholesterol, 1 μg	96.7 ± 3.2	n.s.
Cholesterol, 10 μg	82.5 ± 7.1	**
Cholesterol, 100 μg	54.5 ± 0.9	**
Cholesterol, 1000 μg	31.8 ± 3.3	**

Table 4.4 – Cell viability of Caco-2 cells measured using the MTT assay following treatment with cholesterol.
** Denotes a very significant ($p < 0.01$) difference; n.s. Denotes not significant ($p > 0.05$).

Unexpectedly, only cholesterol, 1μg did not significantly ($p > 0.05$) reduce cell viability (ANOVA, Dunnett's multiple comparison test analysis) compared to cell culture media (Table 4.4; Figure 4.4). Cholesterol, 10, 100 and 1000 μg significantly ($p < 0.01$) reduced cell viability (ANOVA). Cholesterol, 10 μg reduced cell viability to 82.5 per cent which is comparable to the reduction in cell viability observed for DSPC:Chol, 16:4, 100 μg (86.0 per cent; Table 4.3). The reduction in cell viability observed for cholesterol, 100 and 1000 μg was much higher than any lipid or liposome formulation tested (Table 4.2 – Table 4.3). Cholesterol is not water-soluble; therefore, cholesterol was dissolved in ethanol and it is probable that it is the ethanol having an impact on the toxicity level for both cholesterol 100 and 1000 μg. Therefore, the toxicity of ethanol towards Caco-2 cells was determined using the MTT assay. Caco-2 cell viability was significantly ($p < 0.01$; ANOVA) reduced in the presence of the equivalent amount of ethanol used for cholesterol 100 and 1000 μg (56.9 % ± 5.5 and 34.4 % ± 3.9 respectively). The reduction in cell viability in the presence of ethanol was comparable ($p = 0.4972$ and $p = 0.4278$ respectively, two-tailed t-test) to the reduction observed for cholesterol 100 and 1000 μg (Table 4.4) Furthermore, it has previously been shown that ethanol is damaging towards Caco-2 cells (Ma *et al.*, 1999; Banan *et al.*, 1999). Cell viability was not reduced in the presence of cholesterol in conjunction with other lipids *e.g.* PC compared to PC:Chol, DMPC compared to DMPC:Chol, or DSPC compared to DSPC:Chol. Consequently it is the ethanol that caused the decrease in cell viability and not cholesterol (Table 4.4; Figure 4.4).

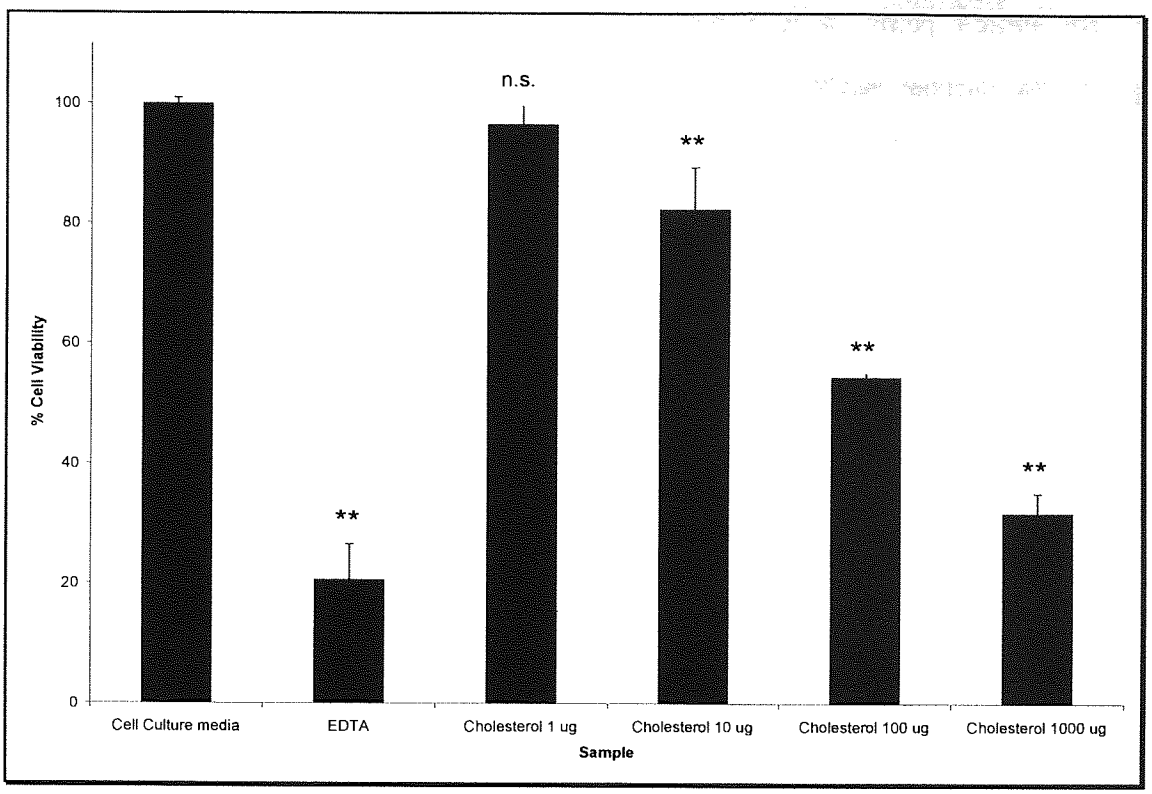

Figure 4.4 – The effect of cholesterol (1 μg, 10 μg, 100 μg and 1000 μg) on Caco-2 cell viability was measured using the MTT assay.

Caco-2 cells, passage 26-40, were seeded onto plastic 24-well plates and used 7-days post seeding. Cholesterol was incubated with the Caco-2 cells for 1 hour at 37°C. Data are mean values and standard deviation of results from four wells. ** Denotes a very significant ($p < 0.01$) difference compared to cell culture media; n.s. Denotes not significant ($p > 0.05$) (ANOVA).

The toxicity of simulated gastric fluids (FaSSIF and SGF) towards Caco-2 cells was measured (Table 4.5; Figure 4.5). The use of simulated gastric fluids would improve the Caco-2 cell model because it would be closer to human model compared to buffer based solutions (*e.g.* HBSS based solutions).

Condition	% Cell Viability ± SD	Statistical Significance (ANOVA)
Cell Culture Media	100 .0 ± 1.0	-
FaSSIF	100.7 ± 3.0	n.s.
SGF	23.1 ± 6.1	**

Table 4.5 – Cell viability of Caco-2 cells measured using the MTT assay following treatment with simulated gastric fluids – FaSSIF and SGF.

** Denotes a very significant ($p < 0.01$) difference; n.s. Denotes not significant ($p > 0.05$).

In agreement with previously reported investigations (Ingels *et al.*, 2002), FaSSIF did not decrease cell viability (Table 4.5; Figure 4.5); however, SGF dramatically reduced cell viability, under similar conditions (Figure 4.5) showing that SGF is toxic to Caco-2 cells and cannot be used in simulated uptake and transport investigations. SGF significantly ($p < 0.01$; ANOVA) reduced cell viability. Cell viability was reduced to almost the same as positive controls *e.g.* EDTA (Table 4.1).

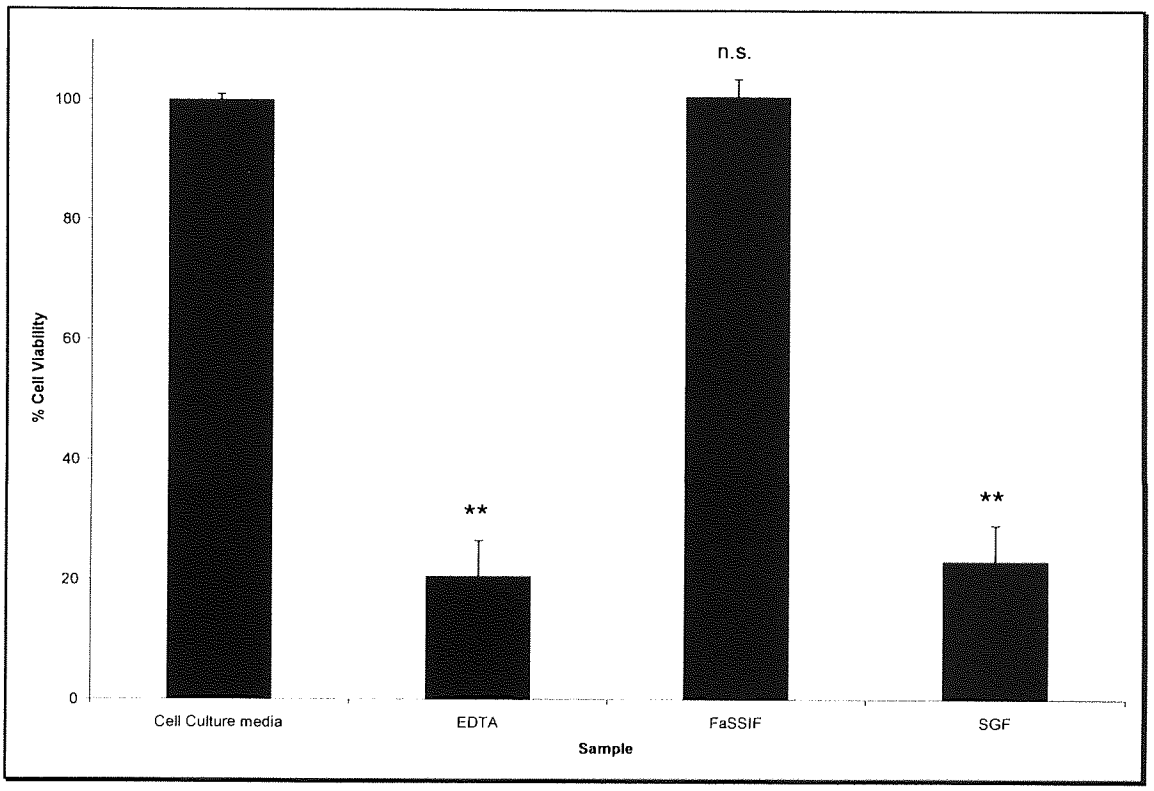

Figure 4.5 – The effect of simulated gastric fluids on Caco-2 cell viability was measured using the MTT assay.

Caco-2 cells, passage 26-40, were seeded onto plastic 24-well plates and used 7-days post seeding. Simulated gastric fluids – FaSSIF and SGF were incubated with the Caco-2 cells for 1 hour at 37°C. Data are mean values and standard deviation of results from four wells. ** Denotes a very significant ($p < 0.01$) difference compared to cell culture media; n.s. Denotes not significant ($p > 0.05$) (ANOVA).

The low pH of SGF (pH 2.0) is the probable cause of the cell damage observed. Ingels *et al.*, (2002) determined the toxicity of FaSSIF (pH 6.8) and FeSSIF (fed state simulated intestinal fluid; pH 5) towards Caco-2 cells and concluded that FaSSIF was not toxic, in contrast, FeSSIF with a much lower pH was toxic. The simulated gastric fluid data provided further verification that the MTT assay was functioning correctly as the data obtained in this study was supported by data obtained by Ingels *et al.*, 2002.

The MTT assay has been utilised to evaluate the toxicity of numerous HPC-*g*-$(POE)_y$-C_n co-polymers towards Caco-2 cells (Francis, *et al.*, 2003). Bromberg and Alakhov (2003), have also used the MTT assay to determine toxicity of microgels composed of cross-linked copolymers of poly(acrylic acid) towards Caco-2 cells. The MTT assay was employed together with the measurement of transepithelial resistance (TEER) and ^{14}C-Mannitol flux (Meaney and O'Driscol, 2000, Bromberg and Alakhov, 2003).

In addition to the MTT assay there are other methods of detecting toxicity including lactate dehydrogenase (LDH) activity and apoptosis assays. Apoptosis assays were not performed because apoptosis was beyond the scope of the thesis. For liposomes to initiate apoptosis they must be taken up by cells. The MLVs used here were too large to be taken up by the Caco-2 cells; however, SUVs and LUVs are smaller and need be used in replacement of MLVs for apoptosis assays. HUVEC and HL60 cells have both been shown to uptake SUVs composed of PC:Cholesterol and PS:Cholesterol (Papadimitriou and Antimisiaris, 2000).

4.3.3 Crystal Violet Assay

The crystal violet assay was chosen to visually confirm the results obtained from the MTT assay in Section 4.3.2; that liposome formulations are non-toxic to Caco-2 cells. The following formulations were tested:

- Transport Medium, pH 7.4 (negative control)
- 40 mM Sodium Azide (positive control)
- 0.05 % v/v Triton X-100 (positive control)
- PC, 500 μg
- PC:Chol, (16:4), 500 μg
- DSPC, 500 μg
- DSPC:Chol, (16:4), 500 μg

Transport medium, pH 7.4 was used as the negative control as it is the base solution used experimentally, therefore should not be toxic towards Caco-2 cells (Figure 4.6). Sodium azide, 40mM and Triton X-100, 0.05 % v/v were used as the positive controls as they are known to be toxic to cells.

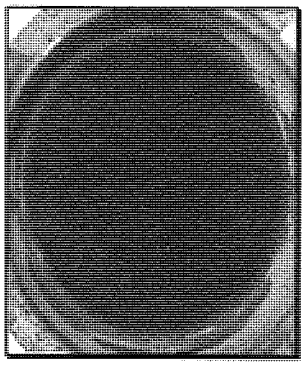

Figure 4.6 – Crystal violet negative control – transport media, pH 7.4.

Non-toxic compounds will not have a detrimental effect on the Caco-2 cell monolayer; therefore following treatment with crystal violet the monolayer should appear as a violet sheet with no holes in the monolayer. An intact monolayer can be observed in Figure 4.6; the Caco-2 cell monolayer has been exposed to transport media (pH 7.4). In contrast, if a substance is toxic towards Caco-2 cell monolayers, cells will lift off the supporting membrane resulting in holes and / or part of the cell monolayer lifting off the supporting membrane and folding over. Non-viable Caco-2 cells will lift off the supporting membrane and where this has occurred the supporting membrane will be very pale compared to the deep violet colour of viable cells. Damage caused to Caco-2 cells by toxic compounds can be observed in Figure 4.7 – Figure 4.10 showing the detrimental effect sodium azide (40 mM) and Triton X-100 (0.05 % v/v) had on Caco-2 cells. Damage caused to the Caco-2 included holes in the monolayer and areas of monolayer that are non-viable and as a result had lifted from the supporting membrane and folded over some of the remaining viable cells – this is observed as very dark purple patches primarily around the edge (Figure 4.7 and Figure 4.9).

Figure 4.11 – Figure 4.14 show Caco-2 monolayers following incubation with the liposome formulations. An intact monolayer is observed – a violet monolayer with no holes. The monolayers are intact therefore confirming that the liposome formulations tested are non-toxic towards Caco-2 cells.

Figure 4.7 – Crystal violet positive control – sodium azide (40 mM).

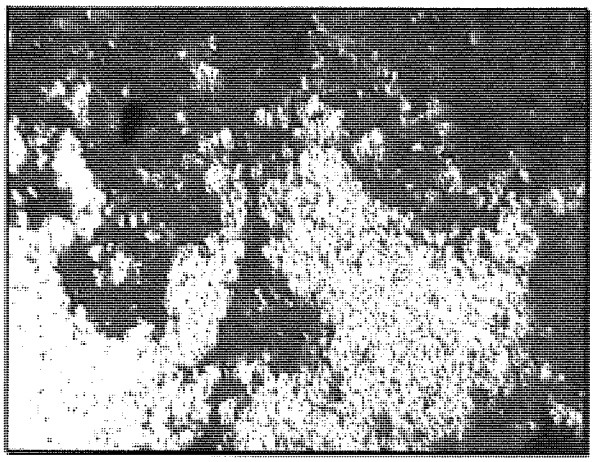

Figure 4.8 – Crystal violet positive control – sodium azide (40 mM) (x10).

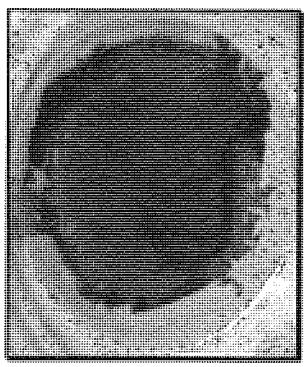

Figure 4.9 – Crystal violet positive control – Triton X-100 (0.05 % v/v).

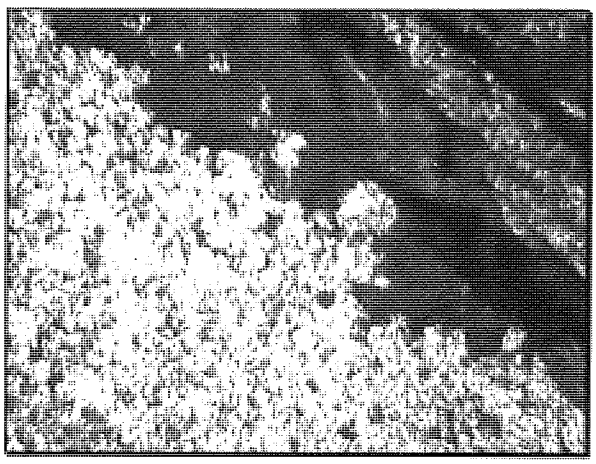

Figure 4.10 – Crystal violet positive control – Triton X-100 (0.05 % v/v) (x10).

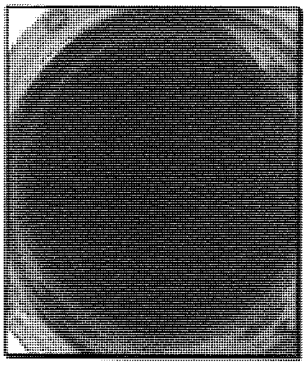

Figure 4.11 – Crystal violet – PC, 500 μg.

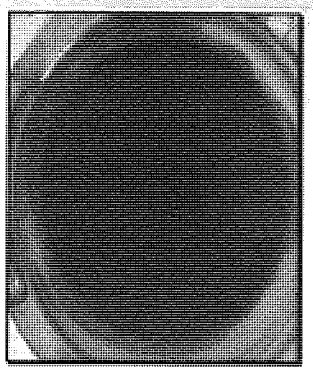

Figure 4.12 – Crystal violet – PC:Chol (16:4), 500 μg.

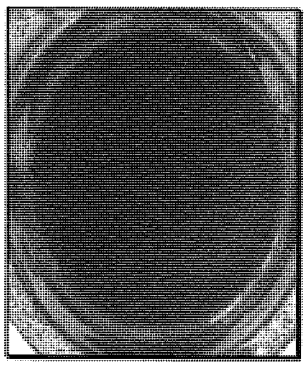

Figure 4.13 – Crystal violet – DSPC, 500 μg.

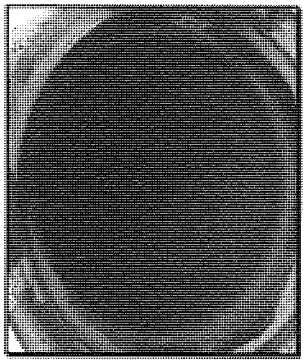

Figure 4.14 – Crystal violet – DSPC:Chol (16:4), 500 μg.

4.4 Conclusion

4.4.1 Monolayer Development and Integrity

Following the measurement of TEER values, it can be concluded that Caco-2 monolayers develop progressively over 21-days, depicting higher TEER values as the monolayer develops (Figure 4.1). On day 14 the TEER value was 374.1 Ω cm^2 exceeding the cut-off value of 250 Ω cm^2; thus, Caco-2 cell monolayers are established from day 14 and can be used experimentally between day 14 and day 21.

The Transwell membrane chosen does not impede the apical to basolateral flux of mannitol as can be seen from Figure 4.2; mannitol flux after the 60-minute time period is 42.0 ± 5.3 % exceeding the accepted 1 % mannitol flux. Thus the Transwell inserts used can be utilised for further experiments. The presence of a developed Caco-2 cell monolayer reduced mannitol flux to 0.28 ± 0.03 per cent which is below the accepted 1 per cent flux over 60 minutes (Figure 4.2).

4.4.2 Toxicity Assays

These investigations indicate that, in the presence of all liposome formulations tested (Table 4.1 – Table 4.4), with the exception of cholesterol alone (dissolved in ethanol), Caco-2 cell viability was maintained above 85 % relative to the control (range, 86.0 ± 1.6 % – 99.5 ± 6.1 %), suggesting that these formulations are non-toxic at the lipid concentrations tested, and can be used in experimental work involving Caco-2 cells.

One exception is cholesterol (Figure 4.4; Table 4.4), for cholesterol to be tested as an individual component, cholesterol had to be dissolved in ethanol resulting in a high concentration of ethanol in the test solution in order to maintain cholesterol in solution (Section 4.3.2). Ethanol is toxic to Caco-2 cells (Ma *et al.*, 1999; Banan *et al.*, 1999), therefore at high cholesterol amounts (100 and 1000 μg) it is probable that the toxicity displayed is due to the amount of ethanol present in the test solution and not the presence of cholesterol; this is due to cholesterol being a natural component of biological membranes and does not produce toxic effects when present with another lipid, *e.g.* PC:Cholesterol.

Moreover, these studies can be performed in the presence of FaSSIF, which had no significant effect on cell viability, allowing the *in vitro* model to closer resemble that of the GI environment (Figure 4.5; Table 4.5).

As can be seen from the photographs of the Transwell inserts (Figure 4.6– Figure 4.14), treated and then stained using crystal violet, that the control inserts and inserts treated with liposomal formulations all have an intact monolayer. In contrast, in the Transwell inserts treated with sodium azide (Figure 4.7 and Figure 4.8) and Triton X-100 (Figure 4.9 and Figure 4.10), the monolayer has been seriously compromised as can be seen by the holes in the monolayer. The magnified (x10) photo(s) of the Transwell inserts treated with sodium azide (Figure 4.8) and Triton X-100 (Figure 4.10) show the damage caused in a more detailed manner. This confirms

the conclusions from the MTT assay data; liposomal formulations are not toxic to Caco-2 cell monolayers and, therefore, can be employed for experimental use.

Chapter 5

Effects of Liposomal Formulations on the Paracellular Route

5. Effects of Liposomal Formulations on the Paracellular Route

5.1 Introduction

For a drug to reach its destination or target organ following oral administration, the drug must navigate the small intestine epithelial cell layer, as previously described (Section 1.3). Thus, the drug must pass through two lipid bilayers; firstly, the apical brush border facing the interior of the small intestine and secondly the basolateral membrane facing the blood vessels (Engvall and Lundahl, 2004). A number of pathways exist that a compound can utilise to traverse the epithelial membrane, including paracellular and transcellular (passive and carrier-mediated) transport (refer to Section 1.3). Physicochemical properties including solubility, lipophilicity and size determine the pathway utilised by molecules to traverse the epithelium. The effect of liposomal formulations on paracellular pathway is discussed within this chapter.

The paracellular route is defined as the passive movement of molecules *via* the tight junctions in the intercellular spaces (see Section 1.3.1). Only small hydrophilic molecules are allowed to pass between the cells and utilise the paracellular route (Hunter and Hirst, 1997). Tight junctions are not static; however, they do inflict a strict size limit for transport. Typically only molecules with a molecular weight less than 200 Da are capable of being transported *via* the paracellular transport (Hochman and Artursson, 1994). Paracellular transport through a healthy epithelium is slow and transport of molecules is minimal compared to other absorption mechanisms including transcellular absorption. Mannitol is commonly used to investigate the paracellular route this is because it is a small molecule – molecular weight of 180 Da and is hydrophilic (low lipophilicity).

Although paracellular and transcellular transport occur, the intestinal epithelium tightly regulates the passage of compounds, resulting in poor absorption of many compounds including peptides and hydrophilic molecules (Artursson, 1990; Sakai *et al.*, 1997; Dorkoosh *et al.*, 2002). Absorption enhancers are being researched to improve transport of drugs (refer to Section 1.9).

Herein, the effect that lipid formulations have on absorption mechanism was investigated, focussing on the paracellular route.

5.1.1 Mannitol

Generally ^{14}C-Mannitol is a typical marker or probe used to investigate paracellular transport experiments across Caco-2 cell monolayers as a method of determining cell monolayer integrity. Typically transport of mannitol across healthy well-formed cell monolayers is very low – < 1 per cent over a 60-minute period (Rao *et al.*, 1999). Apparent permeability (P_{app}) of mannitol reported in the literature varies; for example, Artursson and Karlsson (1991) reported a mannitol P_{app} 0.18 x 10^{-6} cm/second, compared to 1.46 x 10^{-6} cm/second (Lentz *et al.*, 2000). Mannitol transport decreases as Caco-2 cell monolayers develop and conversely mannitol transport across monolayers increases as monolayer integrity decreases due to loss of the tight junction formation. Mannitol is also used as an internal control during transport experiments in order to determine that the monolayer remains intact and undamaged by compounds that are being studied. Mannitol was used as an internal standard when measuring propranolol transport (Chapter 6), glutamine transport (Chapter 7) and digoxin transport (Chapter 8).

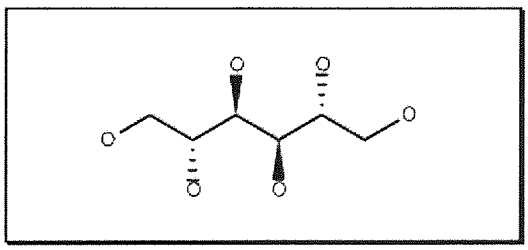

Figure 5.1 – Structure of mannitol ($C_6H_{14}O_6$).

The structure of mannitol is shown in Figure 5.1. Clinically, mannitol is used as an osmotic diuretic (Purcell *et al.*, 1999; Larsen *et al.*, 2002; BNF 46), *i.e.* mannitol remains in the gut and attracts water by osmosis. Mannitol is removed very slowly from the gastrointestinal tract – an indication of the inefficiency of paracellular absorption. Mannitol is also used in cerebral oedema to reduce the elevated pressure in the brain (BNF 46).

5.1.1.1 Alternative Probes

Alternative probes used to investigate the paracellular route include fluorescein. Mannitol was chosen in preference to fluorescein because fluorescence values for fluorescein are strongly pH-dependent. Therefore, the pH of all fluorescein-containing solutions must be altered to a specific pH prior to measurement – pH does not influence mannitol or the radionuclide. Additionally, radioactive mannitol can be measured at the same time as the radioactive test compound, whereas, fluorescein requires an entirely independent assay. Mannitol uses the paracellular route for transport; however, it has not been confirmed that fluorescein exclusively utilises the paracellular route; fluorescein maybe subject to active efflux (Kang, 2001). Apical-to-basolateral flux of mannitol and fluorescein was found to be comparable. However, basolateral-to-apical flux was significantly greater than apical-to-basolateral flux; indicating fluorescein might be subjected to some form of active efflux (Kang, 2001). Consequently, mannitol was chosen as the appropriate probe for the paracellular route for use with Caco-2 cells.

5.1.2 Objective

The objective of this chapter was to use mannitol as a probe for the integrity of tight junctions, and to determine the effect of liposomal formulations on paracellular transport across Caco-2 epithelial monolayers. In addition to mannitol flux being measured, transepithelial resistance (TEER) was measured as an additional indicator of monolayer integrity.

5.2 Material and Methods

General material and methods are described in Chapter 2. Briefly, Caco-2 cells were seeded onto Transwell inserts (2 x 10^5 cells per insert) and used 14 – 21 days post seeding (Section 2.3.3.2). TEERs were measured at the beginning of all experiments using chopstick electrodes. D-[^{14}C]-Mannitol (8 μM) transport was measured over 60 minutes, at 37°C, unless otherwise stated and samples were taken every ten minutes. At designated time points (0, 10, 20, 30, 40, 50 and 60 minutes) samples (100 μl) were collected and placed into scintillation vials; the transport medium was immediately replaced (100 μl) (Section 2.3.5 and Section 2.3.9).

5.3 Results and Discussion

5.3.1 Effect of Temperature on Mannitol Transport

From Chapter 4, Section 4.3.1.2, it was confirmed that mannitol transport at 37°C was detectable over a 60-minute period. Mannitol flux was very low – 0.28 ± 0.03 per cent, with an apparent permeability P_{app} 9.88 x 10^{-7} ± 7.48 x 10^{-8} cm/second. Following confirmation that mannitol flux was detectable at 37°C, the effect of temperature on mannitol flux was determined.

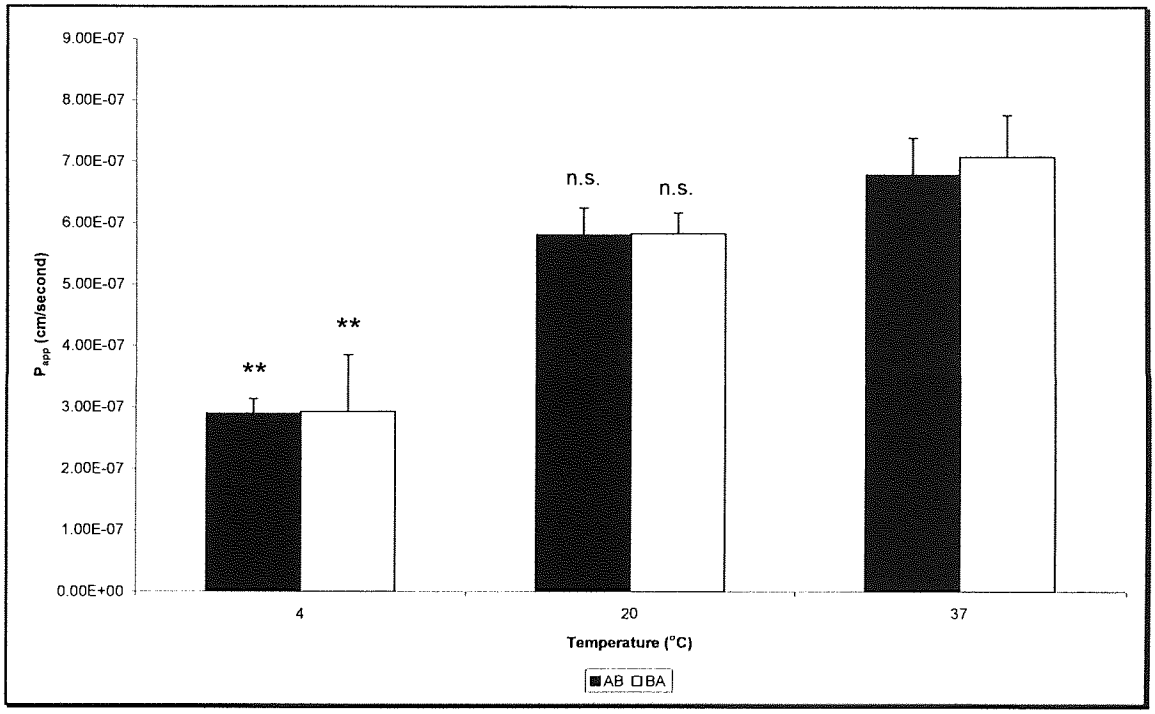

Figure 5.2 – The effect of temperature on the apparent permeability (P_{app}) of mannitol.

Apical-to-basolateral and basolateral-to-apical flux of D-[^{14}C]-Mannitol (8 μM) was measured at 4°C, 20°C and 37°C. Caco-2 cells were seeded onto Transwell inserts, passage 26-40 and used 14-21 days post seeding, n= 4. Transport was measured over 60-minutes ** Denotes a very significant ($p < 0.01$) difference compared to 37°C; n.s. denotes no significant difference ($p > 0.05$) compared to 37°C (ANOVA).

The effect temperature had on mannitol transport was investigated because temperature influences energy-dependent processes – a reduction in temperature decreases active processes (Cogburn *et al.*, 1991; Inui *et al.*, 1992). It remains unclear whether the reduction in transport is because of a reduction in the fluidity of the cellular membrane or inhibition of the active secretion mechanism. Passive paracellular transport is less vulnerable to temperature changes compared to active transport mechanisms. Mannitol transport was measured at 4°C,

20°C and 37°C; transport was determined in both the apical-to-basolateral direction and basolateral-to-apical direction (Figure 5.2).

Increasing the temperature from 4°C to 37°C caused an increase in mannitol flux for both apical-to-basolateral (A – B) flux and basolateral-to-apical (B – A) flux (Figure 5.2). A 2.3–fold increase in mannitol transport was observed after increasing the temperature from 4°C to 37°C in the A – B direction (Figure 5.2); similarly in the B – A direction a 2.4–fold increase in mannitol transport was measured following the increase in temperature (Figure 5.2).

Although temperature affected mannitol transport (Figure 5.2), it was predicted that temperature would have no effect because mannitol is transported *via* the passive paracellular route *i.e.* through tight junctions. The increase in mannitol permeability is because cell bilayers are fluid, not static; therefore, increasing temperature will increase the amount of energy the bilayer has – increasing the fluidity and affecting the tight junctions, resulting in an increase in mannitol flux (Cogburn *et al.*, 1991). Mannitol permeability may have increased with increasing temperature because of changes in the thermodynamics of diffusion.

	P_{app} (Apical-to-Basolateral / Basolateral-to-Apical)		
	4°C	20°C	37°C
Mannitol	0.99	1.00	0.96

Table 5.1 – Ratios calculated from mannitol transport at 4°C, 20°C and 37°C.

Mannitol is transported *via* the passive paracellular route; therefore, apical-to-basolateral flux and basolateral-to-apical flux should be similar. There is no significant difference between apical-to-basolateral flux and basolateral-to-apical flux (Figure 5.2; Table 5.1) at 4°C, 20°C and 37°C (ANOVA) indicating that mannitol transport is passive.

5.3.2 Effect of Liposome Formulations on Mannitol Transport

Transepithelial electrical resistance (TEER) was measured at the beginning of all experiments to ensure the Caco-2 cell monolayer was confluent. TEER measurements were also made at the end of experiments in order to determine if the cell monolayer had been compromised. The reduction in TEER values was calculated as percentage of the control TEER value measured at the end of the experiment (Table 5.2). A significant decrease in TEER measurement was observed following transport experiments in the presence of PC, PC:Cholesterol (16:4), DSPC, 1000 μg and DSPC:Cholesterol, 1000 μg indicating a decrease in monolayer integrity and therefore, a potential increase in membrane permeability (Table 5.2).

	TEER (% of Final Value)	Significance (ANOVA)	
PC (1000 μg)	90.0 ± 2.2	Significant	$p < 0.05$
PC:Cholesterol (1000 μg; 16:4)	90.0 ± 4.4	Significant	$p < 0.05$
DSPC (1000 μg)	86.6 ± 5.8	Very Significant	$p < 0.01$
DSPC:Cholesterol (1000 μg;16:4)	72.6 ± 4.6	Very Significant	$p < 0.01$

Table 5.2 – Percentage reduction in TEER value at the end of the experiment, compared to the control at the end of the experiment.

The effect liposome formulations (PC, PC:Cholesterol, DSPC and DSPC:Cholesterol, 1000 μg) had on TEER measurements (ANOVA with Dunnett's post-test, Instat 3).

TEER measurements are commonly used as a tool in determining if compounds are absorption enhancers or if compounds are toxic towards Caco-2 cells. Borchard *et al.*, (1996) used TEER measurements in conjunction with mannitol transport to determine the effects carbomer and chitosan-glutamate had on Caco-2 cells and if they could be used as absorption enhancers for peptide delivery.

The effect that PC, PC:Cholesterol (16:4), DSPC and DSPC:Cholesterol (16:4), 1000 μg, had on mannitol transport in the both the apical-to-basolateral and basolateral-to-apical direction

was examined (Figure 5.3 – Figure 5.4). Mannitol transport was measured in both directions because mannitol transport is a passive; thus, apical-to-basolateral and basolateral-to-apical transport should be similar and also to determine if the liposome formulations affect the apical and basolateral membranes differently. Mannitol transport was slightly increased in the presence of PC, 1000 μg; however, the increase was not significant ($p > 0.05$) in either the A – B or B – A direction (Figure 5.3).

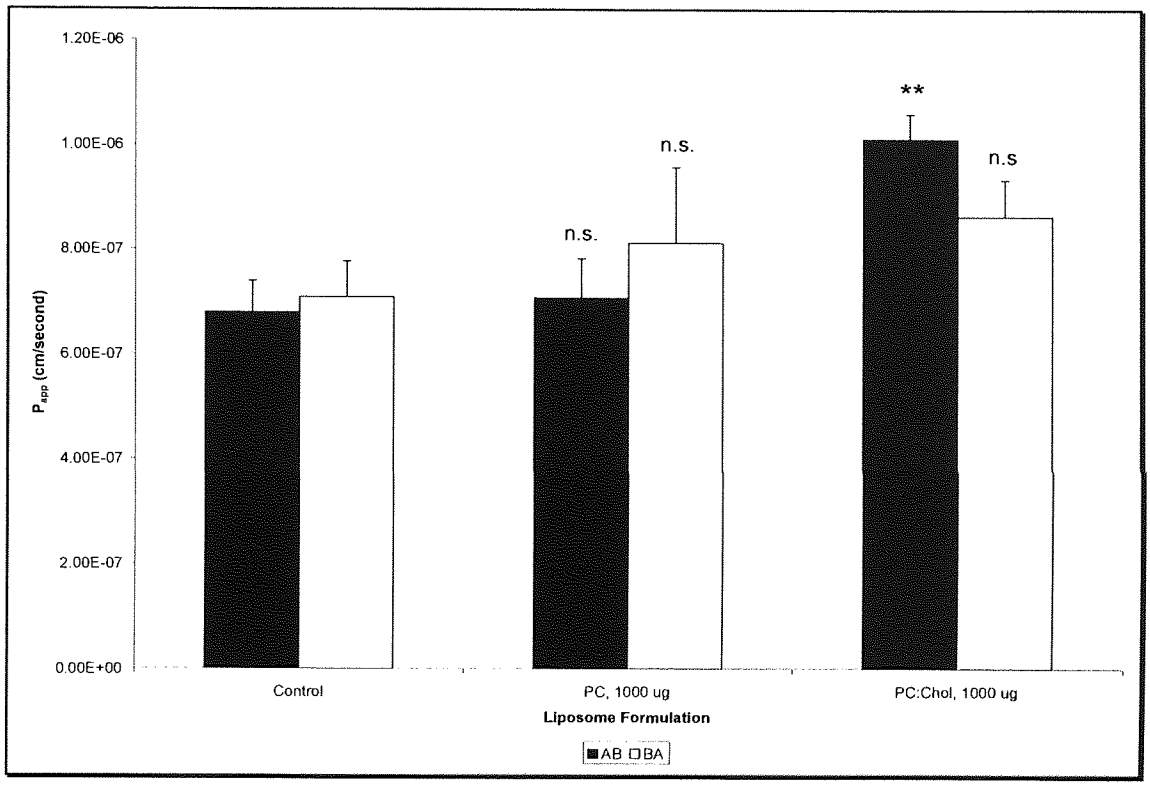

Figure 5.3 – The effect of PC, 1000 μg and PC:Cholesterol (1000 μg; 16:4) on apical-to-basolateral and basolateral-to-apical transport of D-[^{14}C]-Mannitol (8 μM) was measured across Caco-2 cell monolayers.

Caco-2 cells were seeded onto Transwell inserts between passage 26-40 and were used 14-21 days post seeding, n = 4. D-[^{14}C]-Mannitol transport was measured over 60-minutes at 37°C in the presence of PC, 1000g and PC:Cholesterol (16:4), 1000 μg. ** Denotes a very significant ($p < 0.01$) difference compared to the control; n.s. denotes no significant difference ($p > 0.05$) compared to the control (ANOVA).

In contrast to PC, 1000 μg data, PC:Cholesterol (16:4) 1000 μg significantly ($p < 0.01$) increased A – B mannitol transport (Figure 5.3). P_{app} was increased to $1.01 \times 10^{-6} \pm 4.71 \times 10^{-8}$ cm/second. The increase in mannitol transport was expected from the TEER data because at the end of the transport experiment the TEER value was significantly ($p < 0.05$) reduced following incubation with PC:Cholesterol (16:4), 1000 μg (Table 5.2). The reduction in cell

monolayer integrity caused an increase in paracellular transport of mannitol (Figure 5.3). B – A mannitol P_{app} was also increased in the presence of PC:Cholesterol (16:4) 1000 μg; however, the increase was not significant ($p > 0.05$) (Figure 5.3). The increase in mannitol transport was expected from the TEER data because a reduction was observed indicating a decrease in monolayer integrity (Table 5.2).

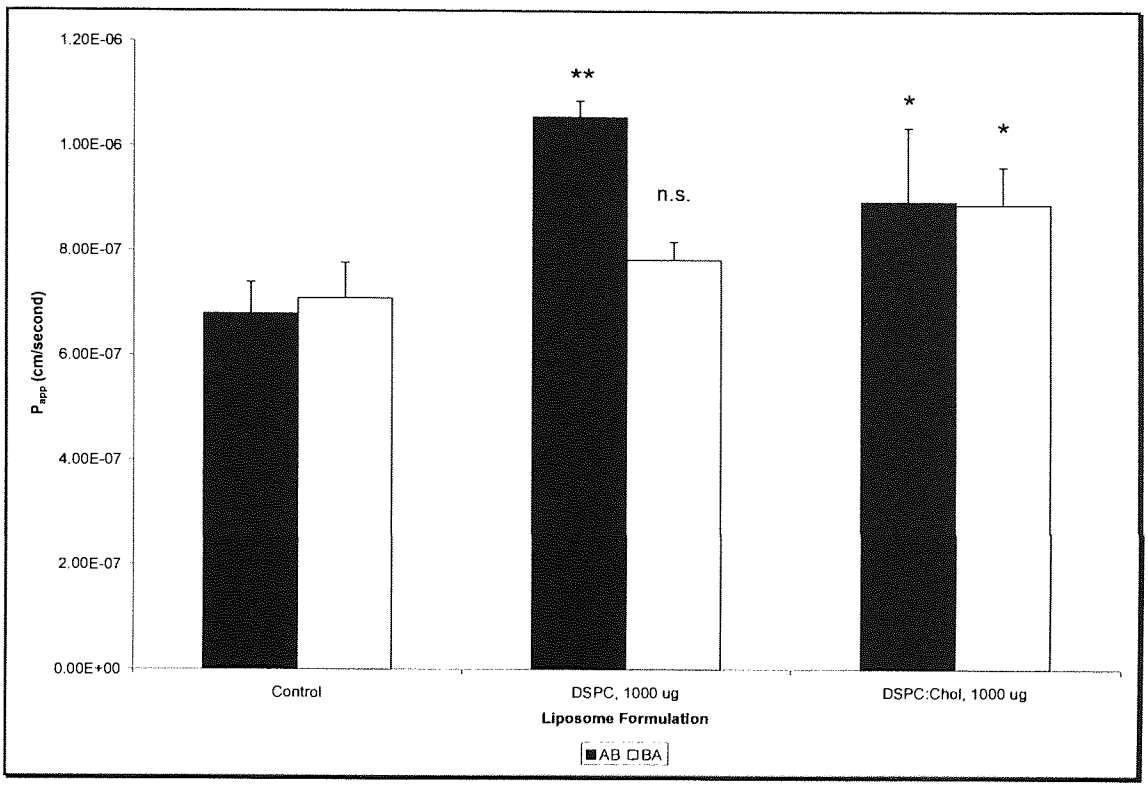

Figure 5.4 – The effect of DSPC, 1000g and DSPC:Cholesterol (16:4), 1000 μg on apical-to-basolateral and basolateral-to-apical transport of D-[^{14}C]-Mannitol (8 μM) was measured across Caco-2 cells.

Caco-2 cells were seeded onto Transwell inserts between passage 26-40 and used 14-21 days post seeding. Data are mean and standard deviation of results from four inserts. D-[^{14}C]-Mannitol transport was measured over 60-minutes at 37°C in the presence of DSPC, 1000g and DSPC:Cholesterol (16:4), 1000 μg. ** denotes a very significant ($p < 0.01$) difference compared to the control; * denotes a significant ($p < 0.05$) difference compared to the control; n.s. denotes no significant difference ($p > 0.05$) compared to the control (ANOVA).

PC was replaced with a higher transition temperature lipid – DSPC. The presence of both DSPC, 1000 μg and DSPC:Cholesterol (16:4) 1000 μg significantly increased paracellular transport of mannitol in the A – B direction (Figure 5.4). P_{app} was increased to $1.06 \times 10^{-6} \pm 3.06 \times 10^{-8}$ cm/second in the presence of DSPC, 1000 μg and $P_{app} = 8.95 \times 10^{-7} \pm 1.41 \times 10^{-7}$ cm/second in the presence of DSPC:Cholesterol, 1000 μg. As with PC:Cholesterol, 1000 μg, an increase in mannitol transport in the presence of DSPC 1000 μg and DSPC:Cholesterol, 1000

μg was expected because there was a significant ($p < 0.01$) decrease in TEER data for both formulations (Table 5.2). B – A mannitol transport was increased in the presence of DSPC, 1000 μg, albeit not significantly ($p < 0.05$); in contrast, B – A mannitol transport was significantly increased ($p < 0.05$) in the presence of DSPC:Cholesterol (16:4) 1000 μg.

Mannitol transport was not increased in the presence of PC, 1000 μg (Figure 5.3), in contrast DSPC, 1000 μg (Figure 5.4) significantly increased mannitol transport. Replacement of PC with a higher transition temperature lipid DSPC caused a significant ($p = 0.0017$, t-test) increase in mannitol transport. The transport experiments were performed at 37°C; therefore, during the experiment PC was at temperature above the transition temperature, conversely DSPC was at a temperature lower than the transition temperature. Consequently, PC liposome bilayers were less rigid compared to DSPC liposome bilayers. The increased rigidity of the DSPC liposomes may have reduced monolayer integrity to a greater extent compared to the less rigid PC bilayers; thus, causing an increase in mannitol transport. Transition temperature of lipids affected the transport of recombinant human epidermal growth factor across Caco-2 cells. PC liposomes had no effect on transport; in contrast, the higher transition temperature lipid DPPC (41°C) increased transport three-fold (Li *et al.*, 2003).

Inclusion of cholesterol in the liposome formulation had very different effects on mannitol transport when included into PC and DSPC liposome formulations. The presence of cholesterol with PC significantly ($p = 0.0040$, t-test) increased mannitol transport compared to PC, 1000 μg (Figure 5.3). In contrast, inclusion of cholesterol with DSPC showed no significant ($p = 0.1259$, t-test) effect on mannitol transport compared to DSPC, 1000 μg (Figure 5.4). There is no significant ($p = 0.2482$, t-test) difference between the increase in mannitol transport in the presence of PC:Cholesterol versus DSPC:Cholesterol. Cholesterol is know to stabilise bilayers (Valenti *et al.*, 2001; Cócera *et al.*, 2003; Sinico *et al.*, 2005); however, due to the different transition temperatures of PC (< 0°C) and DSPC (57°C), DSPC bilayers have a higher rigidity at 37°C compared to PC. Consequently, the inclusion of cholesterol may stabilise PC liposomes to a greater extent, thus increasing mannitol permeability.

DSPC (1000 μg) had no significant effect on mannitol transport (B – A) (Figure 5.4). DSPC:Cholesterol, (1000 μg; 16:4) significantly decreased TEER values (Table 5.2); therefore, an increased cellular permeability towards mannitol was expected in the presence of DSPC:Cholesterol, 1000 μg. This was observed for both A – B and B – A (Figure 5.4). The increase in mannitol cellular permeability in the B – A direction was significant ($p < 0.05$) (Figure 5.4); P_{app} was increased to $8.89 \times 10^{-7} \pm 7.24 \times 10^{-8}$ cm/second.

There was no significant ($p = 0.7461$, t-test) difference between mannitol transport in the presence of PC, 1000 μg and DSPC, 1000 μg. The same was observed for PC:Cholesterol, 1000 μg and DSPC:Cholesterol, 1000 μg ($p = 0.6811$, t-test). Inclusion of cholesterol in the liposome formulation increased mannitol transport compared to the formulation without cholesterol. The increase was not significant ($p = 0.6143$. t-test) for PC nor DSPC ($p = 0.0841$, t-test).

From the data obtained the liposome formulations have different affects on mannitol permeability across Caco-2 cell monolayers. PC:Cholesterol, DSPC, DSPC:Cholesterol all significantly increased A – B mannitol permeability (Figure 5.3 and Figure 5.4). The mechanism of liposome action towards the Caco-2 cell monolayer was beyond the scope of the work presented here; however, possible interactions between liposomes and cell monolayers include phospholipid exchange and the presence of membrane rafts.

Phospholipid exchange between micelles has been investigated using fluorescent phospholipids and has demonstrated that phospholipids are able to exchange between micelles (Thomas *et al.*, 1999). Fluorescent techniques have also been used to measure phospholipid 'flip-flop' between bilayers (Armstrong *et al.*, 2003). Consequently, it is possible that phospholipids present within the liposome formulations and those present in the Caco-2 cell monolayer exchanged positions influencing the fluidity if the Caco-2 cell monolayer thus increasing mannitol permeability.

Membrane rafts or lipid rafts form because lipids do not always mix uniformly in membranes, but they cluster to form microdomains (Brown, 2002; Wassall *et al.*, 2004). Membrane rafts involve more than two molecules and the formation requires the interactions between cholesterol and saturated alkyl chains (Kusumi and Suzuki, 2005). The presence of both alkyl chains and cholesterol in the liposome formulations may have resulted in the formation of membrane rafts in the cellular membrane thus altering the dynamics of the membrane and consequently influencing mannitol permeability across the Caco-2 cell monolayer. NMR, X-ray diffraction and immunofluorescence techniques are employed to study membrane rafts (Wassall *et al.*, 2004; Kusumi and Suzuki, 2005).

TEER measurements and mannitol permeability across Caco-2 cell monolayers were measured in the presence of sodium taurocholate, sodium taurodeoxycholate and sodium taurodihydrofusidate bile salts, sodium dodecyl sulphate and sodium dioctyl sulfosuccinate (anionic surfactants) and polysorbate 80 and polyoxyl 40 hydrogenated castor oil (nonionic surfactants) and sodium dodecyl sulphate. At low concentrations there was no detrimental effect however increasing concentrations resulted in TEER measurements decreasing and mannitol permeability increasing (Anderberg *et al.*, 1992; Anderberg and Artursson, 1992). PEG-4000 is used to measure paracellular transport and tight junction integrity (Haeberlin *et al.*, 1996). The ability of cyclodextrins to enhance drug absorption has been studied by measuring the permeation of PEG-4000 across Caco-2 cell monolayers. In the absence of cyclodextrins permeation varied from 0.1 and 0.4 % per 2 hours, the permeation increased to 3 % in the presence of cyclodextrins (Haeberlin *et al.*, 1996). The mechanism of cyclodextrin action remains unclear; however, possibilities include increased drug aqueous solubility and stability, reduction in the aqueous diffusion layer and / or extraction of membrane components (Haeberlin *et al.*, 1996; Pezron *et al.*, 2002).

5.4 Conclusion

Mannitol transport was determined at 37°C over 60 minutes (unless otherwise stated) and the experimental control had a cellular permeability towards mannitol at 37°C of P_{app} 6.80 x 10^{-7} ±

6.00 x 10^{-8} cm/second in the A – B direction (Figure 5.2) and in the opposite direction P_{app} 7.09 x 10^{-7} ± 6.80 x 10^{-8} cm/second (Figure 5.2). The experimental cellular permeability obtained from mannitol is in agreement with published literature, for example, mannitol P_{app} = 0.18 x 10^{-6} cm/second (Artursson and Karlsson, 1991) and 1.46 x 10^{-6} cm/second (Lentz *et al.*, 2000). Decreasing temperature affects energy-dependent processes to a greater extent compared to passive processes; however, passive processes are affected due to bilayer fluidity. Membrane fluidity decreases with temperature and a decrease in mannitol transport was observed at lower temperatures, probably because the membrane becomes quite static at 4°C. The decrease in mannitol transport at lower temperatures was observed in both directions of transport; even at 37°C mannitol transport was still very low compared to propranolol that is transported *via* the transcellular route 23.4 ± 2.8 x 10^{-6} cm/second (Walgren *et al.*, 1998). A very low P_{app} is typical of the passive paracellular route. Mannitol transport remained less than 1 % over the 60-minute time period at all temperatures; therefore, the monolayers are forming adequately for experimental use.

DSPC:Cholesterol, had the greatest impact on TEER values for Caco-2 monolayers; however, the TEER value was only reduced to 72.6 ± 4.6 per cent of the initial TEER value after 1 hour at 37°C. Although a reduction to 72.6 ± 4.6 per cent of the initial TEER value is a significant ($p < 0.01$) reduction, Caco-2 cells are capable of recovery; Borchard *et al.*, (1996) observed recovery in Caco-2 cells even when the TEER value was reduced to 59.6 ± 4.1 per cent of the initial TEER value. Bromberg and Alahov (2003) investigated the use of polyether-modified poly(acrylic acid) microgels to improve doxorubicin transport. They observed TEER values decreased to 80 per cent of the initial value – the decrease in TEER values was fully reversible, and no significant increase in ^{14}C-Mannitol transport was observed.

Cellular permeability towards mannitol was significantly increased in the presence of PC (1000 μg), PC:Cholesterol (1000 μg; 16:4) and DSPC:Cholesterol, (1000 μg; 16:4) in the A – B direction and DSPC:Cholesterol, 1000 μg in the opposite direction (Figure 5.3 – Figure 5.4). The largest increase was for DSPC, A – B (P_{app} 1.06 x 10^{-6} ± 3.06 x 10^{-8} cm/second), and

DSPC:Cholesterol, B – A (P_{app} 8.89 x 10^{-7} ± 7.24 x 10^{-8} cm/second). Experimentally apparent permeabilities below P_{app} = 1.0 x 10^{-6} cm/second are considered as fully developed monolayers that can be used for experimental use. The P_{app} for DSPC:Cholesterol was less than this value and the P_{app} for DSPC was only slightly greater than this value. Therefore, PC, PC:Chol, (16:4 μM), DSPC, DSPC:Chol, (16:4 μM) (1000 μg) liposome formulations are not detrimental to Caco-2 cell monolayers confirming the findings in Chapter 4.

From the TEER data and mannitol data it can be ascertained that the tight junctions remain intact throughout the 60-minute experimental time period in the presence of the above liposome formulations. The liposome formulations might have an absorption enhancing effect; however, this is not at the detriment of the Caco-2 cell monolayer. Absorption and permeation enhancers must not be toxic or detrimental to either the cell monolayer or the drugs being delivered; commonly used methods to determine toxicity of absorption enhancers include MTT assay, propidium iodide staining, mannitol transport and TEER measurements (Dorkoosh *et al.*, 2002; Meany and O'Driscol, 2000; Bromberg and Alakhov, 2003; Lindhart and Bechgaard, 2003; Palamakula and Khan, 2004). TEER measurements, MTT assay (Chapter 4) and mannitol transport have been employed in this investigation to ensure the liposome formulations are non-toxic towards Caco-2 cells.

In order to verify the mechanism by which liposomes interact and influence membrane dynamics, further investigations need be done. These include repeating the experiments with different total lipid amounts to verify the effect of lipid dose. The series of experiments should be repeated using SUVs or LUVs as a replacement of MLVs because they have a single bilayer opposed to multiple bilayers. Additionally, SUVs and LUVs could be used for mechanistic studies in order to determine the effects liposomes have on the Caco-2 monolayers. Fluorescence studies and X-ray diffraction studies need to be performed in order to verify if phospholipid exchange occurs between the liposome formulations and the Caco-2 monolayer, to determine the presence of membrane rafts (Section 5.3.2) and to determine changes in membrane fluidity (Section 7.4).

In summary, the four liposome formulations tested are suitable for use with the Caco-2 cell line and for investigating their potential as permeation enhancers for propranolol, glutamine and digoxin.

Chapter 6

Effects of Liposomal Formulations on the Passive Transcellular Route

6. Effects of Liposomal Formulations on the Passive Transcellular Route

6.1 Introduction

The movement of a solute along a gradient, either concentration or electrical is known as the passive transcellular route, the rate of transport is directly proportional to the gradient (see Section 1.3.2.1). The process is passive; therefore, requires no expenditure of energy. Absorption of hydrophobic drugs principally occurs by the transcellular route (Kararli *et al.*, 1989). For passive transcellular flux, the molecule must have an appropriate set of physicochemical properties, because of the lipophilic bilayer. Size, lipophilicity, hydrogen bonding and conformation determine whether the compound will be able to cross the lipophilic cellular barrier (Hunter and Hirst, 1997).

Lipid-partitioning is a part of passive transcellular transport and involves the incorporation of a hydrophobic drug molecule into the lipid membrane of the cell and then partitioning of the molecule into the cell. Generally, small amphipathic drugs move efficiently through the transcellular route by partitioning into and out of the lipid bilayers (Daugherty and Mrsny, 1999). The lipid bilayer is highly impermeable to charged molecules, where charge and high degree of hydration prevent them from entering the hydrocarbon phase of the bilayer. The 'rule of 5' is a general rule of thumb based on physicochemical properties of a compound which can be used to estimate a drugs permeability. Lipinski *et al.*, (2001), analysed a large number of compound libraries and created the 'rule of 5' which defines a poorly absorbed compound as, one with five or more hydrogen bond donors, a molecular weight greater than 500, Moriguchi LogP over 4.15 (or a LogP greater than 5; Figure 6.1) and the sum of nitrogens and oxygens to be greater than ten.

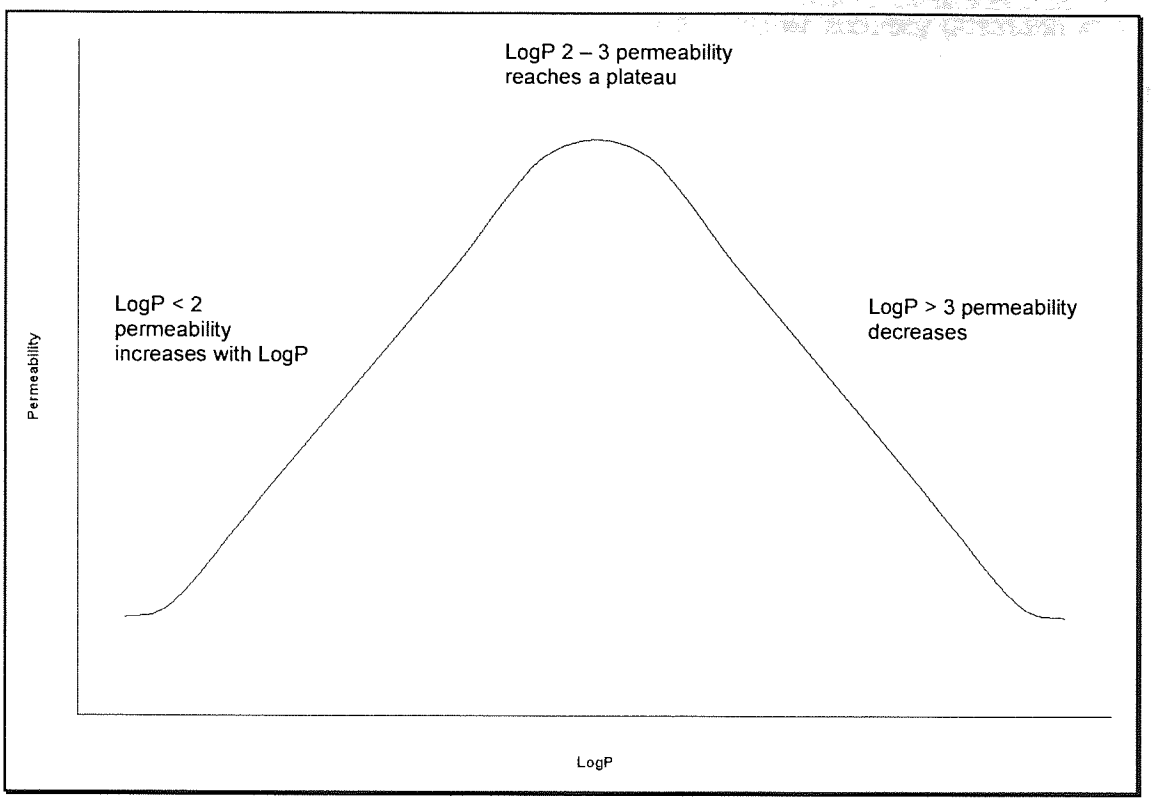

Figure 6.1 - The relationship between LogP values and permeability.

The absorption of drugs through biological membranes is directly proportional to the value of the partition coefficient. Increasing lipophilicity of a compound results in increasing permeability across the intestinal epithelium until a plateau is reached at a LogP value of approximately 2 – 3 (Martin, 1981; Ungell *et al.*, 1998). Compounds with a LogP of about 2– 3 display the highest absorption and are generally predicted to be completely absorbed in humans. Permeability begins to decrease for compounds with a LogP value greater than 4 (Wils *et al.*, 1994; Artursson *et al.*, 2001) (Figure 6.1). At low LogP values, a drug cannot penetrate the lipid membrane because the drug is too hydrophilic (Daugherty and Mrsny, 1999). However, at high LogP values, the drug becomes so lipophilic that diffusion through the unstirred water layer will become the rate-limiting step for absorption. Drug molecules that are too lipophilic tend not leave the lipid bilayer (Daugherty and Mrsny, 1999). LogP is a useful determinant for predicting drug absorption; however, LogP is a single physicochemical property. The dynamic polar molecular surface area is a new theoretical method that takes into account several physicochemical properties of the drug molecule to predict drug absorption. The dynamic polar

molecular surface area can predict drug absorption with a higher accuracy (Artursson *et al.*, 2001).

6.1.1 Propranolol

The probe chosen to investigate the effect of liposomal formulations on the passive transcellular route of drug uptake was propranolol (Figure 6.2). Propranolol has previously been used as a passive transcellular marker with Caco-2 cells (Krishna *et al.*, 2001). Propranolol is a highly lipophilic and permeant compound; thus, following oral administration is almost completely absorbed (Taylor *et al.*, 1985; Krishna *et al.*, 2001). However, propranolol is subjected to extensive and highly variable hepatic first-pass metabolism following oral administration, with a reported systemic bioavailability of 20 – 70 % (Johnsson and Regardh, 1976; Ghabrial *et al.*, 1994; Taylan *et al.*, 1996; Agoram *et al.*, 2001). Due to the high hepatic clearance, the plasma half-life is short – varying from 1.5 to 6 hours (Taylan *et al.*, 1996; Agoram *et al.*, 2001).

Propranolol is a widely used non-selective β-adrenergic receptor (beta-blocker) blocking agent and is used to treat some heart conditions, reduce the symptoms of angina pectoris, lower blood pressure in people with hypertension, used to prevent migraines, and to reduce performance anxiety (Gopalaswamy *et al.*, 1997; BNF, 2003). Propranolol is a P-gp substrate (Yang *et al.*, 2000) and inhibitor (von Moltke and Greenblatt, 2001).

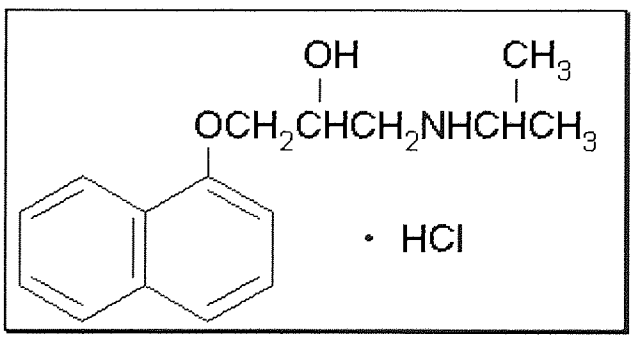

Figure 6.2 – Structure of propranolol hydrochloride.

Propranolol is administered as propranolol hydrochloride tablets (Eddington *et al.*, 1998) and like many other drugs, propranolol is a substrate for the efflux pump P-glycoprotein (Yang *et al.*, 2000). Since this thesis was started, alternative methods have been developed to improve propranolol delivery, for example, using a dendrimer-propranolol prodrug (D'Emanuele *et al.*, 2004). To produce the prodrug, propranolol was conjugated to generation 3 dendrimers (G3) and lauroyl-G3 PAMAM (polyamidoamine) dendrimers. Apical-to-basolateral transport of propranolol was increased when propranolol was covalently bound to the dendrimers, thus increasing bioavailability. However, it has been argued that as propranolol has a high membrane permeability it is considered to be "transparent" to P-glycoprotein because P-glycoprotein does not significantly limit absorption or elimination (Collet *et al.*, 2004). This can be explained because propranolol diffuses so rapidly across the cell membrane that, even though propranolol is been actively transported out of the cell by P-glycoprotein, it immediately diffuses back (Collet *et al.*, 2004).

6.1.1.1 Physicochemical Properties of Propranolol

Physicochemical properties of a compound are important in determining the route of absorption, for example, for a compound to be able to utilise the passive paracellular route size is an important restriction – only compounds with a molecular weight < 200 Da can be transported *via* the paracellular route. In addition to size, other physicochemical properties are involved in determining the route of absorption; these include lipophilicity and p*K*a. Propranolol is transported *via* the passive transcellular route, having a molecular weight of 259.3, a pK_a value of 9.45, and a LogP value of 3.10 (Mannhold *et al.*, 1990; Moriguchi *et al.*, 1994; Leo, 1995; Mannhold *et al.*, 1998; Hung *et al.*, 2001). Although stated that the LogP value of propranolol is 3.10 there is a range of LogP values found within the literature; these include both experimental and calculated LogP values, in the range 2.15 – 3.46 (Yee, 1997; Wang *et al.*, 1997).

6.1.2 Objective

The objective of this chapter was to determine the effect of liposomal formulations (MLVs) on passive transcellular transport using propranolol as a chosen probe. Propranolol uptake and

transport across Caco-2 cell monolayers was measured using 24-well plates and Transwell inserts.

6.2 Material and Methods

General material and methods were previously described in Chapter 2. Briefly, to measure DL-Propranolol [4-^{3}H] Hydrochloride (14 nM) uptake, Caco-2 cells were seeded on 24-well plates. The Caco-2 cell monolayers were used 7-days post-seeding. DL-Propranolol [4-^{3}H] Hydrochloride (14 nM) uptake was measured over 10 minutes and at 37°C unless otherwise stated (Section 2.3.3.1 and Section 2.3.8) For DL-Propranolol [4-^{3}H] Hydrochloride (14 nM) transport experiments, Caco-2 cells were seeded onto Transwell inserts (2 x 10^5 cells per insert) and used 14 – 21 days post seeding. TEERs were measured using chopstick electrodes. DL-Propranolol [4-^{3}H] Hydrochloride (14 nM) transport was measured over 60 minutes, at designated time points (0, 10, 20, 30, 40, 50 and 60 minutes) samples (100 μl) were collected and placed into scintillation vials; the transport medium was immediately replaced (100 μl) (Section 2.3.3.2 and Section 2.3.9).

6.3 Results and Discussion

6.3.1 Propanolol Uptake

Propranolol uptake was measured using Caco-2 cells in 24-well plates over time (5, 10, 20, 30, 45 and 60-minutes) (Figure 6.3). Propranolol uptake is a passive process; therefore, the process is not dependent on energy expenditure and a steady consistent rate of propranolol uptake was expected which would be expressed as a linear rate of uptake.

Propranolol uptake was linear between 5 minutes and 60 minutes (R^2 = 0.9403; however, if 0 minutes *i.e.* the beginning of the experiment is included the R^2 value = 0.5625. The decrease in linear uptake is probably due to the rapid uptake of propranolol (Collet *et al.*, 2004) resulting in an abolishment of sink conditions and an equilibrium being established. Consequently, further uptake experiments were measured over a maximum of 10 minutes.

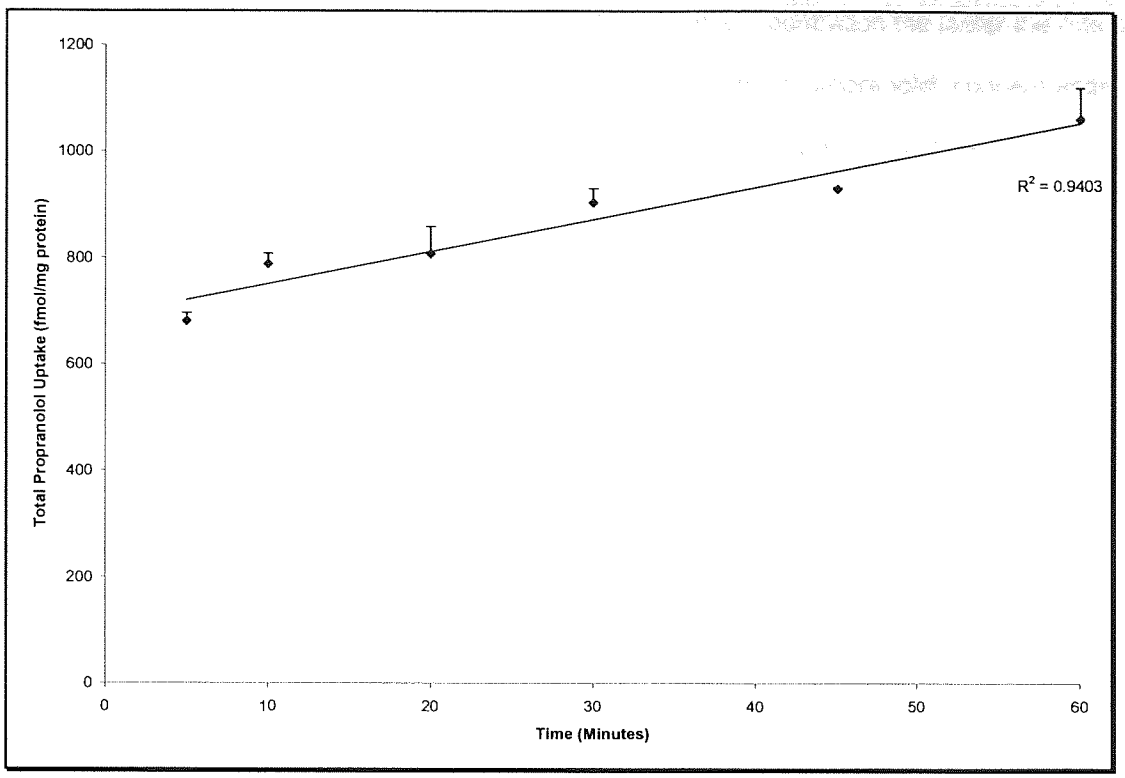

Figure 6.3 – DL-Propranolol [4-^{3}H] Hydrochloride (14 nM) uptake was measured over time.

Caco-2 cells, passage 26-40, were seeded onto plastic 24-well plates and were used 7-days post seeding. Uptake was measured at 37°C. Data are mean and standard deviation of results from four wells.

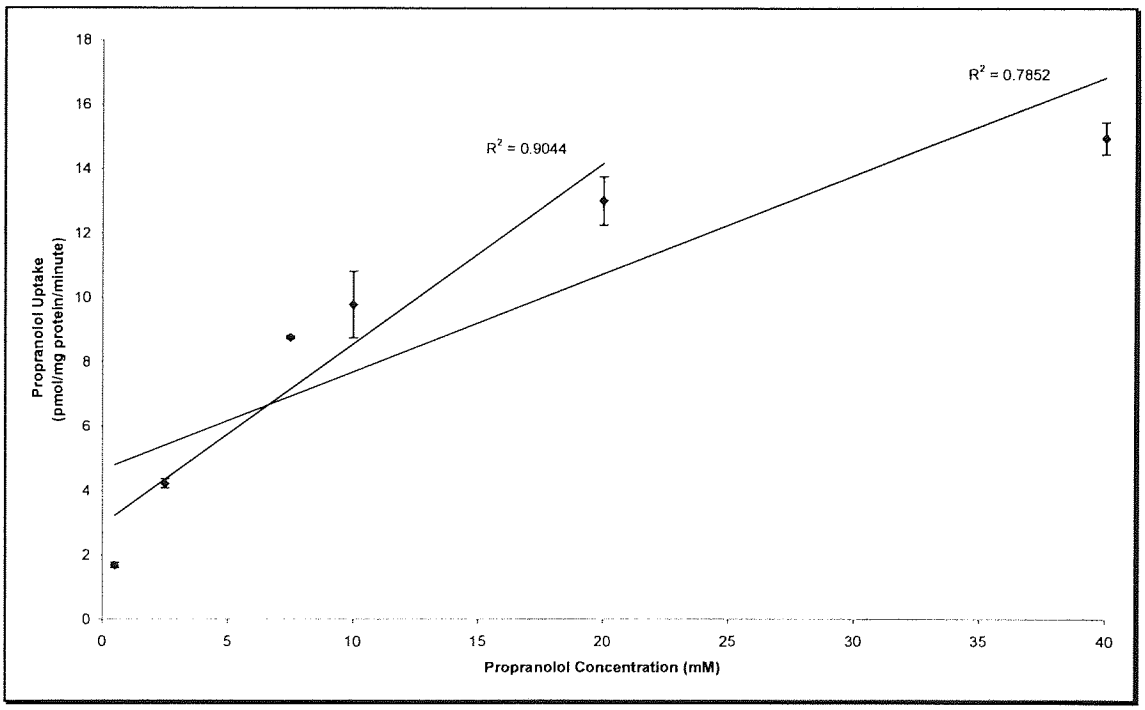

Figure 6.4 – The effect of increasing propranolol concentration on DL-Propranolol [4-^{3}H] Hydrochloride (14 nM) uptake.

Caco-2 cells, passage 26-40, were seeded onto plastic 24-well plates and used 7-days post seeding. Uptake was measured over ten minutes at 37°C. Data are mean and standard deviation of results from four wells.

Passive transport is gradient driven, therefore, the higher the concentration the faster the rate of transport. Herein, propranolol uptake was measured at different propranolol concentrations. Increasing propranolol concentration resulted in an increase in propranolol uptake (Figure 6.4) where the increase in uptake was linear up to 20 mM (R^2 = 0.9044).

The rate of propranolol uptake decreased between 20 and 40 mM (Figure 6.4). As with propranolol uptake over time, the decrease in the rate of propranolol uptake is likely to be due to an equilibrium being established and thus loss of sink conditions at this high propranolol concentration. Therefore, in subsequent experiments propranolol concentration was always lower than 20 mM.

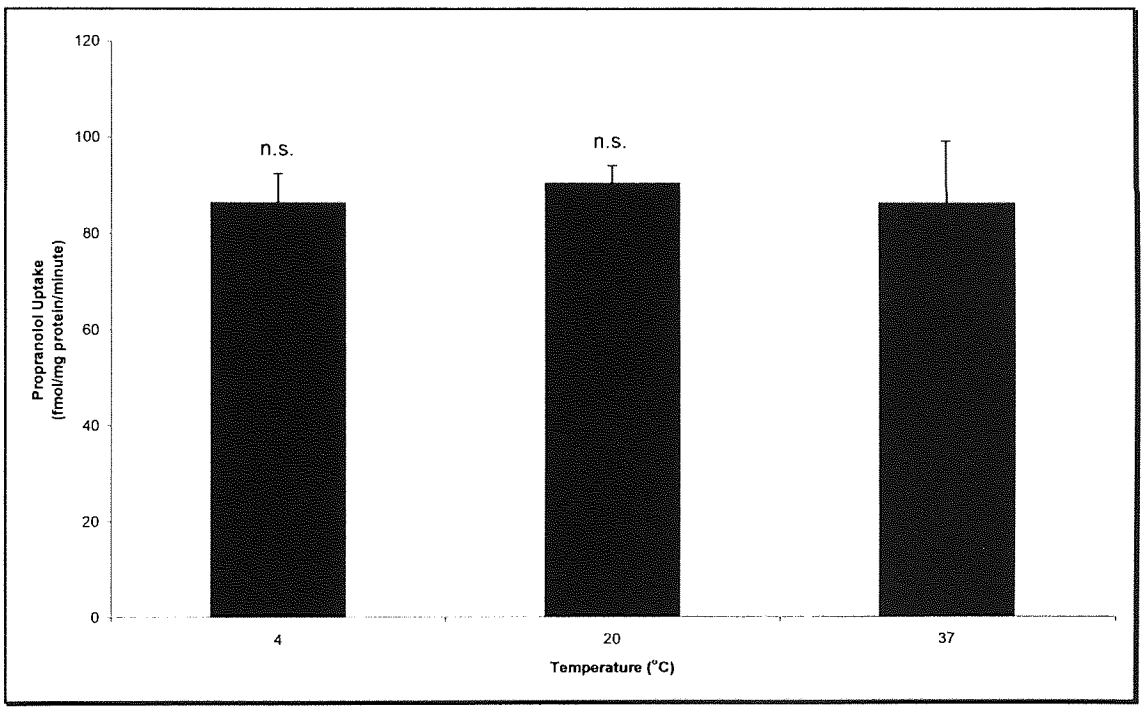

Figure 6.5 – The effect of temperature (4°C, 20°C and 37°C) on DL-Propranolol [4-^{3}H] Hydrochloride (14 nM) uptake into Caco-2 cell monolayers.

Caco-2 cells were seeded onto plastic 24-well plates between passage 26-40 and used 7-days post seeding. Data are mean and standard deviation of results from four wells. n.s. denotes no significant difference ($p > 0.05$) compared to 37°C (ANOVA).

As mentioned in Chapter 5, passive processes are affected to a lesser extent by changes in temperature, compared to energy-dependent processes. Propranolol uptake was measured at 4°C, 20°C and 37°C to determine the effect of temperature on propranolol uptake (Figure 6.5).

A reduction in temperature should not result in reduced passive processes because no expenditure of energy is required. However, propranolol is a P-gp substrate and efflux *via* P-gp is an active process. Active processes are sensitive to changes in temperature, a reduction in temperature will reduce active processes; consequently, temperature may have been expected to influence propranolol uptake. Propranolol uptake did not increase with increasing temperature (Figure 6.5). Uptake remained constant at all temperatures confirming that propranolol uptake is a passive transport process. Although propranolol is a P-gp substrate propranolol has such a high membrane permeability it is considered to be essentially "transparent" to P-gp and hence temperature will not affect propranolol uptake (Collett *et al.*, 2004). P-gp expression and activity in Caco-2 cells was not measured within the scope of the thesis; however, P-gp expression has been confirmed in Caco-2 cells within the passage window used (26 – 40) (Hosoya *et al.*, 1996). Western blotting analysis was used to verify the expression of P-gp in Caco-2 monolayers. The functionality of P-gp was measured on the basis of CsA and verapamil transport being greater in the B – A direction opposed to the A – B direction (Hosoya *et al.*, 1996).

Compounds that are toxic to Caco-2 cells will reduce propranolol uptake because uptake will only occur if Caco-2 cells are viable and have an intact membrane. Non-viable cells will not be able to participate in propranolol uptake, even though it is a passive process, because non-viable cells will lift off the base of the well and will therefore be removed during the cell washing stage (Section 2.3.8). The effect of temperature on uptake or transport of a compound is a simple approach to distinguishing between the different absorption routes, however, additional experiments must be performed to verify the route used (Cogburn *et al.*, 1991), including the use of metabolic inhibitors. Sodium azide was the metabolic inhibitor used.

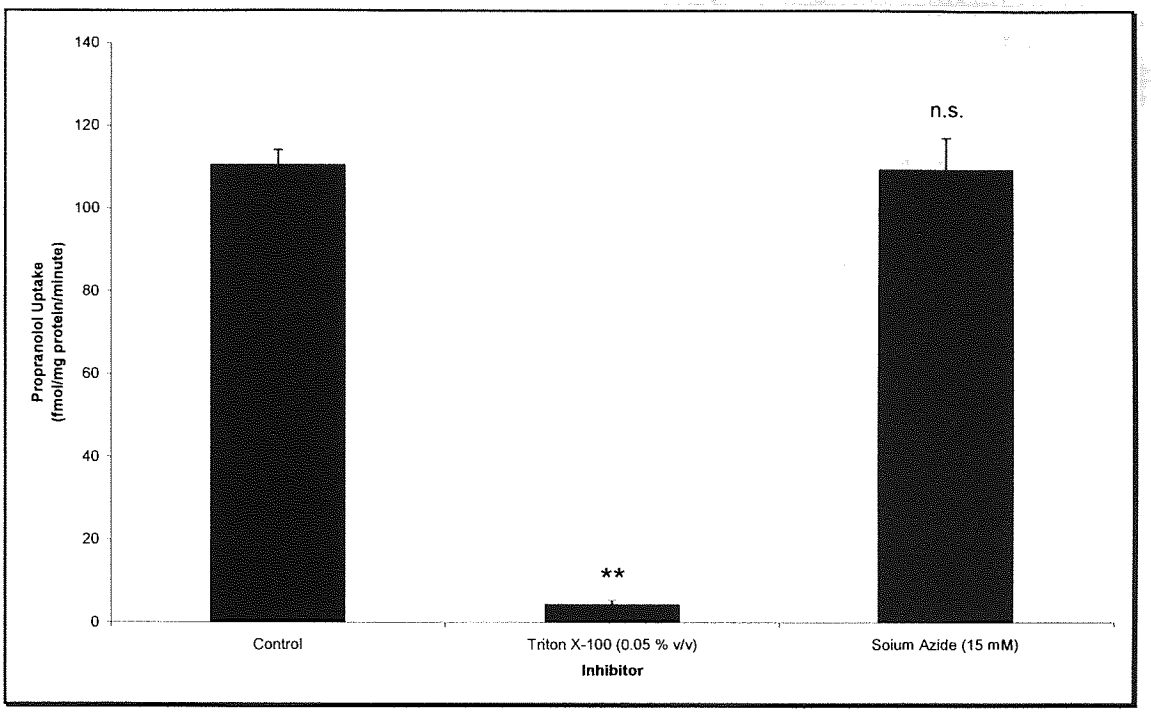

Figure 6.6 – Uptake of DL-Propranolol [4-^{3}H] Hydrochloride (14 nM) in the presence of Triton X-100 (0.05 % v/v) and sodium azide (15 mM) was measured.

Caco-2 cells (passage 26-40) were seeded onto plastic 24-well plates and used 7-days post seeding. Uptake was measured at 37°C. Data are mean and standard deviation of results from four wells. ** denotes a very significant difference ($p < 0.01$) compared to the control; n.s. denotes no significant difference ($p > 0.05$) compared to the control (ANOVA).

The effect of Triton X-100 (0.05 % v/v) on propranolol uptake was determined because Triton X-100 is known to be toxic to Caco-2 cells. Triton X-100 is a P-gp inhibitor; however, Triton-X-100 was used at a concentration that was known to be detrimental to Caco-2 cell monolayers. Triton X-100 is used to solubilise cell membranes, therefore, as expected Triton X-100 (0.05 % v/v) was toxic to Caco-2 cells (Section 4.3.3) and propranolol uptake was significantly ($p < 0.01$) decreased. Metabolic inhibitors, including sodium azide, do not affect passive processes, and did not affect propranolol uptake in these experiments ($p > 0.05$) (Figure 6.6), implying that propanolol uptake is *via* a passive process. Propranolol uptake was decreased to 4.0 per cent of the control in the presence of Triton X-100 compared to 99.0 per cent in the presence of sodium azide (Figure 6.6; Table 6.1).

The effect liposomes formulations – PC, PC:Cholesterol (16:4), DSPC, and DSPC:Cholesterol (16:4) had on propranolol uptake was investigated. For the uptake experiments two total lipid amounts were used – 500 and 1000 μg (Figure 6.7).

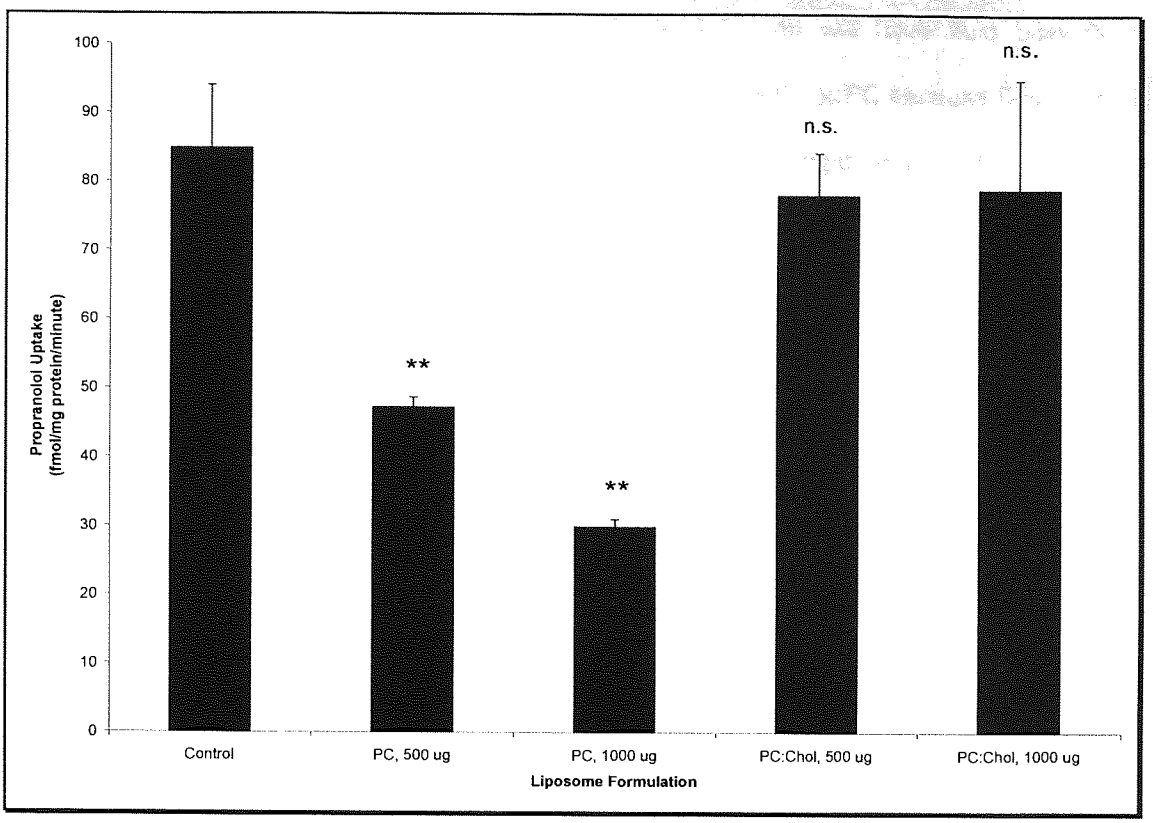

Figure 6.7 – Uptake of DL-Propranolol [4-^{3}H] Hydrochloride (14 nM) into Caco-2 cell monolayers in the presence of PC (500 and 1000 μg) and PC:Cholesterol (500 and 1000 μg; 16:4) liposomes was measured.

Caco-2 cells (passage 26-40) were seeded onto plastic 24-well plates and used 7-days post seeding. Uptake was measured at 37°C. Data are mean and standard deviation of results from four wells. ** denotes a very significant difference ($p < 0.01$) compared to the control; n.s. denotes no significant difference ($p > 0.05$) compared to the control (ANOVA).

PC, 500 and 1000 μg significantly ($p < 0.01$) reduced propranolol uptake (Figure 6.7). PC, 500 μg reduced propranolol uptake to 55.7 per cent of the control (Table 6.1). Increasing the total lipid content from 500 μg to 1000 μg further reduced propranolol uptake (Figure 6.7); uptake was reduced to 35.3 per cent of the control (Table 6.1). Although PC reduced propranolol uptake (Figure 6.7), the decrease in uptake was not to the same extent as Triton X-100 – 4.0 per cent of the control (Figure 6.6; Table 6.1). The Caco-2 cell monolayer remained intact on the base of the 24-well plate, indicating a viable monolayer.

Including cholesterol in the liposome formulation reversed the decrease in propranolol uptake elicited by liposomes (Figure 6.7). In the presence of PC:Cholesterol, propranolol uptake was not significantly ($p > 0.05$) reduced (Figure 6.7; Table 6.1). Propranolol uptake was not detrimentally affected by PC:Cholesterol (16:4) liposomes.

PC has a low transition temperature (< 0°C; Table 2.1) and will have fluid bilayers at physiological temperature (37°C); therefore, PC was replaced with DSPC because DSPC has a higher transition temperature (57°C; Table 2.1) and thus will have rigid bilayers at physiological temperatures. In contrast to PC, propranolol uptake was not significantly ($p > 0.05$) decreased in the presence of DSPC, (500 and 1000 μg) (Figure 6.8; Table 6.1).

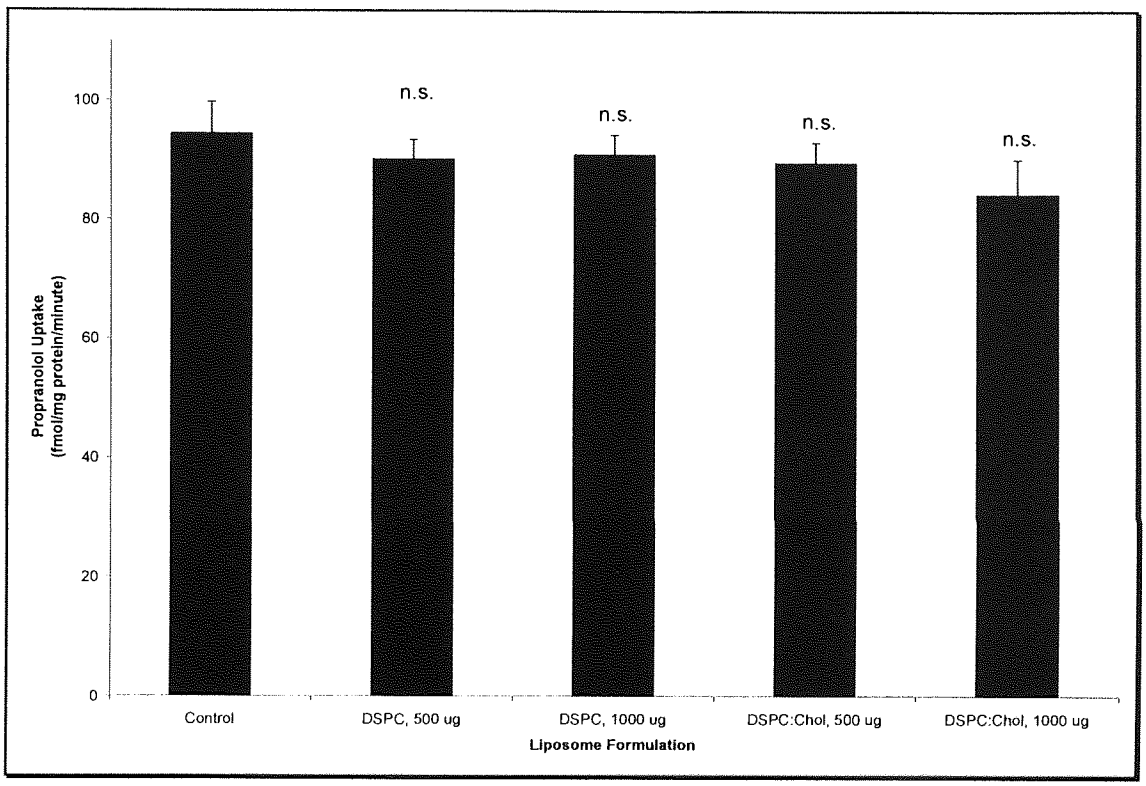

Figure 6.8 – Uptake of DL-Propranolol [4-^{3}H] Hydrochloride (14 nM) into Caco-2 cell monolayers in the presence of DSPC (500 and 1000 μg) and DSPC:Cholesterol (500 and 1000 μg; 16:4) liposomes was measured.

Caco-2 cells (passage 26-40) were seeded onto plastic 24-well plates and used 7-days post seeding. Uptake was measured at 37°C. Data are mean and standard deviation of results from four wells. n.s. denotes no significant difference ($p > 0.05$) compared to the control (ANOVA).

As with PC, cholesterol was included in the formulation because cholesterol modulates membrane fluidity (Section 3.1.1). The inclusion of cholesterol into the formulation had no significant ($p > 0.05$) effect on propranolol uptake (Figure 6.8). The decrease in propranolol uptake in the presence of DSPC:Cholesterol was comparable to the decrease in the presence of PC:Cholesterol (Figure 6.7; Figure 6.8; Table 6.1). Replacement of the lipid PC with DSPC, in the absence of cholesterol eliminated the inhibition of propranolol uptake (Figure 6.8; Table 6.1).

Formulation	Propranolol Uptake – % of Control	Significance
Control	100.0	-
Triton X-100	4.0	**
Sodium Azide	99.0	n.s.
PC, 500 μg	55.7	**
PC, 1000 μg	35.3	**
PC:Chol, 500 μg	92.2	n.s.
PC:Chol, 1000 μg	93.2	n.s.
DSPC, 500 μg	95.4	n.s.
DSPC, 1000 μg	96.3	n.s.
DSPC:Chol, 500 μg	94.9	n.s.
DSPC:Chol, 1000 μg	89.3	n.s.

Table 6.1 – Summary of DL-Propranolol [4-^{3}H] Hydrochloride (14 nM) uptake in the presence of liposome formulations.

** denotes a very significant difference ($p < 0.01$) compared to the control; n.s. denotes no significant difference ($p > 0.05$) compared to the control (ANOVA).

6.3.2 Propranolol Transport

6.3.2.1 Effect of Temperature on Propranolol Transport

Propranolol transport was measured using Transwell inserts, in both directions – apical-to-basolateral and basolateral-to-apical. Propranolol is transported *via* the passive transcellular route; therefore, propranolol transport was determined at 4°C and 37°C in order to verify that propranolol is transported passively.

Propranolol transport was marginally decreased at 4°C in both directions of transport (Figure 6.9), however, the decrease was not significant ($p > 0.05$). For passive transport processes A – B and B – A transport should be similar; there was no significant difference between A – B and B – A (Figure 6.9) transport at 37°C ($p > 0.05$; t-test) indicating transport occurred by passive means. The ratio of transport (AB/BA) was 1.19 at 37°C.

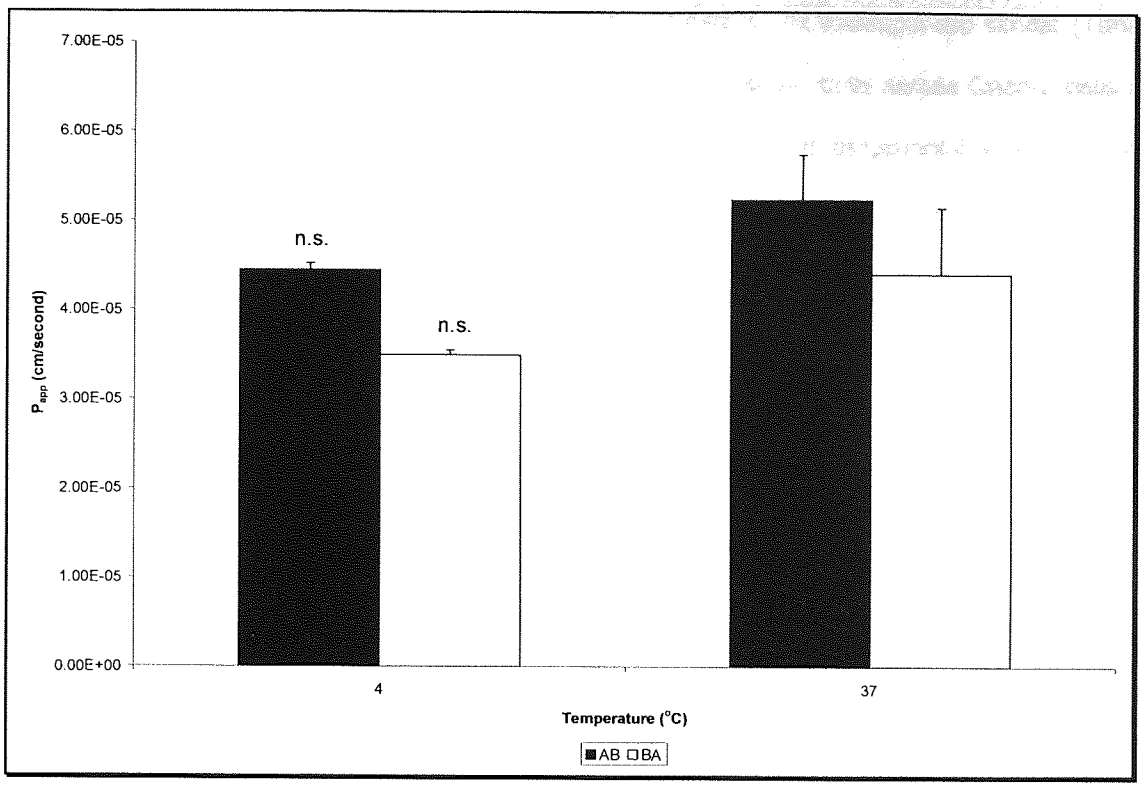

Figure 6.9 – The effect of temperature (4°C and 37°C) on DL-Propranolol [4-^{3}H] Hydrochloride (14 nM) transport (A – B and B – A) was measured using Transwell inserts (n = 4).

Caco-2 cells were seeded on Transwell inserts, passage 26-40 and used 14-21 days post-seeding, transport was measured over 60-minutes. n.s. denotes no significant difference ($p > 0.05$) compared to 37°C (t-test).

From the uptake results, it was expected that temperature would not affect propranolol transport, however, for both apical-to-basolateral transport and basolateral-to-apical transport (Figure 6.9), temperature had a slight affect on propranolol transport; a non-significant ($p > 0.05$) increase for both A – B and B – A was observed. The lower transport at 4°C is probably due to the fluidity of the membrane increasing with temperature resulting in higher transport rates (Cogburn *et al.*, 1991). The P_{app} values obtained for propranolol indicate that propranolol is transported *via* a passive process *i.e.* A – B and B – A transport are similar; however, as propranolol is a substrate for P-gp it might have been expected for B – A transport to be greater than A – B, this was not observed. A higher B – A transport was not measured because propranolol is essentially "transparent" to P-gp because it has high membrane permeability and therefore P-gp does no limit absorption (Collett *et al.*, 2004).

Mannitol is transported *via* the passive paracellular route (Chapter 5) and propranolol is transported *via* the passive transcellular route. As both mannitol and propranolol are transported

via passive processes it has been observed that A – B and B – A transport are similar (Table 6.2). However, these data show that transport *via* the paracellular route across Caco-2 cells is less than the transcellular route. Consequently, if mannitol and propranolol were actually transported *via* different mechanisms it was expected that propranolol transport would be greater in both directions compared to mannitol (Table 6.2).

	P_{app} (cm/second)	
	A – B	B – A
Mannitol	$6.8 \times 10^{-7} \pm 6.0 \times 10^{-8}$	$7.1 \times 10^{-7} \pm 6.8 \times 10^{-8}$
Propranolol	$52.5 \times 10^{-6} \pm 50.1 \times 10^{-7}$	$44.1 \times 10^{-6} \pm 74.4 \times 10^{-7}$
Significant Difference	$p < 0.0001$	$p = 0.0005$

Table 6.2 – Comparison of the apparent permeabilities of mannitol and propranolol at 37°C.
$P < 0.0001$ and $p = 0.0005$ are both considered extremely significantly different (t-test).

It was confirmed that although both mannitol and propranolol transport are transported *via* passive processes the processes involved are different. Mannitol transport is significantly lower in both directions compared to propranolol (Table 6.2). The lower permeability of mannitol across Caco-2 cell monolayers suggests it uses the paracellular route, consistent with its physicochemical properties [molecular weight of 180 Da and low lipophilicity (LogP -3.10 and MLogP -2.5)] (Meaney and O'Driscoll, 1999; Lipinski *et al.*, 2001). The higher P_{app} for propranolol and its high molecular weight of 259.3 and lipophilicity (LogP 2.53 – 3.46) suggest that this compound should utilise the passive transcellular route for transport.

Apparent permeability (P_{app}) coefficients are commonly used in literature; published P_{apps} for propanolol in the apical-to-basolateral directions include $13.5 \pm 0.34 \times 10^{-6}$ cm/second (Asano *et al.*, 2003); $23.4 \pm 2.8 \times 10^{-6}$ cm/second (Walgren *et al.*, 1998); $36.6 \pm 0.9 \times 10^{-6}$ cm/second (Markowska *et al.*, 2001); $41.9 \pm 4.3 \times 10^{-6}$ cm/second (Artursson, 1990); $43.0 \pm 3.6 \times 10^{-6}$ cm/second (Artursson and Magnusson, 1990); and $28.0 \pm 1.8 \times 10^{-6}$ cm/second (Walgren *et al.*, 1998) for basolateral-to-apical transport. The experimental P_{app} calculated from this investigation

is 52.5 x 10^{-6} ± 50.1 x 10^{-7} (Table 6.2; Figure 6.9) for apical-to-basolateral transport and 44.1 x 10^{-6} ± 74.4 x 10^{-7} for basolateral-to-apical transport. Both calculated P_{apps} are slightly higher than values stated above, however, the range of P_{apps} quoted for propranolol is quite varied: 13.5 ± 0.34 x 10^{-6} – 43.0 ± 3.6 x 10^{-6} cm/second. Although apparent permeability coefficients are frequently quoted there are discrepancies in calculated P_{app} values (Caldwell *et al.*, 1998). Caldwell *et al.*, (1998) calculated P_{app} coefficients for β-adrenoceptor antagonists and the P_{apps} showed significant discrepancies from those determined in other laboratories. They concluded that several experimental factors directly affect the P_{app} value including pH gradients, additional diffusion barriers (i.e. unstirred water layer and type of filter support), analyte concentration, detection method and possibly cell culture variations.

6.3.2.2 Effect of Liposome Formulations on Propranolol Transport

As stated in Chapter 5 TEER measurements were taken at the beginning and end of all transport experiments. Table 6.3 shows the TEER data as a percentage of the control at the end of the experiment. TEER values decreased following incubation with PC, PC:Cholesterol (16:4), DSPC, or DSPC:Cholesterol, 1000 μg (Table 6.3) indicating that monolayer integrity was compromised.

	TEER (% of Initial Value)	Significance (ANOVA)	
PC (1000 μg)	96.9 ± 1.4	n.s.	p > 0.05
PC:Cholesterol (1000 μg; 16:4)	94.2 ± 5.4	n.s.	p > 0.05
DSPC (1000 μg)	93.7 ± 2.8	n.s.	p > 0.05
DSPC:Cholesterol (1000 μg: 16:4)	93.7 ± 2.1	n.s.	p > 0.05

Table 6.3 – Percentage of initial TEER value at the end of the experiment, compared to the control at the end of the experiment.

The effect of liposome formulations (PC, PC:Cholesterol, DSPC and DSPC:Cholesterol, 1000 μg) on TEER measurements (ANOVA with Dunnett's post-test, Instat 3).

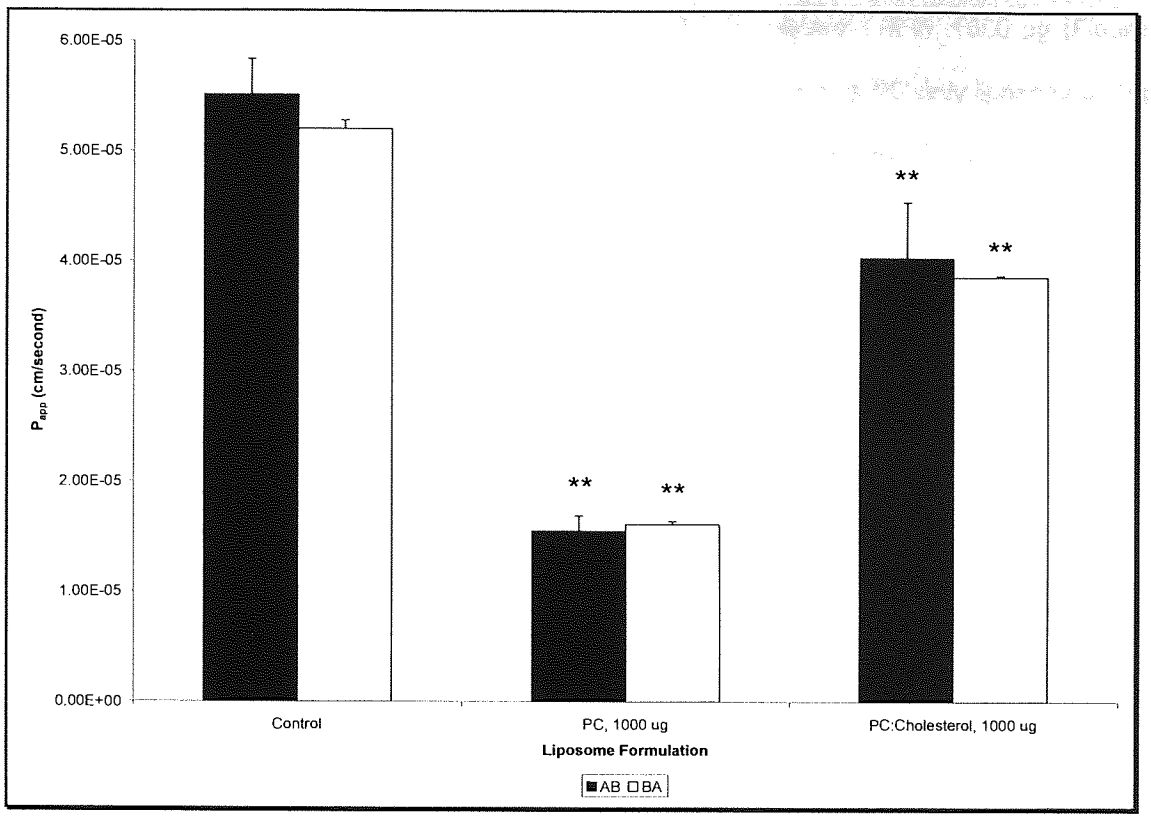

Figure 6.10 – DL-Propranolol [4-^{3}H] Hydrochloride (14 nM) transport in both the apical-to-basolateral and basolateral-to-apical direction in the presence of PC (1000 μg) and PC:Cholesterol (1000 μg; 16:4) liposomes was measured across Caco-2 cell monolayers.

Transport was measured at 37°C over 60 minutes, n = 4. Caco-2 cells were seeded onto Transwell inserts, passage 26-40 and used 14-21 days post seeding. ** denotes a very significant difference ($p < 0.01$) compared to the control (ANOVA).

The effect liposome formulations (PC, PC:Cholesterol (16:4), DSPC, and DSPC:Cholesterol (16:4) 1000 μg) had on propranolol transport was investigated over 60-minutes. In the presence of PC liposomes, (1000 μg) propranolol transport was significantly ($p < 0.01$) reduced in the apical-to-basolateral direction (Figure 6.10). Apparent permeability was reduced 3.5-fold, to P_{app} $15.5 \times 10^{-6} \pm 13.8 \times 10^{-7}$ cm/second. Propranolol transport was expected to similar to the control or slightly increased because the TEER value was reduced indicating that the cell monolayer had been compromised during the transport experiment (Table 6.3). Compounds that do not utilise the paracellular route of transport maybe able to utilise the paracellular route if the monolayer integrity is compromised and the tight junctions have become 'leaky', thus increasing their transport. However, a decrease in propranolol transport was measured (Figure 6.10).

Propranolol transport was reduced in the presence of PC:Cholesterol (16:4), 1000 μg (Figure 6.10). Although the decrease in cellular permeability was not as great as PC-only liposomes, the decrease was significant ($p < 0.01$). Propanolol transport was reduced to 73.3 per cent of the control. Additionally, the inclusion of cholesterol with PC further enhanced the effect PC-only liposomes had on mannitol permeability (Section 5.3.2). The observed difference between PC and PC:Cholesterol on propranolol permeability is probably due to cholesterol stabilising the lipid bilayers resulting in a propanolol permeability being similar to the more rigid DSPC liposome formulation (Figure 6.11).

The effect of the liposome formulations on propranolol transport was measured in the basolateral-to-apical direction. PC, 1000 μg significantly ($p < 0.01$) reduced propranolol transport (Figure 6.10). PC reduced propranolol to approximately the same extent in both directions of transport – 28.2 per cent reduction for A – B transport and 31.0 per cent reduction for B – A transport (Figure 6.10).

Propranolol transport (B – A) was reduced to 74.3 per cent of the control in the presence of PC:Cholesterol, (16:4) 1000 μg (Figure 6.10). The reduction in cellular permeability of propranolol is considered significant ($p < 0.01$) and is comparable to the reduction in A – B propranolol transport (73.3 per cent; Figure 6.10).

As an additional comparison, PC was replaced with the lipid DSPC. Propranolol transport (A – B) was decreased to approximately 74.1 per cent in the presence of DSPC, 1000 μg (Figure 6.11), which is considered significant ($p < 0.05$). However, cellular permeability was not reduced to the same extent as with PC, 1000 μg liposomes (Figure 6.10).

The inclusion of cholesterol into the liposome formulation had no additional effects compared to DSPC-only liposomes. DSPC:Cholesterol, 16:4 liposomes reduced propranolol transport to 72.1 per cent of the control (Figure 6.11) which is considered significant ($p < 0.05$).

DSPC:Cholesterol and PC:Cholesterol reduced propranolol transport (A – B) by a similar amount, 72.1 per cent and 73.3 per cent relatively. No additional effects were observed following the inclusion of cholesterol in the liposome formulation possibly because DSPC is a high transition temperature lipid and therefore will have rigid bilayers at 37°C because it is below its transition temperature, both DSPC and DSPC:Cholesterol liposome formulations also had similar effects on mannitol transport (Section 5.3.2).

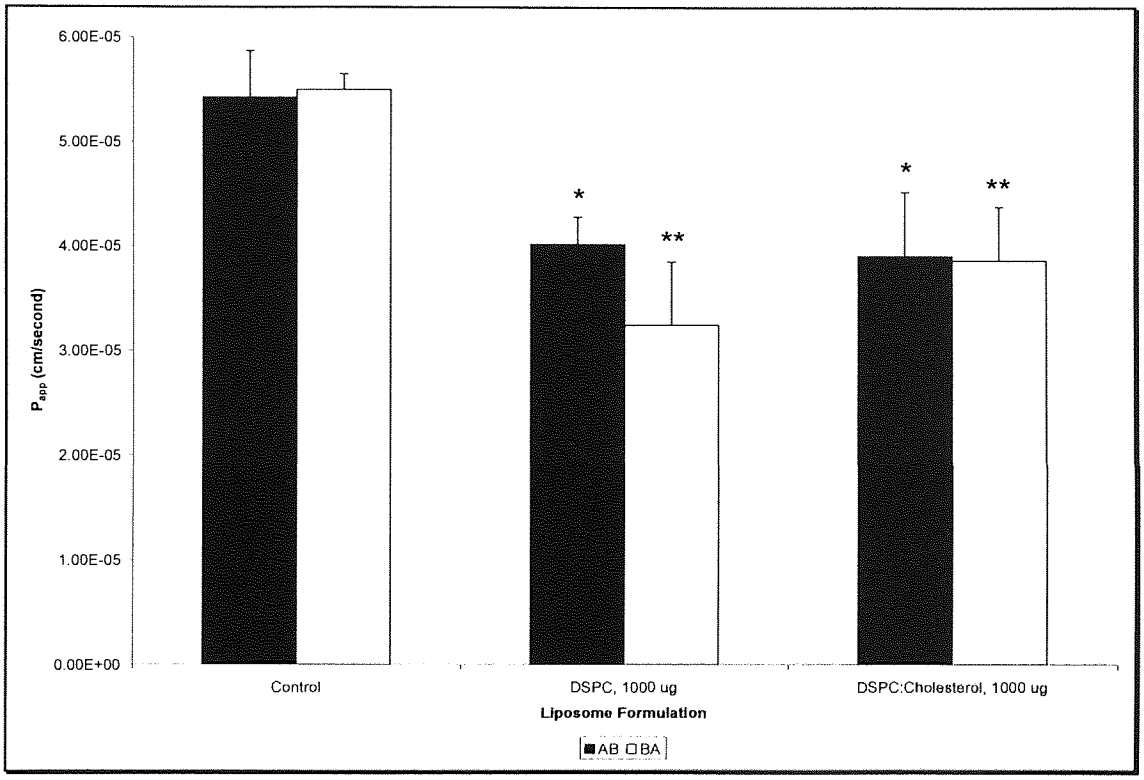

Figure 6.11 – DL-Propranolol [4-^{3}H] Hydrochloride (14 nM) transport in the apical-to-basolateral and basolateral-to-apical direction in the presence of DSPC (1000 μg) and DSPC:Cholesterol (16:4; 1000 μg) liposomes was measured across Caco-2 cell monolayers.

Transport was measured at 37°C over 60 minutes, n = 4. Caco-2 cells were seeded onto Transwell inserts, passage 26-40 and used 14- 21 days post seeding. ** denotes a very significant ($p < 0.01$) difference; * denotes a significant difference ($p < 0.05$) compared to the control (ANOVA).

Replacement of PC with DSPC resulted in a decrease in propranolol transport in the basolateral-to-apical direction. DSPC, 1000 μg significantly ($p < 0.01$) reduced propranolol transport by 59.0 per cent (Figure 6.11). The presence of DSPC:Cholesterol, (16:4), 1000 μg, liposomes resulted in a 70.3 per cent decrease in propranolol transport in the B – A direction

which is not dissimilar to the 72.1 per cent reduction in propranolol transport observed in the A – B direction (Figure 6.11).

In agreement with the A – B propranolol transport data and mannitol (Section 5.3.2) PC:Cholesterol, DSPC and DSPC:Cholesterol liposomes had a similar influence on permeability compared to PC-only liposomes. The observed differences in permeability are due to bilayer rigidity and stability. DSPC is a high transition lipid consequently at 37°C the bilayers will be in the rigid gel phase and cholesterol is known to improve stability. In contrast, PC-only liposomes will be in the liquid crystal state (Figure 3.5). The differences in rigidity will influence the interaction with the Caco-2 cell monolayer and thus the differences in propranolol transport.

The decrease in propranolol transport that was brought about in the presence of PC, PC:Cholesterol, DSPC and DSPC:Cholesterol was not in agreement with the TEER data (Table 6.3) – a small reduction was measured. The decrease in propranolol transport was not predicted from the mannitol data (Chapter 5) because mannitol transport was enhanced in the presence of the liposome formulations, thus an increase in propranolol transport was also expected. There are two possible interactions the liposome formulations can have with the Caco-2 cell line: firstly, they can be taken up intact by the monolayer, and secondly, the component lipids might partition into the cell membrane (Westergaard and Dietschy, 1976; Udata *et al.*, 2003). It has not been confirmed from this study if intact liposomes are taken up by the Caco-2 cell line. Propranolol is a lipophilic compound with a high permeability (Taylor *et al.*, 1985; Krishna *et al.*, 2001), consequently propranolol diffusion into the liposome formulations cannot be ruled out. If propranolol diffused into the liposome formulations the amount of propranolol available to the Caco-2 cell monolayer would be decreased. The possibility that propranolol diffused into the liposome formulations is re-iterated by the fact that all liposome formulations reduced propranolol transport to approximately the same extent in both directions of transport (Figure 6.10 – Figure 6.11). To rule out the possibility that propranolol has entered the lipophilic bilayer of the liposomes both uptake and transport experiments would need to be repeated using liposomes loaded with propranolol. Propranolol transport in both directions was significantly

reduced in the presence of simulated intestinal fluid; furthermore, propranolol permeability remained similar in both directions (FaSSIF) (Ingels *et al.*, 2004). The reduction in propranolol transport was not due to toxicity towards Caco-2 cells because FaSSIF has been shown to be compatible with the Caco-2 cell monolayers (Ingels *et al.*, 2002). FaSSIF contains sodium taurocholate and lecithin and the decrease in transport may have been due to propranolol being encapsulated or interacting with the sodium taurocholate/lecithin mixed micelles (Ingels *et al.*, 2004).

Propranolol transport was significantly decreased in the presence of all formulations (Figure 6.10 and Figure 6.11) compared to only PC, 500 μg and 1000 μg in the uptake studies (Figure 6.7). Therefore, if the decrease was due to propranolol diffusion into the liposome formulations a decrease in all uptake experiments would have been expected. A slight decrease was observed in the presence of all liposome formulations (Figure 6.7 and Figure 6.8); however, only a significant decrease in propranolol uptake was observed in the presence of PC, 500 μg and 1000 μg. The observed differences between the uptake and transport experiments may be due to the time period the experiments were measured over – the uptake experiments were measured over 10 minutes compared to 60 minutes for the transport experiments (Section 6.2). Consequently, the uptake experiments may have been too short for a significant amount of propranolol to diffuse into the liposomes and thus significantly reduce propranolol uptake. Moreover, both propranolol uptake and transport were decreased to a greater extent in the presence of PC-only liposomes; therefore, propranolol may diffuse into PC-only liposomes to a greater extent compared to the other liposome formulations used, thus reducing propranolol uptake and transport the most.

As previously stated in Section 5.3.2 and Section 5.4 liposomes have the potential to interact with the Caco-2 cell monolayer in a number of ways – phospholipid exchange and membrane rafts. The use of SUVs and LUVs in replacement of MLVs needs to be investigated and the mechanism of liposome action on the Caco-2 cell monolayer needs to be established (Section 7.4).

6.4 Conclusion

From the uptake studies, it can be concluded that none of the four liposome formulations enhanced propranolol uptake. PC had a detrimental effect on overall uptake. PC:Chol, (16:4), DSPC and DSPC:Chol (16:4) had neither beneficial or detrimental effect on propranolol uptake as propranolol uptake was not significantly altered. All four liposome formulations significantly reduced propranolol transport in both directions (Figure 6.10 – Figure 6.11). The presence of cholesterol in PC formulations had a greater impact on both propranolol uptake and transport compared to DSPC formulations. This difference can be explained by the fact that cholesterol alters membrane fluidity and during experiments the temperature is 37°C and PC is at a temperature higher than its transition temperature, whereas DSPC is at a temperature below its transition temperature; therefore, the liposome bilayers have different rigidities and incorporation of cholesterol will influence rigidity of the bilayers (Valenti *et al.*, 2001; Cócera *et al.*, 2003; Sinico *et al.*, 2005). The different rigidity of the liposome bilayers may alter the interaction between the liposomes and the Caco-2 cell monolayer and the more fluid PC bilayers may have an increased detrimental effect compared to DSPC bilayers.

From the data obtained it can be concluded that PC is a less suitable lipid compared to DSPC because DSPC had less detrimental effects on propranolol uptake and transport. Consequently, DSPC liposome formulations are more appropriate liposome formulations that are intended to aid the delivery of drugs that utilise the passive transcellular route. The advantage of DSPC as a lipid compared to PC may be because DSPC has a higher transition temperature (57°C) compared to PC (below 0°C); consequently DSPC forms more rigid bilayers. However, the experiments must be repeated in the presence of propranolol loaded liposomes before either PC or DSPC are excluded as potential permeation enhancers for use with drugs that use the passive transcellular route.

Chapter 7

Effects of Liposomal Formulations on the Carrier-Mediated Route

7. Effects of Liposomal Formulations on the Carrier-Mediated Route

7.1 Introduction

In contrast to passive transcellular transport, active transport requires energy expenditure, that is generated from the hydrolysis of ATP or high-energy compounds on the surface of the protein serving as a pump. The carrier-mediated pathway is saturable, is sensitive to temperature changes, presence of competitors and metabolic inhibitors including sodium azide. For more detailed information regarding active transport see Section 1.3.2.2.

7.1.1 Amino Acid Absorption

Amino acids are the building blocks of life; twenty common amino acids exist, all with a common structure and a differing side-chain making them unique. Amino acid and dipeptide absorption is vital because the protein humans consume is degraded to the basic components, those being amino acids, di- and tri-peptides. Amino acids, dipeptides and tripeptides are transported *via* different transporters – numerous amino acid transporters exist. Amino acids are transported into cells *via* specific proteins on the cell surface that specifically recognise the amino acids and bind with them and then transport the amino acids between the extracellular medium and inside the cell.

Initially, the amino acid transporters were defined using the following criterion:

i. The type of amino acid side-chain that the protein transporter is capable of moving across the membrane, according to substrate specificity and kinetic properties.

ii. The thermodynamic properties of transport.

Expression of transporters varies from cell to cell; different amino acid transporters are expressed on different cells. Common or almost ubiquitous systems include systems A, ASC, L, y^+ and X^-_{AG}, and tissue-specific transport systems include $B^{o,+}$, N^m, and $b^{o,+}$ (Palacin *et al.*, 1998; Refer to Section 7.1.2).

7.1.1.1 Molecular Biology and Amino Acid Transporters

More than twenty amino acid transporters have been isolated using molecular biology techniques. These transporters belong to four protein families. Additionally, the gene (*γBAT*) for responsible cystinuria has been identified; cystinuria is an inherited disease that affects amino acid transport. The nomenclature of amino acid transporters has thus been updated and modified accordingly.

7.1.2 Glutamine

The probe chosen to investigate the active transport route across gut epithelial cells was glutamine (Figure 7.1). Glutamine is one of the twenty amino acids that the human body utilises in protein synthesis. Glutamine is classified as a non-essential amino acid. However, it is the most abundant amino acid in the body, comprising approximately 60 per cent of the circulating amino acids (Epler *et al.*, 2003). Glutamine is readily synthesised by various tissues including skeletal muscles, liver and adipose tissue. Research suggests that glutamine is conditionally essential when the metabolic demand for glutamine exceeds the amount available in the free glutamine pool and that, which can be provided by *de novo* synthesis. Skeletal muscles are the primary site for glutamine synthesis and storage. Amino-acid transport is important because enterocytes require glutamine, together with other amino acids for cellular metabolism (Wasa *et al.*, 2004). The rapid turnover rate of enterocytes results in glutamine being an essential metabolic precursor in nucleotide biosynthesis (glutamine provides the nitrogen for the nucleotide bases) (Costa *et al.*, 2000), glucose and amino sugar biosynthesis, protein synthesis and for glutathione homeostasis in these cells (Bode, 2001; Wasa *et al.*, 2004).

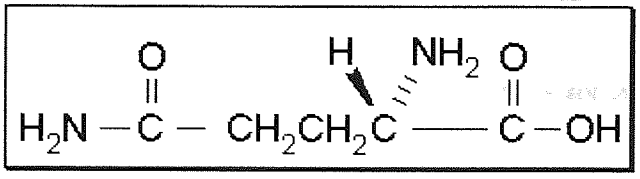

Figure 7.1 – Structure of glutamine.

Uptake of glutamine is mediated *via* both sodium (Na^+)-dependent and Na^+-independent saturable transporters (Souba *et al.*, 1992; Costa *et al.*, 2000). Several systems have been reported to transport glutamine these include B^o, $B^{o,+}$, y^+L, A, and N (Na^+-dependent systems) and include $b^{o,+}$ and L (Na^+-independent systems) (Costa *et al.*, 2000; Bode *et al.*, 2001). Approximately 90 per cent of glutamine transport is by the Na^+-dependent transport System B^o (Costa *et al.*, 2000; Pan *et al.*, 2002). Alanine, leucine and phenylalanine inhibit glutamine uptake (Costa *et al.*, 2000).

Glutamine is transported *via* active carrier-mediated transporters; however, a passive diffusion component exists (Souba *et al.*, 1992; Costa *et al.*, 2000). In Caco-2 cells, passive glutamine diffusion contributes up to 30 per cent of the Na^+-independent uptake for 1 mM glutamine (Costa *et al.*, 2000). At low glutamine concentrations (below 1 mM) passive diffusion is limited, whereas at high glutamine concentrations (above 3 mM) passive diffusion plays a major role in glutamine uptake (Costa *et al.*, 2000).

Glutamine transport by the basolateral membrane has been characterised using rabbit epithelial cells (Wilde and Kilberg, 1991). Both Na^+-dependent (System A) and Na^+-independent mediate glutamine transport on the basolateral membrane (Wilde and Kilberg, 1991; Bode, 2001). Glutamine transporters are located on the basolateral membrane to ensure a continuous supply of glutamine to the enterocyte in the post-absorptive state (Wilde and Kilberg, 1991; Epler *et al.*, 2003).

7.1.3 Objective

The objective of this chapter was to determine the effect of liposomal formulations on the carrier-mediated route where glutamine was the chosen probe. Glutamine uptake and transport across Caco-2 cell monolayers was measured using plastic 24-well plates and Transwell inserts.

7.2 Material and Methods

General material and methods were previously described in Chapter 2. Statistical test performed include the one-way analysis of variance (ANOVA with Dunnett's post-test) and unpaired t-test using *Graphpad Instat 3.0*). To measure ^{14}C-Glutamine (2 μM) uptake, Caco-2 cells were seeded on 24-well plates (Section 2.3.3.1 and Section 2.3.8) or seeded on Transwell inserts to measure transport (Section 2.3.3.2 and Section 2.3.9). ^{14}C-Glutamine (2 μM) uptake was measured over ten minutes at 37°C unless otherwise stated. ^{14}C-Glutamine (2 μM) transport was measured over 60-minutes. Inhibitors of glutamine uptake were prepared using transport media – serine (1 mM); alanine (1 mM); phenylalanine (1 mM), Gly-Tyr (1 mM); Ala-Ala (1 mM); des Tyr Enkephalin (1 mM); sodium azide (15 mM) and Triton X-100 (0.05 % v/v).

7.3 Results and Discussion

7.3.1 Glutamine Uptake by Caco-2 Cells

Glutamine uptake was measured over time (5, 10, 20, 30, 45 and 60-minutes) (Figure 7.2); as expected glutamine uptake increased with time. The results show an initial linear phase (0 – 30 minutes) followed by a slower rate of uptake (30 – 60 minutes). The decrease in rate of uptake may be due to a decreased concentration gradient across the membrane over time or because the amino acid transporters became saturated thus reducing uptake. For future experiments that are not performed over time an incubation time of ten minutes was chosen as that duration falls within the initial linear uptake phase.

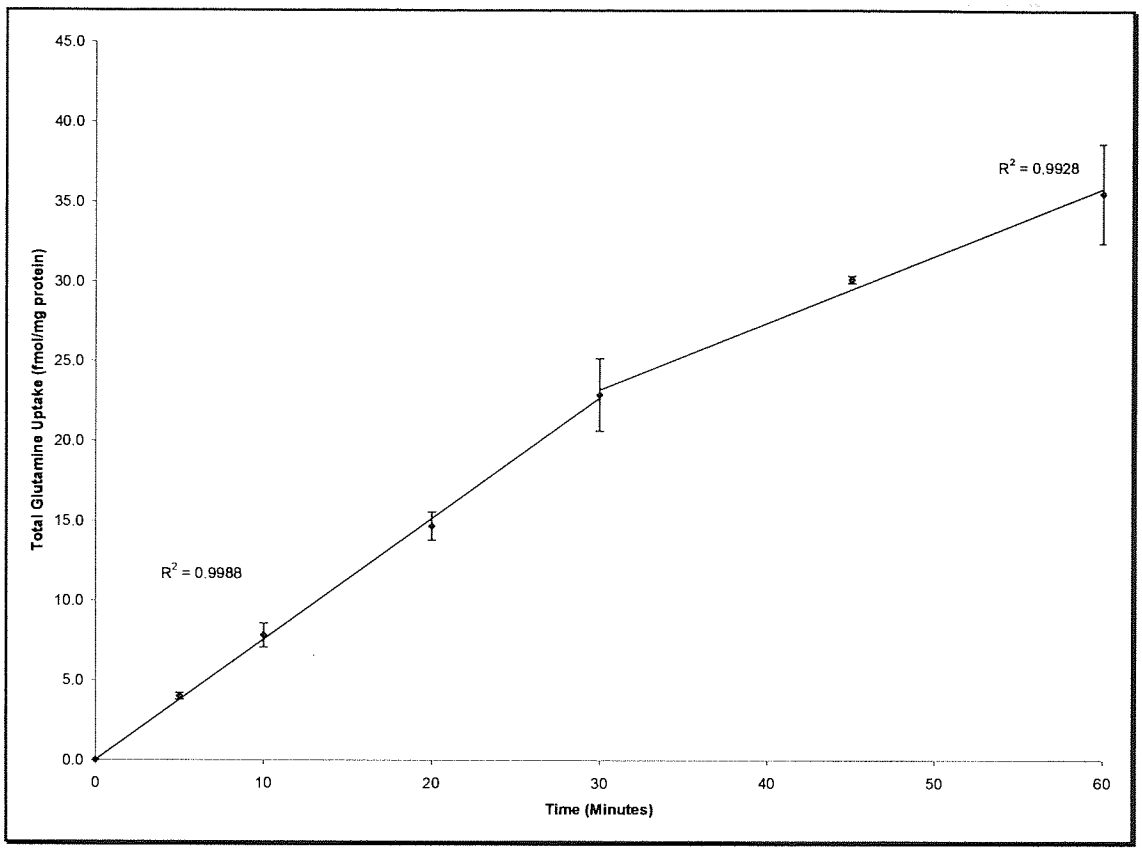

Figure 7.2 – Uptake of ^{14}C-Glutamine (2 μM) was measured over time.

Caco-2 cells were seeded onto plastic 24-well plates between passage 26 and 40. The Caco-2 cell monolayers were used 7-days post seeding and uptake was measured at 37°C. Data are mean and standard deviations of results from four wells.

Glutamine uptake was calculated over a range of concentrations (Figure 7.3). Glutamine uptake occurs *via* both activate and passive uptake processes (Souba *et al.*, 1992; Costa *et al.*, 2000). This study confirmed that increasing glutamine concentration caused an increase in glutamine uptake. Uptake was linear up to 20 mM glutamine, followed by a plateau. Active uptake is a saturable pathway therefore the plateau at higher glutamine concentrations may be due to the saturation of the active pathway (Figure 7.3). To eliminate the active component of glutamine uptake and therefore calculate the passive component, the experiment was repeated in the presence of an inhibitor of glutamine uptake – 1 mM serine (Figure 7.4 and Figure 7.5). Figure 7.4 shows that serine (1 mM) is an inhibitor of glutamine when glutamine concentration is ≥ 7.5 mM. Serine (1 mM) was present in excess and reduced glutamine uptake by eliminating active uptake (Figure 7.4).

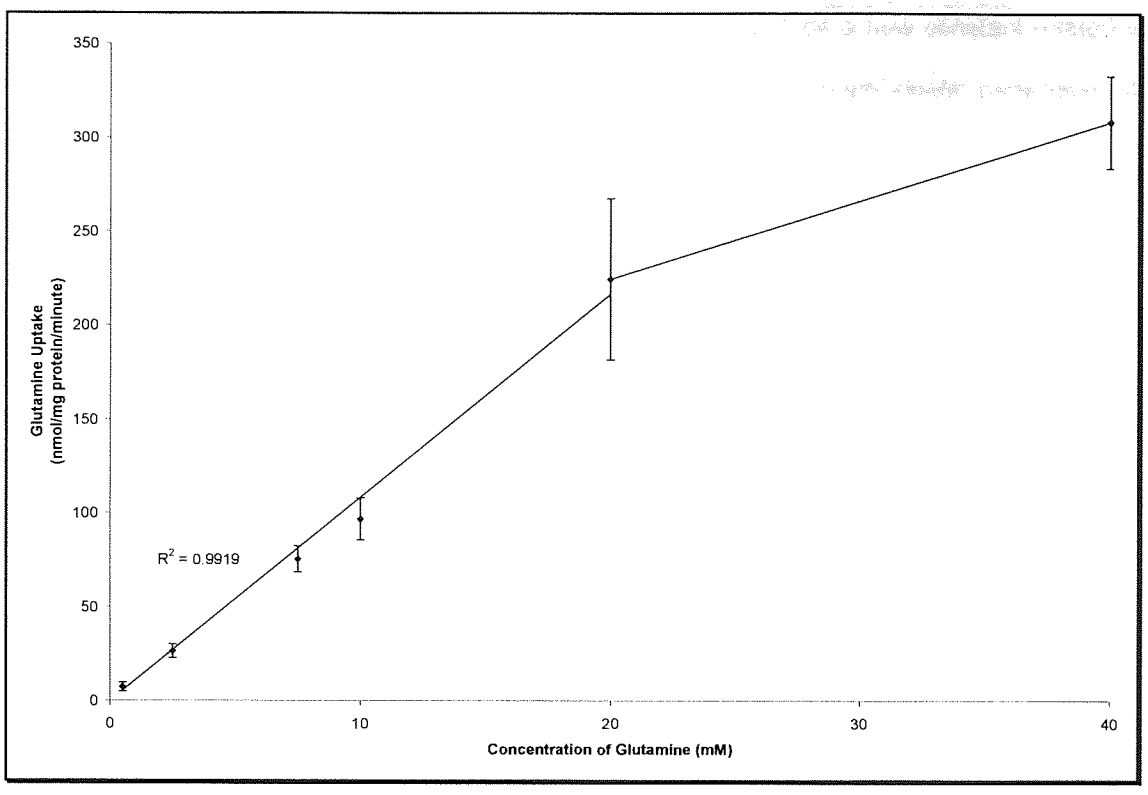

Figure 7.3 – The effect of increasing glutamine concentration on ^{14}C-Glutamine (2 μM) uptake was measured using Caco-2 cells.

Caco-2 cells, passage 26-40, were seeded onto plastic 24-well plates and used 7-days post seeding. ^{14}C-Glutamine uptake was measured over 10 minutes at 37°C. Data are mean and standard deviations of results from four wells.

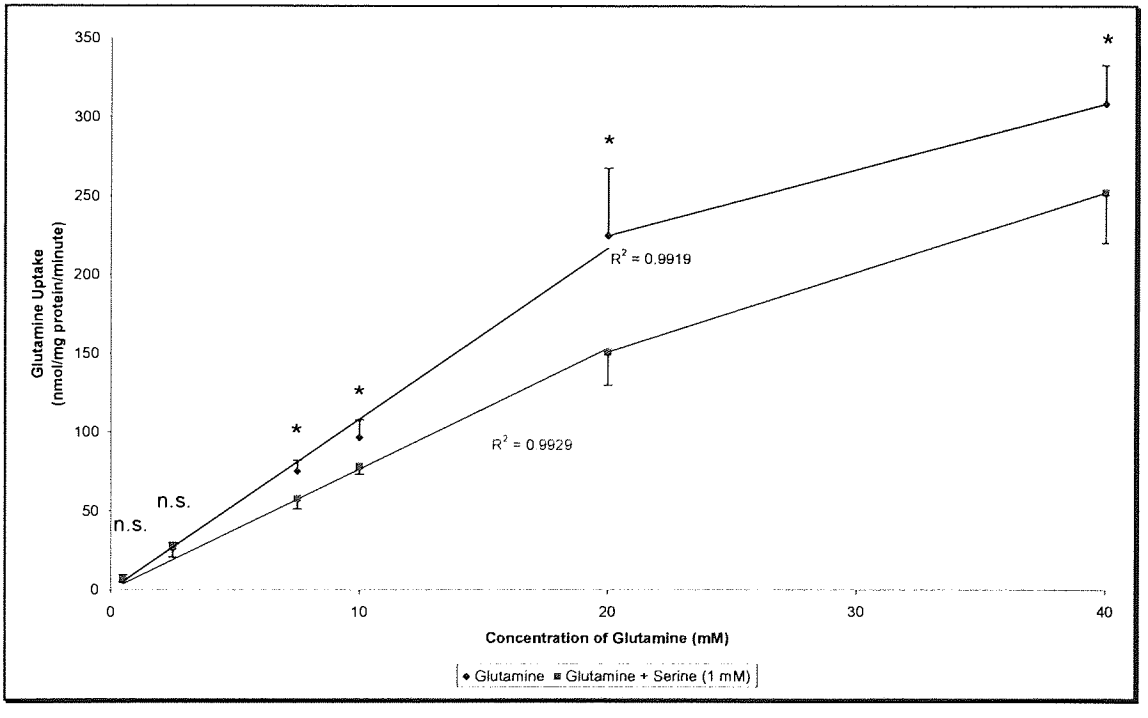

Figure 7.4 – Uptake of ^{14}C-Glutamine (2 μM) in the presence of 1 mM serine was measured using Caco-2 cell monolayers.

Caco-2 cells, passage 26-40, were seeded onto plastic 24-well plates and used 7-days post seeding. Uptake was measured at 37°C over ten minutes. Data are mean and standard deviations of results from four wells. * Denotes a significant ($p < 0.05$) difference compared to glutamine alone; n.s. denotes no significant difference compared to glutamine alone (t-test).

The Michaelis-Menten equation (Appendix 1) was adapted to include a new constant related to passive uptake (K_d) (Equation 7.1) and was therefore used to calculate kinetic parameters of ^{14}C-Glutamine uptake.

$$V = \left(\frac{(V_{max} \times [S])}{(K_m + [S])} \right) + (K_d \times [S])$$

Equation 7.1 – Adapted Michaelis-Menten equation for total uptake to include a passive component (K_d).

Where:

V	=	Uptake (moles/mg protein/minute)
V_{max}	=	Maximum uptake (moles/mg protein/minute)
[S]	=	Initial substrate concentration (mM)
K_m	=	Michaelis constant (mM)
K_d	=	Passive uptake constant (nmol/mg protein/minute/mM)

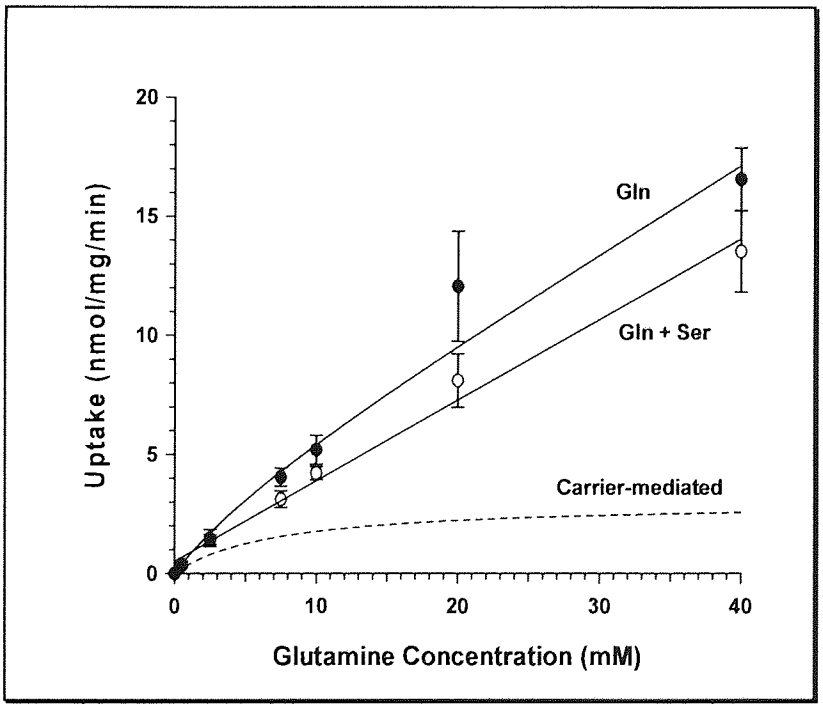

Figure 7.5 – Uptake of ^{14}C-Glutamine (2 μM) in the presence of serine.

The active component of uptake has been calculated using Fig P software. Caco-2 cells, passage 26-40 were seeded onto plastic 24-well plates and used 7-days post seeding. ^{14}C-Glutamine was measured over ten minutes at 37°C. Data are mean and standard deviations of results from four wells.

Uptake of ^{14}C-Glutamine involved both carrier-mediated (saturable) and passive processes (non-saturable) (Figure 7.5). Using Fig P software, V_{max} was calculated to be 3.0 nmol/mg protein/minute, K_m 7.1 mM and K_d 0.36 nmol/mg protein/minute/mM (Figure 7.5) with serine (1 mM) largely eliminating the active component. V_{max} values for glutamine reported in the literature include 1.39 ± 0.12 nmol/mg protein/minute (Pan *et al.*, 2002); 3.213 ± 0.025 nmol/mg protein/minute (Costa *et al.*, 2000) and 3.730 ± 0.316 nmol/mg protein/minute (Wasa *et al.*, 2004) thus the V_{max} value calculated here falls within the values found within the literature.

7.3.1.1 Effect of Temperature on Glutamine Uptake

Glutamine uptake is an active process; active processes are sensitive to temperature changes, where a reduction in temperature reduces active processes. Uptake of glutamine was measured at 4°C, 20°C and 37°C (Figure 7.6). Glutamine uptake increased with increasing temperature confirming that glutamine uptake is an active transport process. Increasing the temperature from 4°C to 37°C resulted in a 4-fold increase in glutamine uptake (Figure 7.6). Reducing temperature from 37°C to 4°C significantly reduced (approximately 4-fold) both glutamine uptake and transport (Section 7.3.2.1) indicating an active component. However, a 2-fold decrease in mannitol transport (Section 5.3.1) was observed following a decrease in temperature; consequently, a greater than 4-fold decrease in glutamine uptake and transport was expected. Active and passive components contribute to glutamine uptake and transport and the reduction in temperature will only reduce the active component, consequently the passive component appears to be important in glutamine uptake and transport. Although, a greater reduction in glutamine uptake and transport was expected compared to the mannitol data, the 4-fold decrease is comparable to the 4.4-fold reduction in the active uptake of Gly-L-Pro following a reduction in temperature (Biggs, 2003).

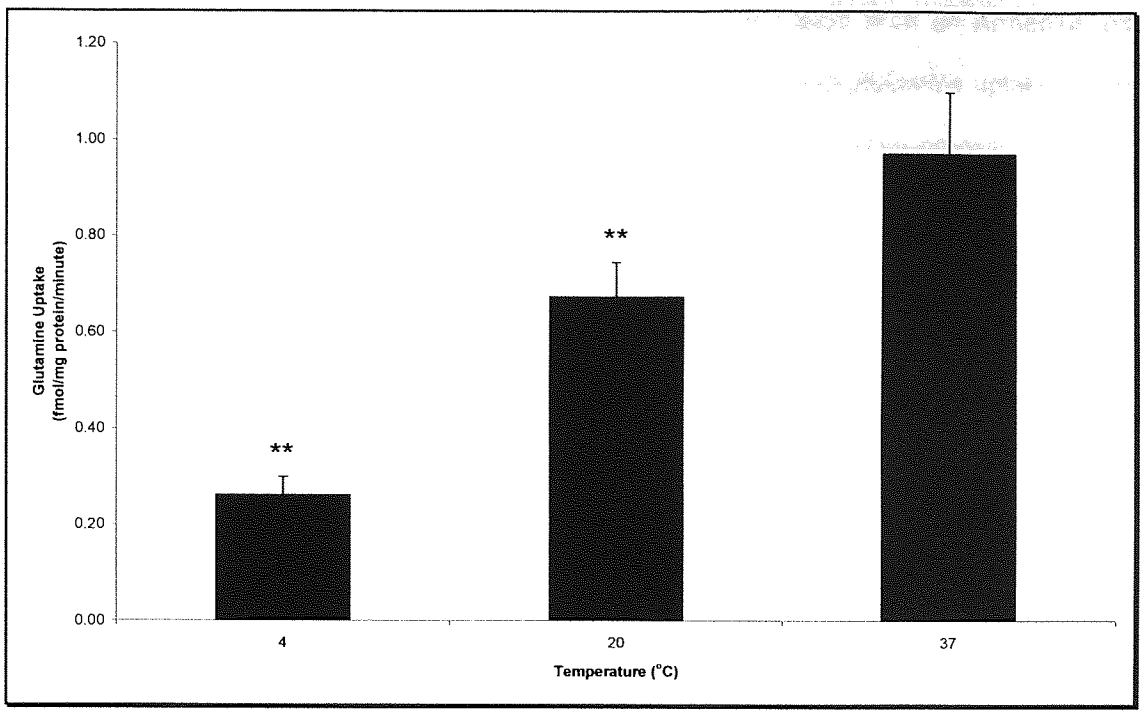

Figure 7.6 – Uptake of ^{14}C-Glutamine (2 μM) was measured at different temperatures using Caco-2 cell monolayers.

Caco-2 cells were seeded onto plastic 24-well plates, monolayers were used 7-days post seeding. ^{14}C-Glutamine uptake was measured over ten minutes at 37°C. Data are mean and standard deviations of results from four wells. ** denotes a very significant difference ($p < 0.01$) compared to 37°C (ANOVA).

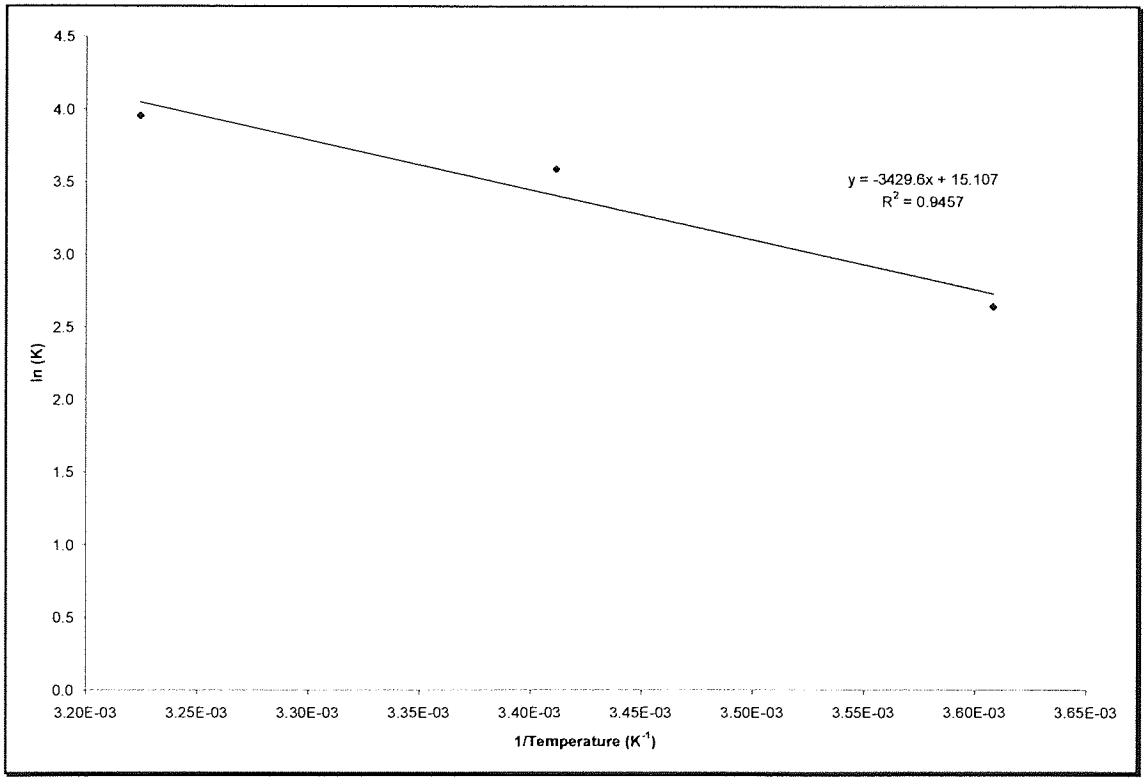

Figure 7.7 – Arrhenius plot for ^{14}C-Glutamine (2 μM).

The activation-energy for ^{14}C-Glutamine was calculated from the Arrhenius plot using the temperature data.

The activation energy (E_a) for ^{14}C-Glutamine uptake was calculated from an Arrhenius plot (Figure 7.7) giving a calculated E_a of 28.51 kJ/mol – further indicating glutamine uptake has an active component as the E_a is higher than the E_a expected for a passive process (less than 16.38 kJ/mol) (Hopfer and Hogget, 1981).

7.3.1.2 Effect of Competitors and Metabolic Inhibitors on Glutamine Uptake

To verify that glutamine uptake is an active process, glutamine uptake was measured in the presence of metabolic inhibitors because they decrease active uptake processes. Sodium azide (15 mM) was used to deplete ATP, an energy source of active processes, thus decreasing active processes. Uptake of glutamine was significantly ($p < 0.01$) reduced in the presence of sodium azide (Figure 7.8) thus confirming that uptake of glutamine is active. Sodium azide reduced the active uptake of glutamine, thereby reducing glutamine uptake by 27.1 per cent. Glutamine uptake may have been reduced further in the presence of a glycolysis inhibitor, for example 2-deoxy-D-glucose. Depleting both ATP stores using sodium azide and inhibiting glycolysis using 2-deoxy-D-glucose would have depleted the Caco-2 cell monolayer of energy reserves thus reducing active processes.

The effect of Triton X-100 (0.05 % v/v) on glutamine uptake was determined because Triton X-100 is toxic to Caco-2 cells. Therefore, uptake of glutamine should be reduced because in the presence of Triton X-100 the Caco-2 cells will either be non-viable or in the process of becoming non-viable cells and consequently all uptake and transport activities will cease. This is illustrated in Figure 7.8, Triton X-100 significantly ($p < 0.01$) reduced uptake of glutamine.

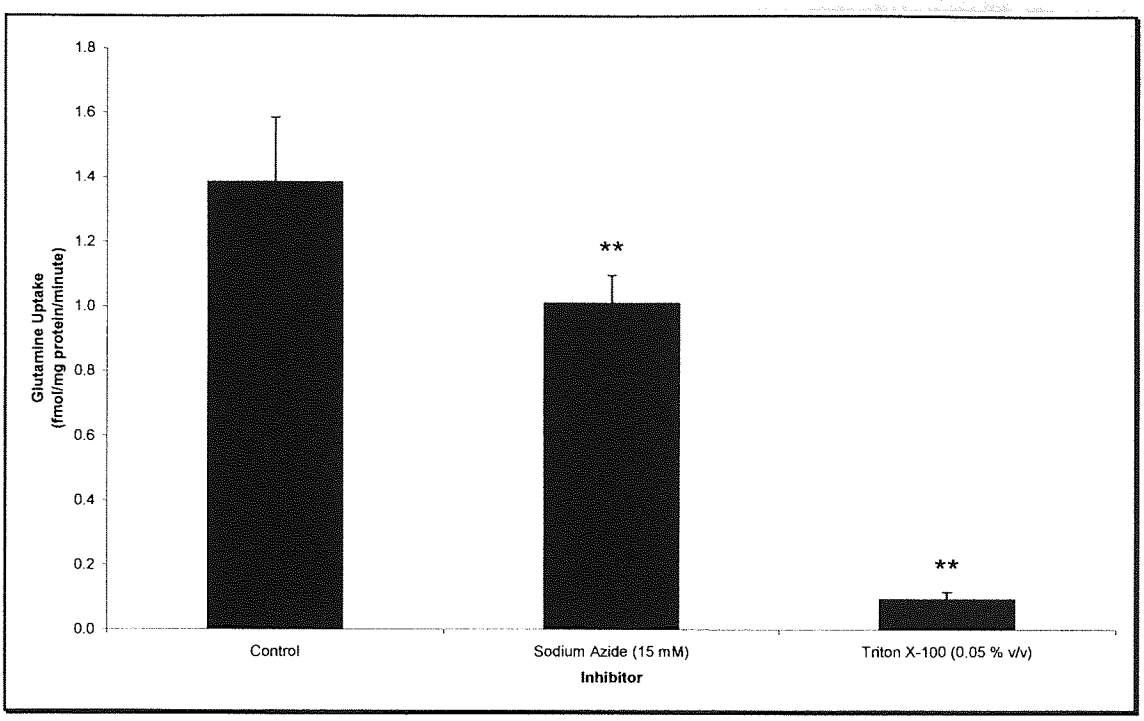

Figure 7.8 – Uptake of ^{14}C-Glutamine (2 μM) into Caco-2 cell monolayers was measured in the presence of sodium azide (15 mM) and Triton X-100 (0.05 % v/v).

Caco-2 cells were seeded onto plastic 24-well plates between passage 26 and 40, monolayers were used 7-days post seeding. Uptake was measured over ten minutes at 37°C. Data are mean and standard deviations of results from four wells. ** Denotes a very significant difference ($p < 0.01$) compared to the control (ANOVA).

Several transport systems exist in the human intestine; these include the amino acid transport system, the dipeptide and tripeptide transport systems and the monocarboxylic acid transporter. Glutamine is transported using the amino acid transport system. Amino acids that use the same transport system will compete for the transporter, resulting in reduced glutamine transport and uptake. Compounds that do not use the amino acid transporter and use alternative transport systems (*e.g.* the dipeptide transporter) will have no impact on the rate of glutamine uptake.

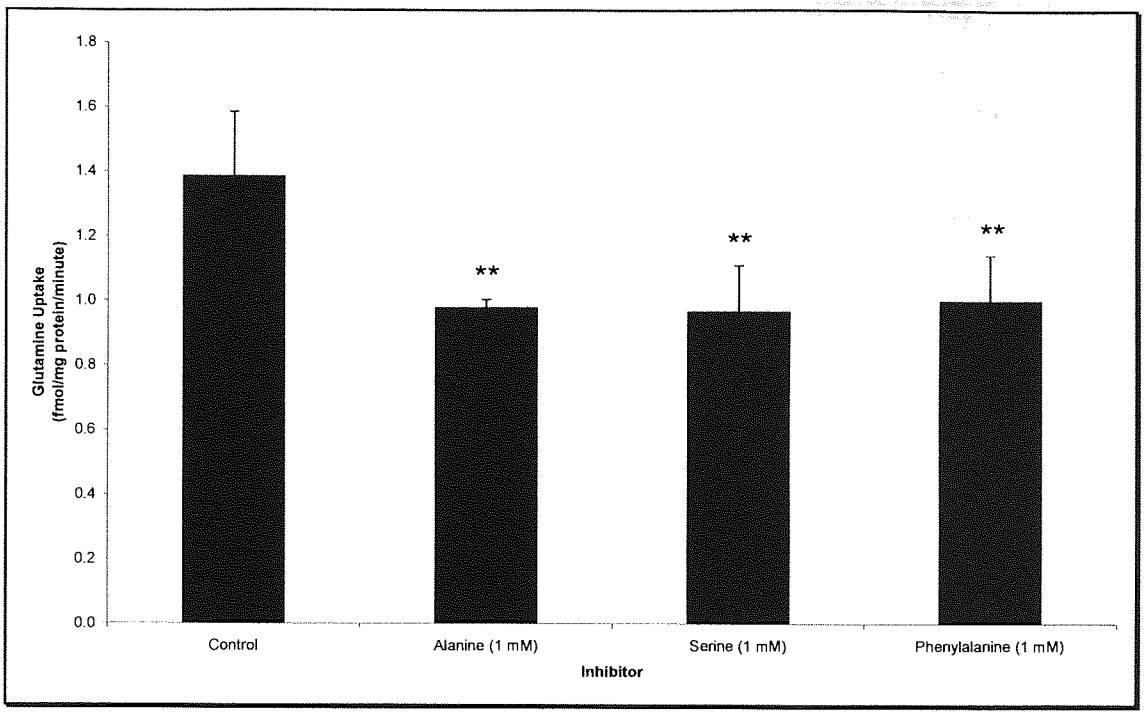

Figure 7.9 – Uptake of ^{14}C-Glutamine (2 μM) into Caco-2 cell monolayers was measured in the presence of alanine, serine and phenylalanine (1 mM).

Caco-2 cells, passage 26-40 were seeded onto plastic 24-well plates and used 7-days post seeding. Uptake was measured at 37°C over ten minutes. Data are mean and standard deviations of results from four wells. ** Denotes a very significant ($p < 0.01$) difference compared to the control (ANOVA).

Alanine, serine and phenylalanine (1 mM) were chosen as competitors for glutamine uptake; Figure 7.9 shows that all three competitors significantly ($p < 0.01$) reduced glutamine uptake, where alanine, serine and phenylalanine all inhibited ^{14}C-Glutamine uptake by approximately 30 per cent. This is in agreement with Costa *et al.*, 2000, who showed that alanine and phenylalanine significantly inhibited glutamine uptake.

Compounds transported using different transport systems from the amino acid transport system should have no effect on the rate of glutamine uptake. Gly-Try and Ala-Ala and des-Tyr-Leucine Enkephalin (Gly-Gly-Phe-Leu) are not transported *via* the amino acid transporters; therefore, they should have no effect on glutamine uptake. Figure 7.10 illustrates that the Gly-Try, Ala-Ala or des-Tyr-Leucine Enkephalin (Gly-Gly-Phe-Leu; 1 mM) had no effect on the rate of glutamine uptake; the rate of glutamine uptake is not significantly different from the control ($p > 0.05$).

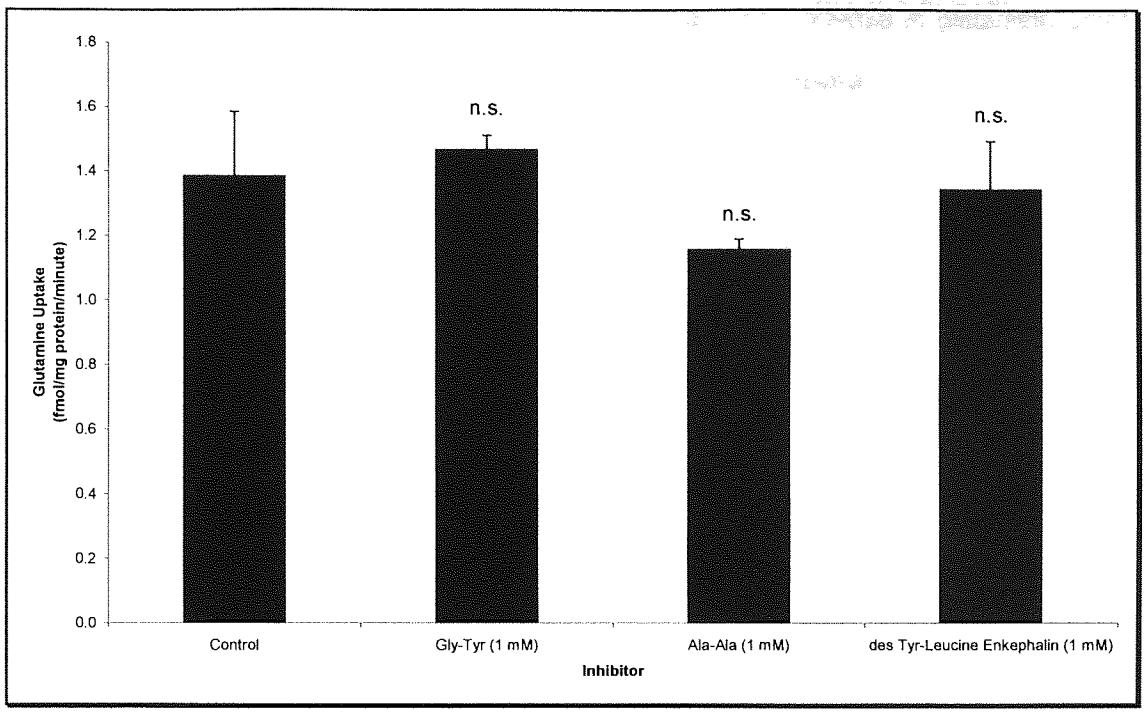

Figure 7.10 – Uptake of ^{14}C-Glutamine (2 μM) into Caco-2 cell monolayers was measured in the presence of glycine-tyrosine, alanine-alanine, and des-tyrosine-leucine enkephalin (1 mM).

Caco-2 cells, passage 26-40 were seeded onto plastic 24-well plates and used 7-days post seeding. Uptake was measured at 37°C over ten minutes. Data are mean and standard deviations of results from four wells. n.s. denotes no significant ($p > 0.05$) difference compared to the control (ANOVA).

From these experiments, the observations of reduced glutamine uptake in the presence of other amino acids (Figure 7.9) and unaffected glutamine uptake in the presence of di-peptides and des-Tyr-Leucine Enkephalin (Figure 7.10) provides further evidence for a carrier-mediated pathway.

7.3.1.3 Effect of Liposome Formulations on Glutamine Uptake

The effect that PC (500 μg) liposome formulation had on glutamine uptake was determined (Figure 7.11). Glutamine uptake significantly ($p < 0.001$) increased in the presence of PC (500 μg) over the initial 20 minutes of the experiment. However, between 30 and 60 minutes the same enhancement of glutamine uptake was not observed (Figure 7.11). Although glutamine uptake was not significantly increased between 30 and 60 minutes glutamine uptake was not detrimentally affected compared to the control. The loss of enhancement of glutamine uptake post 30 minutes may be due to saturation of the glutamine transporters, and this is

substantiated by the data in Figure 7.2 that illustrates a linear increase in glutamine uptake between 0 and 30 minutes followed by a slower rate of glutamine uptake.

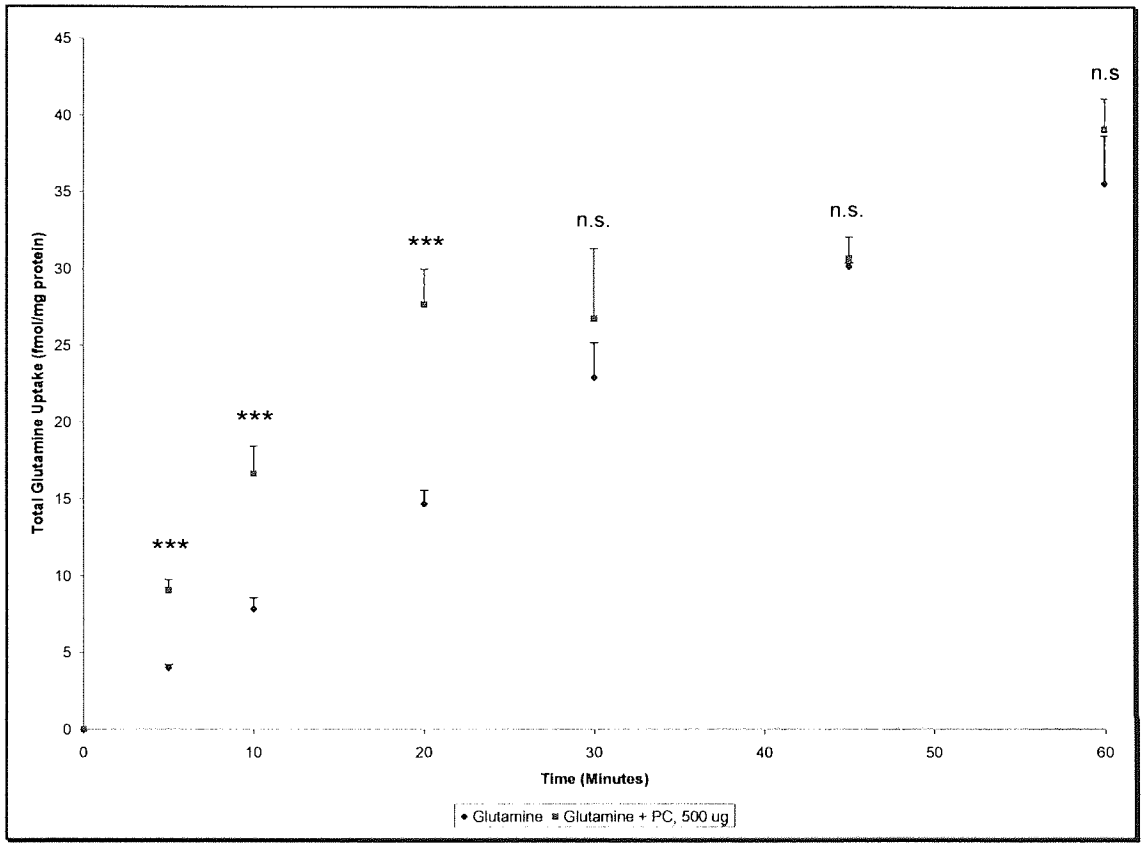

Figure 7.11 – Uptake of ^{14}C-Glutamine (2 μM) was measured over time in the presence of PC, 500 μg liposomes.

Caco-2 cells, passage 26-40 were seeded onto plastic 24-well plates and used 7-days post seeding. Uptake was measured at 37°C. Data are mean and standard deviations of results from four wells. *** Denotes an extremely significant ($p < 0.0001$) different compared to glutamine alone; n.s. denotes no significant ($p < 0.05$) difference compared to glutamine alone (t-test).

Cholesterol was included in the liposome formulation and the effect of PC:Cholesterol, (16:4; 500 μg) liposome formulation on glutamine uptake was determined (Figure 7.12). PC:Cholesterol, (16:4) significantly increased glutamine transport up to 20 minutes, however, after 20 minutes glutamine uptake was not enhanced (Figure 7.12). The presence of cholesterol had no additional effect on uptake as PC and PC:Cholesterol, 500 μg both had the same effect on glutamine uptake.

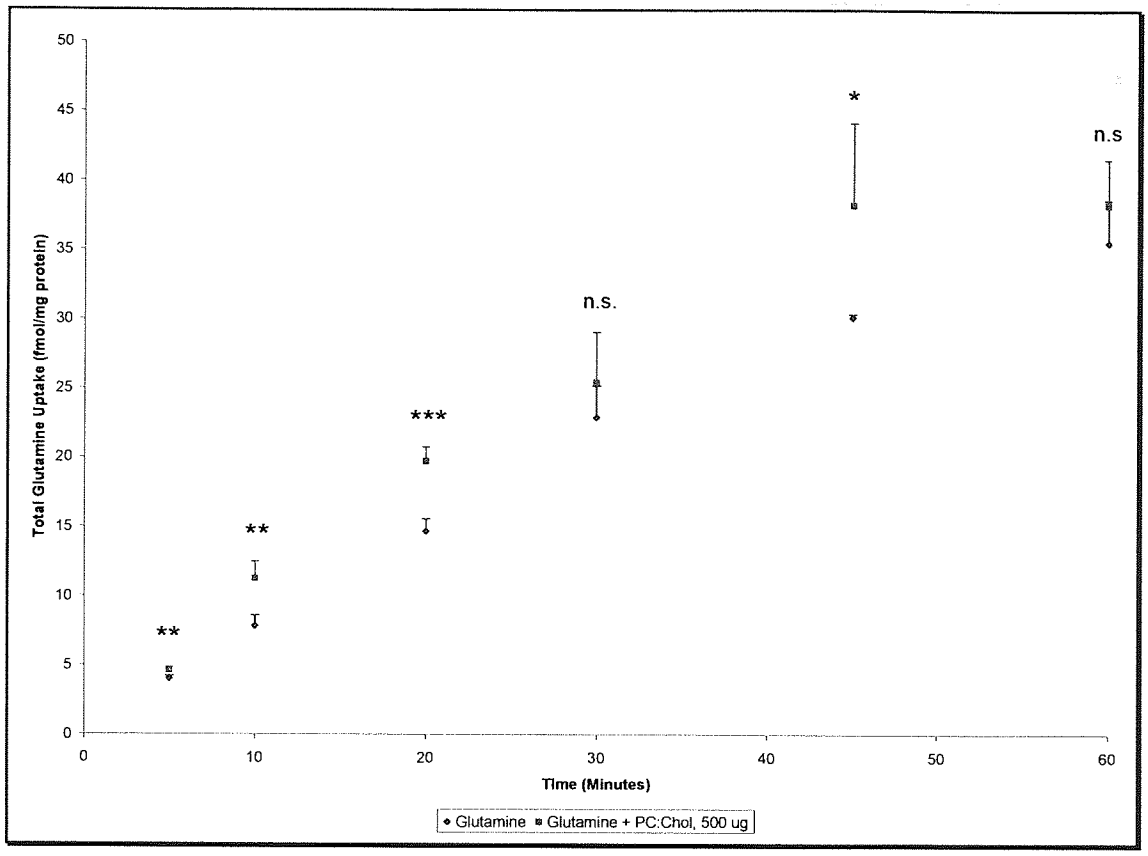

Figure 7.12 – Uptake of ^{14}C-Glutamine (2 μM) was measured over time in the presence of PC:Cholesterol, (16:4), 500 μg liposomes.

Caco-2 cells, passage 26-40 were seeded onto plastic 24-well plates and used 7-days post seeding. Uptake was measured at 37°C. Data are mean and standard deviations of results from four wells. *** Denotes an extremely significant difference compared to glutamine alone; ** Denotes a very significant difference compared to glutamine alone; * Denotes a significant difference compared to glutamine alone; n.s. denotes no significant difference compared to glutamine alone (t-test).

PC was replaced with DSPC because DSPC has a higher transition temperature (57°C; Table 2.1) and the uptake experiments were repeated. DSPC, 500 μg liposome formulation significantly increased glutamine uptake over the first 20 minutes (Figure 7.13). In agreement with PC (Figure 7.11) and PC:Cholesterol, 500 μg (Figure 7.12) glutamine uptake was not detrimentally affected by the presence of DSPC liposomes between 30 and 60 minutes.

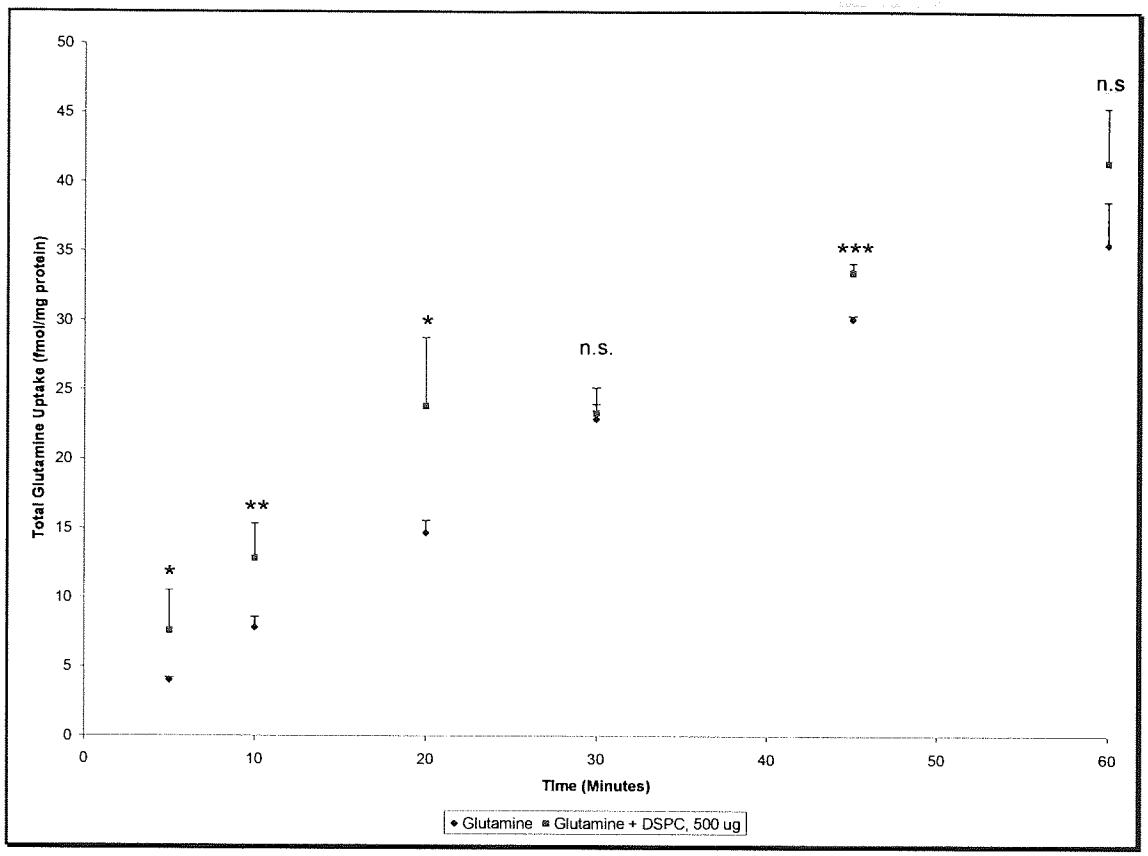

Figure 7.13 – Uptake of ^{14}C-Glutamine (2 μM) was measured over time in the presence of DSPC, 500 μg liposomes.

Caco-2 cells, passage 26-40 were seeded onto plastic 24-well plates and used 7-days post seeding. Uptake was measured at 37°C. Data are mean and standard deviations of results from four wells. *** Denotes an extremely significant difference compared to glutamine alone; ** Denotes a very significant difference compared to glutamine alone; * Denotes a significant difference compared to glutamine alone; n.s. denotes no significant difference compared to glutamine alone (t-test).

In contrast to the other liposome formulations tested, DSPC:Cholesterol (16:4), 500 μg significantly increased glutamine uptake at all time points (0 – 60 minutes) (Figure 7.14). The presence of a high transition temperature lipid (DSPC) and cholesterol in the liposome formulation may have increased glutamine uptake between 30 and 60 minutes by altering the membrane fluidity to a greater extent than PC, PC:Cholesterol and DSPC resulting in significantly higher glutamine uptake throughout the duration of the experiment.

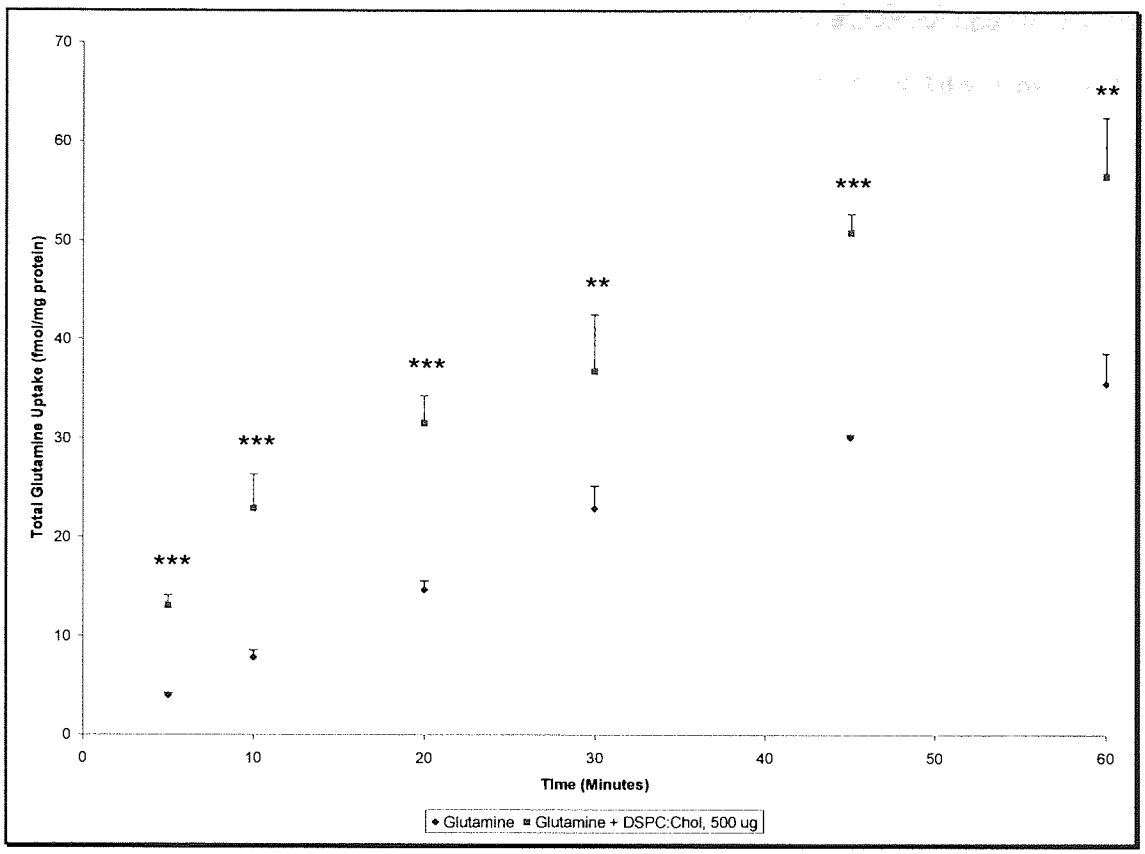

Figure 7.14 – Uptake of ^{14}C-Glutamine (2 μM) was measured over time in the presence of DSPC:Cholesterol, (16:**4**), 500 μg liposomes.

Caco-2 cells, passage 26-40 were seeded onto plastic 24-well plates and used 7-days post seeding. Uptake was measured at 37°C. Data are mean and standard deviations of results from four wells. *** Denotes an extremely significant difference compared to glutamine alone; ** Denotes a very significant difference compared to glutamine alone (t-test).

Liposome Formulation	Rate of Glutamine Uptake (0 – 20 minutes) (fmol / mg protein / minute)	R^2 Value	Significance (ANOVA)	
Control	0.71 ± 0.04	0.9992	-	-
PC, 500 μg	1.22 ± 0.10	0.993	$p < 0.01$	**
PC:Cholesterol, 500 μg	0.98 ± 0.04	0.9859	$p < 0.01$	**
DSPC, 500 μg	1.08 ± 0.15	0.9999	$p < 0.01$	**
DSPC:Cholesterol, 500 μg	1.18 ± 0.09	0.9483	$p < 0.01$	**

Table 7.1 – Comparison of the rate of glutamine uptake in the presence of all four liposome formulations.

The rate of glutamine uptake was calculated over the first 20 minutes. ** denotes a very significant ($p < 0.01$) difference compared to the control (ANOVA).

As a comparison between liposome formulations and their effect on glutamine uptake, the rate of glutamine transport was calculated for the first twenty minutes of the uptake experiment – twenty minutes was chosen because uptake was linear as judged by R^2 values (Table 7.1) and because glutamine uptake was significantly increased in the presence of all liposome formulations over the first 20 minutes of the experiment.

The rate of glutamine uptake was significantly increased in the presence of all liposome formulations compared to the control.

7.3.2 Glutamine Transport

7.3.2.1 Effect of Temperature on Glutamine Transport

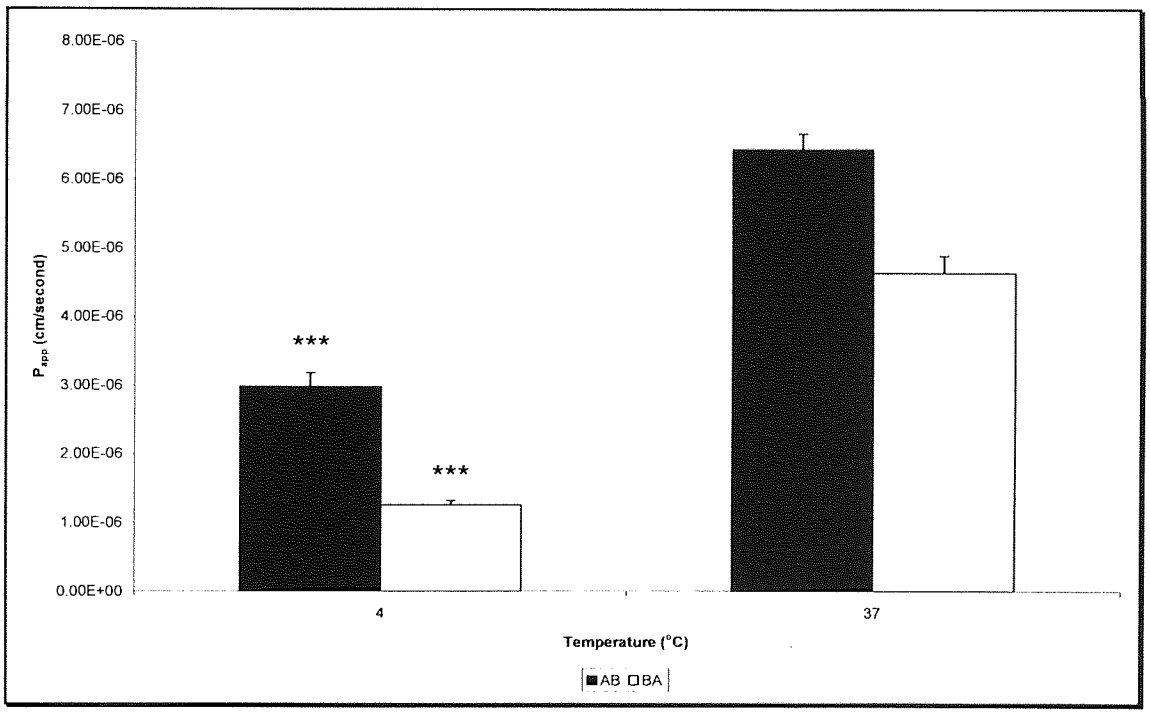

Figure 7.15 – The effect of temperature (4°C and 37°C) on ^{14}C-Glutamine transport (2 μM) (A – B and B – A) was measured using Transwell inserts (n = 4).

Caco-2 cells were seeded on Transwell inserts, between passage 26 and 40, and used 14-21 days post-seeding, transport was measured over 60-minutes. *** denotes an extremely significant difference ($p < 0.0001$) compared to 37°C (t-test).

Glutamine transport was measured at 4°C and 37°C in both directions of transport. The reduction in temperature reduced glutamine transport in both directions (Figure 7.15). Glutamine transport has both an active and passive component (Souba *et al.*, 1992; Costa *et al.*, 2000) and the reduction in temperature will reduce the active component of glutamine transport. The reduction in glutamine transport observed in Figure 7.15 is in parallel with the data obtained from the uptake study (Figure 7.6).

7.3.2.2 Effect of Inhibitors and Metabolic Poisons on the Transport of Glutamine

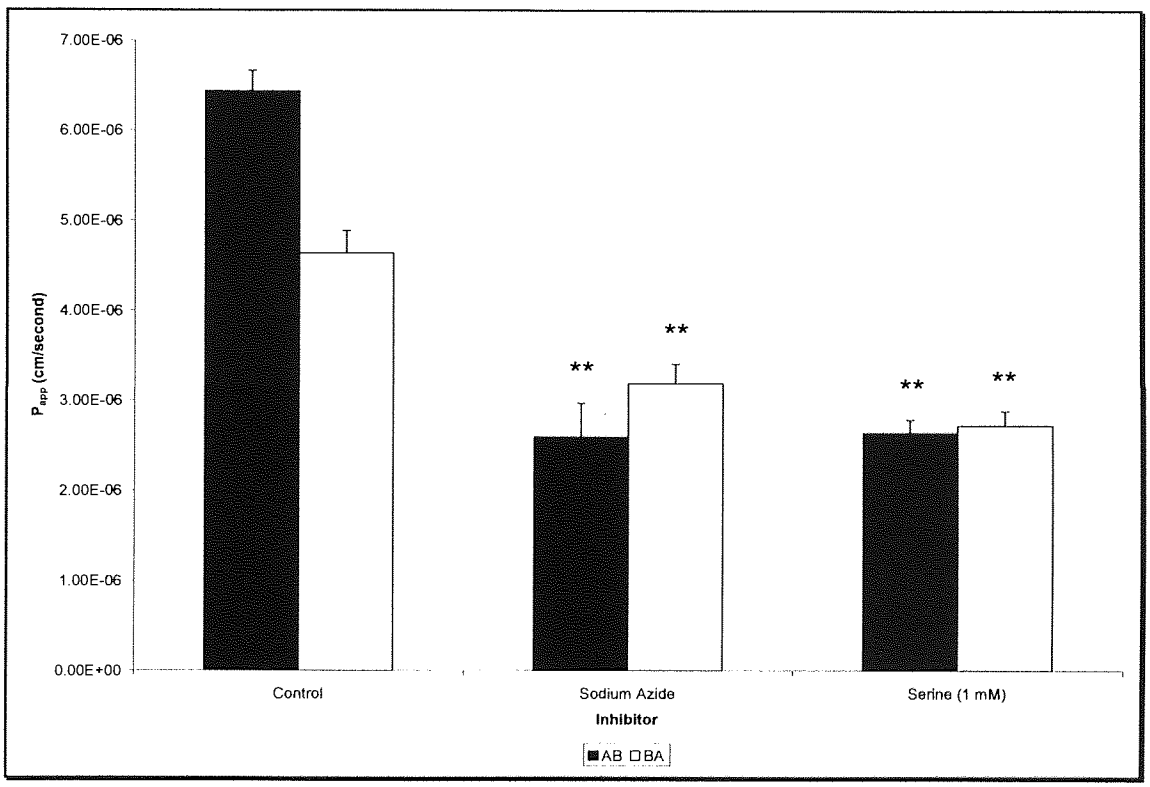

Figure 7.16 – Effect of the metabolic inhibitor sodium azide (15 mM) and the inhibitor serine (1 mM) on ^{14}C-Glutamine (2 μM) transport (A – B and B – A).

Caco-2 cells were seeded onto Transwell inserts, passage 26-40 and used 14-21 days post seeding. Transport was measured at 37°C over 60 minutes, n = 4. ** denotes a very significant ($p < 0.01$) difference compared to the control (ANOVA).

Glutamine transport was measured in the presence of the metabolic inhibitor sodium azide (15 mM) and serine (1 mM) to confirm the results obtained from the uptake experiment that both sodium azide (Figure 7.8) and serine (Figure 7.9) reduce active glutamine transport in Caco-2 cells. Glutamine transport was significantly ($p < 0.01$) decreased in the presence of sodium azide and serine in both directions of transport (Figure 7.16).

7.3.2.3 Effect of Liposome Formulations on Glutamine Transport

TEER measurements were made at the beginning and the end of all transport experiments to verify if test compounds had compromised the integrity of the Caco-2 cell monolayer. The presence of the liposome formulations significantly reduced the TEER measurements indicating a decrease in monolayer integrity (Table 7.2). The decrease in membrane integrity means that tight junctions open and compounds that are not normally transported *via* the paracellular route are able to be transported through the tight junctions, thus causing an increase in overall transport. The observed reduction in TEER measurements following incubation with the liposomes formulations is in agreement with the findings that Myri 52 (Lo, 2003) chitosan-glutamate and carbomer (Borchard *et al.*, 1996) have been shown to reduce TEER measurements of Caco-2 cell monolayers.

	TEER (% of Initial Value)	Significance (ANOVA)	
PC (1000 μg)	91.1 ± 3.0	Very Significant	$p < 0.01$
PC:Cholesterol (1000 μg; 16:4)	90.2 ± 3.3	Very Significant	$p < 0.01$
DSPC (1000 μg)	87.8 ± 4.6	Very Significant	$p < 0.01$
DSPC:Cholesterol (1000 μg: 16:4)	92.7 ± 2.9	Significant	$p < 0.05$

Table 7.2 – Percentage reduction in TEER value at the end of the experiment, compared to the control at the end of the experiment.

The effect liposome formulations (PC, PC:Cholesterol, DSPC and DSPC:Cholesterol, 1000 μg) had on TEER measurements (ANOVA with Dunnett's post-test, Instat 3).

Glutamine transport was measured in both directions of transport in the presence of PC, 1000 μg and PC:Cholesterol, 1000 μg (Figure 7.17). Both liposome formulations significantly increased apical-to-basolateral transport of glutamine; however, PC, 1000 μg increased transport 3-fold compared to a 2.1-fold increase for PC:Cholesterol, 1000 μg (Figure 7.17). Basolateral-to-apical transport of glutamine was significantly increased in the presence of PC and PC:Cholesterol (16:4; 1000 μg) (Figure 7.17).

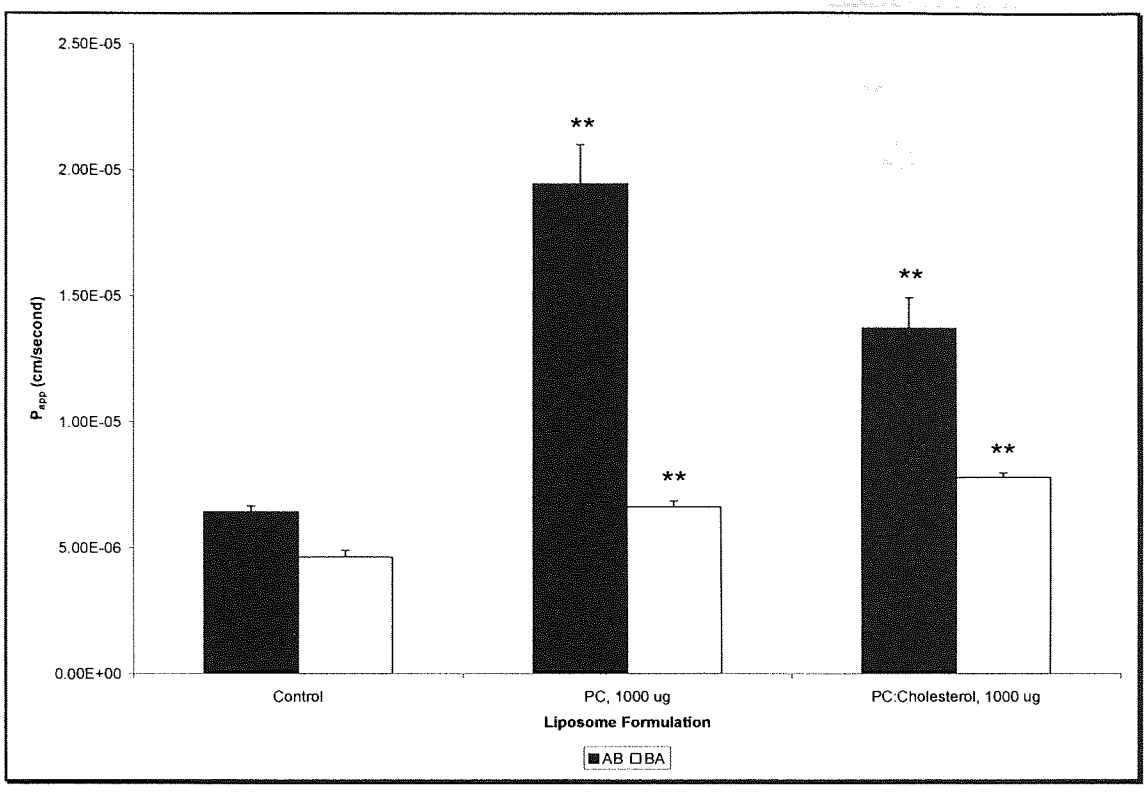

Figure 7.17 – ^{14}C-Glutamine (2 μM) transport in the apical-to-basolateral and basolateral-to-apical direction was measured in the presence of PC (1000 μg) and PC:Cholesterol (1000 μg; 16:4) liposomes.

Transport was measured at 37°C over 60 minutes, n = 4. Caco-2 cells were seeded onto Transwell inserts, passage 26-40 and used 14-21 days post seeding. ** denotes a very significant ($p < 0.01$) difference compared to the control (ANOVA).

PC was replaced with DPSC and the transport experiments were repeated (Figure 7.18). DSPC, 1000 μg and DSPC:Cholesterol, 1000 μg both significantly increased apical-to-basolateral transport of glutamine. However, in contrast to the PC results, DSPC:Cholesterol, 1000 μg increased A – B glutamine transport to a greater extent compared to DSPC, 1000 μg. Glutamine transport was increased 1.3-fold in the presence of DSPC, 1000 μg compared to 2.2-fold increase for DSPC:Cholesterol, 1000 μg (Figure 7.18). DSPC and DSPC:Cholesterol (16:4; 1000 μg) significantly increased the B – A transport of glutamine (Figure 7.18). The increase in B – A glutamine transport in the presence of PC and DSPC was comparable, where both formulations increased glutamine transport by 1.4-fold. The inclusion of cholesterol in both formulations caused a further increase in glutamine transport and, in agreement with the formulations without cholesterol, the increase in glutamine was comparable for PC:Cholesterol and DSPC:Cholesterol – 1.7-fold increase (Figure 7.17) compared to a 1.8-fold increase (Figure 7.18).

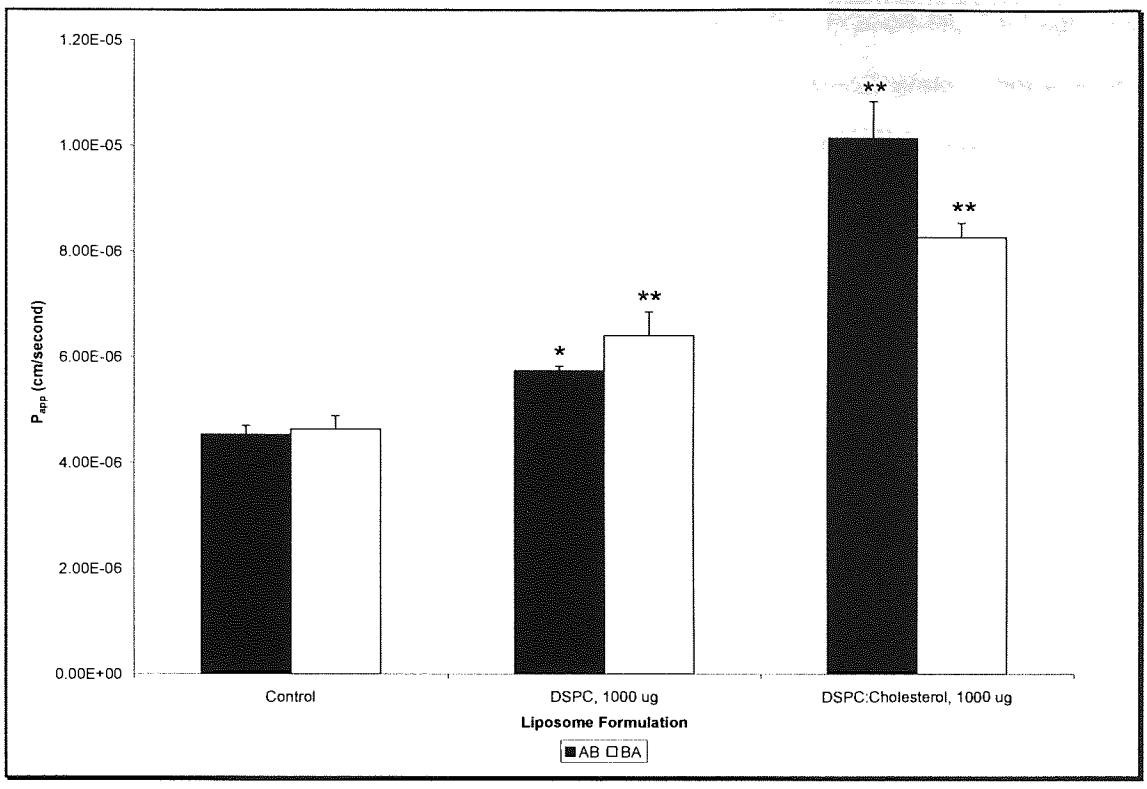

Figure 7.18 – ^{14}C-Glutamine (2 μM) transport in the apical-to-basolateral and basolateral-to-apical direction was measured in the presence of DSPC (1000 μg) and DSPC:Cholesterol (16:4; 1000 μg) liposomes.

Transport was measured at 37°C over 60 minutes, n = 4. Caco-2 cells were seeded onto Transwell inserts, passage 26-40 and used 14-21 days post seeding. ** denotes a very significant ($p < 0.01$) difference compared to the control; * denotes a significant ($p < 0.05$) difference compared to the control (ANOVA).

The increase in glutamine transport was expected for all the liposome formulations due to the reduction of TEER values (Table 7.2). Previous studies have shown a correlation between a reduction in TEER values and increased permeability across Caco-2 cells *e.g.* permeability of Lucifer Yellow CH was increased in the presence of discodermin A (Okada *et al.*, 2000) and cyclopeptidic $\alpha_v\beta_3$-anatgonist was increased in the presence of sodium taurocholate, sodium caprate and sodium laurate (Kamm *et al.*, 2000). Peptide delivery across Caco-2 cell monolayers has been significantly enhanced in the presence of lysophosphatidylcholines (Hovgaard *et al.*, 1995; Brøndsted *et al.*, 1995).

The effects of non-ionic surfactants on the carrier mediated transport of ceftibuten have been investigated. Ceftibuten is transported *via* a proton-coupled peptide transporter. It was found that some surfactants had no effect on ceftibuten uptake, some increased or decreased ceftibuten uptake. Tween 20, Tween 40 and sucrose stearate palmitate (7:3) monoester

significantly suppressed ceftibuten uptake. Tetraglycerol monooleate, tetraglycerol monostearate, hexaglycerol monostearate, hexaglycerol sesquistearate, hexaglycerol tristearate, and decaglycerol tristearate significantly enhanced ceftibuten uptake. Membrane fluidity was measured using fluorescence anisotropy. Surfactants that enhanced ceftibuten uptake decreased fluorescence anisotropy of TMA-DPH (*i.e.* increased membrane fluidity), and the surfactants that inhibited drug uptake increased the fluorescence anisotropy of TMA-DPH (i.e. decreased membrane fluidity). These results indicated that each surfactant modulates the fluidity of the membrane outer lipid layer; however, the mechanism of action of the surfactants could not be clarified (Koga *et al.*, 1998; Koga *et al.*, 1999a; Koga *et al.*, 1999b; Koga *et al.*, 2000).

7.4 Conclusion

From the uptake studies, it can be concluded that all liposome formulations; PC, PC:Cholesterol, (16:4), DSPC and DSPC:Cholesterol, (16:4) had a positive effect on glutamine uptake over the initial 20 minutes of the experiment. Only DSPC:Cholesterol (Figure 7.14) significantly increased glutamine uptake over the entire 60-minute duration of the experiment. In agreement with the uptake data all liposome formulations had a positive effect on glutamine transport in both directions; however, the increase in A – B transport was greater than B – A transport. The increase in glutamine uptake and transport is probably due to alteration in membrane fluidity and thus permeability causing an increase in glutamine uptake. Membrane fluidity could be investigated using fluorescence polarisation studies (Rege *et al.*, 2002; Nano *et al.*, 2003). DPH (1,6-Diphenyl-1,3,5-hexatriene) and TMA-DPH (1-(4-trimethylammonium)-6-phenyl)-1,3,5-hexatriene) are fluorescent membrane probes used in the fluorescence polarisation studies (Illinger and Kuhry, 1994). TMA-DPH became widely used as a probe for determining plasma membrane fluidity by measuring the fluorescence anisotropy on living cells (Illinger and Kuhry, 1994). The fluidity of the hydrophobic core of the lipid bilayers is measured using the fluorescent probe DPH, whereas TMA-DPH measures the fluidity of the polar head group (Rege *et al.*, 2002). With fluorescence anisotropy, membrane fluidity is interpreted in terms of hindrance to rotational motion of the probe, since it is embedded in the constraining phospholipid membrane (Illinger and Kuhry, 1994).

Steady-state fluorescence anisotropy is a sensitive technique for determining lipid fluidity and has been employed to determine changes in membrane fluidity of Caco-2 cells (Illinger and Kuhry, 1994; Rege *et al.*, 2002; Nano *et al.*, 2003). Caco-2 cells have previously been incubated with the experimental compounds (*e.g.* surfactants), were then labelled with either DPH or TMA-DPH, and the fluorescence intensity was measured. The effect of Tween 80, Cremophor EL, vitamin E TPGS, *N*-octyl glucoside, benzyl alcohol and cholesterol had on membrane fluidity have all been investigated using steady-state fluorescence anisotropy (Rege *et al.*, 2002). Cholesterol is known to increase membrane rigidity; therefore, as expected cholesterol caused a decrease in membrane fluidity (*i.e.* increase in anisotropy) of the hydrophobic core as measured using DPH. However, the anisotropy of TMA-DPH remained unaffected by cholesterol suggesting that polar head group fluidity was not affected. Benzyl alcohol is known to increase membrane fluidity; thus, as expected a decrease in anisotropy of both DPH and TMA-DPH was measured suggesting an increase in the fluidity of both the hydrophobic region and the polar head group region of the bilayer (Rege *et al.*, 2002). A significant decrease in the anisotropy of DPH was measured in the presence of Tween 80 and Cremophor EL. In contrast, vitamin E TPGS significantly increased anisotropy of DPH, suggesting rigidisation of the hydrophobic portion of the bilayer. The anisotropy of TMA-DPH was not affected by Tween 80, Cremophor EL or vitamin E TPGS. *N*-octyl glucoside did not alter the fluorescence anisotropy of either DPH or TMA-DPH, indicating that it has no effect on Caco-2 cell membranes fluidity (Rege *et al.*, 2002). The effect fatty acids have on Caco-2 cell membrane fluidity has also been investigated using steady-state fluorescence anisotropy. Membrane fluidity was not effected by the addition of palmitic acid; whereas, membrane fluidity was enhanced following the addition of unsaturated fatty acids (Nano *et al.*, 2003).

Alterations in membrane fluidity will influence the permeability of the cellular membrane and may induce changes in receptors and transporters. Therefore, the presence of the liposome formulations may have a two-fold impact on glutamine uptake and transport. Firstly, if the liposomes increase cellular permeability the passive component of glutamine uptake and transport will increase, and secondly, if the amino acid transporters are altered following a change in membrane fluidity, the active component of glutamine uptake and transport will

increase resulting in the observed increase in glutamine uptake and transport (Figure 7.11 – Figure 7.14; Figure 7.17 – Figure 7.18). Since this work presented here began, Tween 80, Cremophor EL and vitamin E TPGS have all been shown to modify membrane fluidity. Membrane fluidity being increased in the presence of Tween 80 and Cremophor EL and was decreased in the presence of vitamin E TPGS. All of these non ionic surfactants inhibited P-gp efflux, however, the mechanism was different, Tween 80 and Cremophor EL increased membrane fluidity and increased A – B transport, conversely, vitamin E TPGS decreased membrane fluidity and decreased B – A transport. Although Tween 80 and Cremophor EL both increased membrane fluidity and had the same effect on P-gp, only Tween 80 inhibited transport of the dipeptide glycyl sarcosine, and only Cremophor EL inhibited the monocarboxylic acid transporter, as measured using benzoic acid (Rege *et al.*, 2002). These non ionic surfactants share a common functional feature in their ability to modulate membrane fluidity; however, other factors may also influence the effect non ionic surfactants and excipients have on membrane transporter activity. The permeability of the model peptide $Acf(NMef)_2NH_2$ across Caco-2 cell monolayers is increased in the presence of Cremophor EL and polysorbate 80 (Nerurkar *et al.*, 1996; Nerurkar *et al.*, 1997). In contrast, *N*-octyl glucoside, a non ionic surfactant does not modulate membrane fluidity and does not affect membrane transporter functioning (Rege *et al.*, 2002).

Alterations in the physical state of plasma membrane lipids can influence a number of important carrier-mediated transport processes (Sinicrope *et al.*, 1992). Changes in membrane fluidity have been shown to alter the activity of sodium and proton-coupled membrane transporters (Nabekura *et al.*, 1996; Lee *et al.*, 1999).

The presence of the liposome formulations brought about different effects on glutamine uptake and transport. Whilst the general effect was positive, there were differences. The mechanism by which the liposome formulations increase glutamine uptake and transport requires further investigation. Cholesterol has been shown to decrease membrane fluidity (Rege *et al.*, 2002) however, the effect of PC and DSPC have on Caco-2 cell membrane fluidity has not been

measured. The influence that the presence of cholesterol within the liposome formulation has on the membrane fluidity also needs to be measured. MLVs were used in the studies presented here; therefore, it needs to be established if LUVs, SUVs and micelles have the same affect on glutamine uptake and transport and membrane fluidity. From these initial investigations it can be concluded that all four liposome formulations tested, are suitable candidates for use as delivery vehicles for drugs that utilise a carrier-mediated route.

Chapter 8

Effects of Liposomal Formulations on Efflux

8. Effects of Liposomal Formulations on Efflux

8.1 Introduction

Efflux is defined as the active removal of drugs from within cells, *i.e.* drugs are actively pumped out of cells. Efflux is a major problem for the pharmaceutical industry because drugs which are targeted to specific areas where pharmacological effects are required; can be eliminated by the tissue itself. However, once this has been achieved and the drugs have entered the cells *via* one of the absorption mechanisms (Section 1.3), there is still the possibility that the drug will be actively pumped out of the cell and hence, unable to bring about their pharmacological effects (see Section 1.4). Efflux pumps located in the intestine include P-glycoprotein, members of the multidrug resistance associated protein family (MRP), breast cancer resistance protein (BCRP), organic cation transporters and members of the organic polypeptide family (Wagner *et al.*, 2001). P-glycoprotein, MRP's and BCRP are members of the ATP-binding cassette (ABC) family; thus efflux *via* these pumps is an active process and ATP-dependent. Active processes, both non-specific and specific are susceptible to inhibitors. For example, verapamil and cyclosporin A inhibit P-glycoprotein; probenecid and benzbromarone inhibit MRP (Schinkel and Jonker, 2003); BCRP is inhibited by fumitremorgin C (Lage and Dietel, 2000), and all are inhibited by sodium azide.

8.1.1 P-Glycoprotein (P-gp)

P-glycoprotein is an ATP-driven efflux pump and has received much attention with regard to its role in restricting drug absorption and distribution and as a potential source for variability in drug pharmacokinetics and pharmacodynamics. P-glycoprotein is the focus of this chapter's research (Section 1.4.1), and was chosen because it is expressed in the main absorptive organ, the intestine, and because P-glycoprotein has a broad substrate specificity (Wagner *et al.*, 2001).

The broad substrate specificity of P-gp is a major problem for the pharmaceutical industry. Detailed or specific structural requirements for P-gp are not known, thus intensifying the

problem and making a solution harder to find. However, P-gp inhibitors do exist with varying degrees of effectiveness. For a comprehensive list of P-gp inhibitors, substrates and inducers refer to Appendix 4 and for a number of examples refer to Table 8.1. Inhibitors of P-gp that were chosen for experimental work include cyclosporin A, verapamil and nifedipine. These inhibitors were chosen because they have previously been shown to modulate P-glycoprotein (Pedersen *et al.*, 1983; Cavet *et al.*, 1996; Wacher *et al.*, 1996; Hamilton *et al.*, 2001).

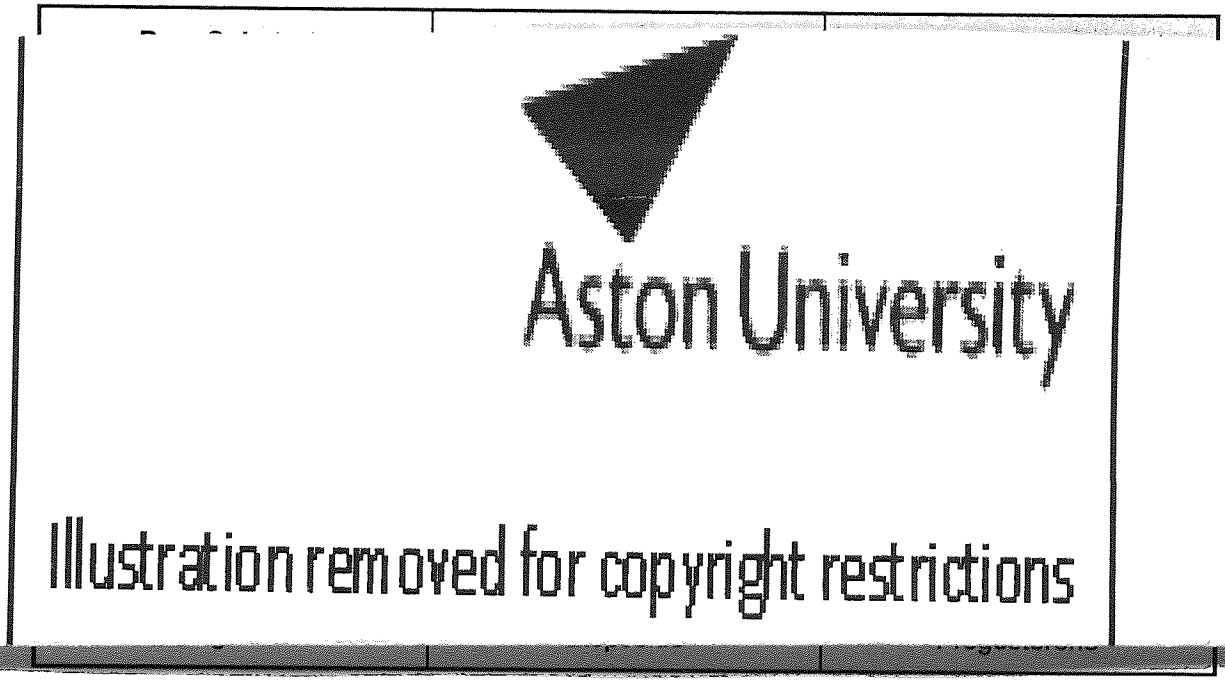

Table 8.1 – Examples of P-glycoprotein inhibitors, substrates and inducers.

(Belz *et al.*, 1983; Pedersen *et al.*, 1983; Tanigawara *et al.*, 1992; Spatzenegger and Jaeger, 1995; Hollo *et al.*, 1996; Pascaud *et al.*, 1998; Ambudkar *et al.*, 1999; Eagling *et al.*, 1999; Ho *et al.*, 2000; Katoh *et al.*, 2000; Hamilton *et al.*, 2001; Matheny *et al.*, 2001; Weber *et al.*, 2001).

8.1.1.1 P-Glycoprotein Expression in Caco-2 Cells

Expression of a polarised efflux pump in Caco-2 cells was reported by Augustijns *et al.*, (1993). Cyclosporin A (CsA) transport was greater in the secretory direction (basolateral-to-apical) compared to the absorptive flux (apical-to-basolateral). The presence of P-glycoprotein inhibitors chlorpromazine and progesterone caused an increase in CsA absorptive flux and a decrease in secretory flux. These results indicated the presence of an efflux pump, such as P-glycoprotein. Immunoblotting was employed to demonstrate the presence of P-gp in Caco-2 cells (Peters and Roeloefs, 1992). Expression of P-glycoprotein on the apical membrane of

Caco-2 cells has been confirmed by immunohistochemical localisation. The presence of polarised expression of P-glycoprotein in Caco-2 cell monolayers also supports the observation that the secretory flux of vinblastine sulphate (a P-gp substrate) was greater than absorptive flux (Hunter *et al.*, 1993).

P-glycoprotein is expressed in Caco-2 cell monolayers; however, the level of P-gp expression in the Caco-2 cell line is important because they are used as model of intestinal absorption. Consequently, human intestinal permeability may be underestimated if P-gp is expression is higher in the Caco-2 cell line compared to P-gp expression in humans. Conversely, if P-gp is under expressed a P-gp substrate may not be identified (Anderle *et al.*, 1998). Variation in P-gp expression can be due to time in culture and culturing conditions (Anderle *et al.*, 1998). Trypsinising Caco-2 cells can alter P-gp expression; P-gp expression is decreased if Caco-2 cells are trypsinised after reaching confluence, whereas if Caco-2 cells are trypsinised before reaching confluence (approximately 70 per cent) resulted in an increase in P-gp expression after long-term cultivation. P-gp expression changes with time in culture, P-gp expression increased until day 21 followed by a decrease in P-gp expression (Behrens and Kissel, 2003). Previous exposure to drugs including celiprolol and vinblastine induce P-gp expression (Anderle *et al.*, 1998). To reduce variations within the data obtained experiments were limited to Caco-2 cells passage 26 to 40 and the same cell culture conditions were applied throughout. P-gp expression in Caco-2 cells was not measured within this study; however, P-gp expression and activity has been confirmed by Hosoya *et al.*, 1996 and Anderle *et al.*, 1998 within the passage window (26 – 40) used.

8.1.2 Digoxin

The probe chosen to investigate efflux in Caco-2 cells was digoxin (Figure 8.1). Digoxin was chosen because it is a P-glycoprotein substrate (Matheny *et al.*, 2001) and has previously been used as a probe in *in vitro* models and in pharmacokinetic drug-drug interactions in healthy volunteers (Kovarik *et al.*, 1999; Westphal *et al.*, 2000; Becquemont *et al.*, 2001; Verstuyft *et al.*, 2003).

Digoxin is a drug originally derived from the foxglove plant (*Digitalis lanata*) (Goldman, 2001; Eichhorn and Gheorghiade, 2002). Digoxin is a cardiac glycoside; the cardiac glycosides are a group of drugs that have a common specific effect on the myocardium. Digoxin has been used to treat congestive heart failure, primarily by improving the pumping ability of the heart, for more than 200 years (Collins *et al.*, 2003; Hood Jr. *et al.*, 2004) Additionally, digoxin is used to normalise some dysrhythmias (abnormal type of heartbeat).

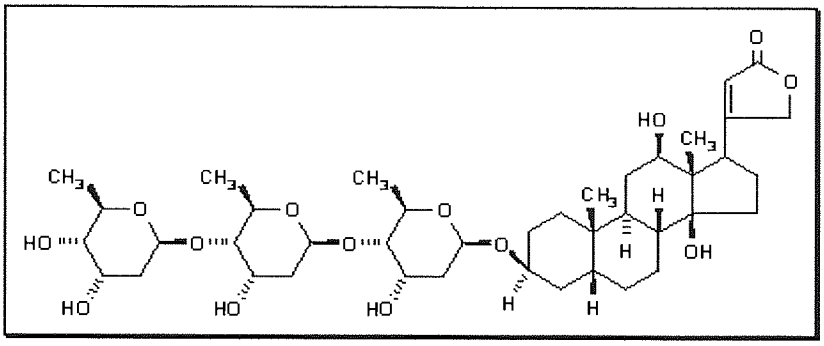

Figure 8.1 – Structure of digoxin ($C_{41}H_{64}O_{14}$) molecular weight is 780.95.

Digoxin is administered both orally and by intravenous injection. Digoxin is characterised by good oral absorption and a long half-life (Zussman *et al.*, 2001). Approximately 70 – 80 % of an oral dose of digoxin is absorbed *via* transcellular transport (Katoh *et al.*, 2001), mainly in the proximal part of the small intestine (Agoram *et al.*, 2001). Digoxin bioavailability is affected by genetic polymorphism of the human *MDR1* gene that encodes P-glycoprotein (Kurata *et al.*, 2002). Digoxin has a narrow therapeutic index; consequently small changes in digoxin blood levels can result in adverse drug reactions; any co-administered medication that modifies plasma digoxin levels may have an adverse effect on efficacy and toxicity (Zussman *et al.*, 2001). Digoxin is a lipophilic drug and therefore has a high oral bioavailability (Hunter and Hirst, 1997), however, bioavailability is affected by the presence of P-gp inducers and inhibitors. When digoxin is administered with rifampin, a known inducer of gut (but not renal) P-gp, oral bioavailability was found to decrease. Conversely, when digoxin is administered with quinidine, a known inhibitor of both intestinal and renal P-gp, oral bioavailability was found to increase (Agoram *et al.*, 2001). Digoxin has an intermediate lipophilicity (Molin *et al.*, 1983; Sababi *et al.*, 2001) and is lipophilic enough to be absorbed from the colon – approximately one third of that

from the small intestine (Sababi *et al.*, 2001). Digoxin is sparsely soluble in water and slightly soluble in diluted (50%) alcohol and in chloroform (Molin, 1986).

Previous work has shown that Caco-2 cell monolayers show greater basolateral-to-apical flux of digoxin than apical-to-basolateral flux – producing a net secretory flux (Cavet *et al.*, 1996). P-gp inhibitors verapamil (100 μM), nifedipine (50 μM) abolished net secretion of digoxin – by decreasing basolateral-to-apical flux and increasing apical-to-basolateral flux (Cavet *et al.*, 1996).

8.1.3 Mechanism of Excipient Action

Excipients have not only been used to improve solubility (Section 1.9) but they have also been investigated to reverse or reduce the effects of P-glycoprotein. The mechanism of how non-ionic surfactants inhibit P-gp remains unclear (Rege *et al.*, 2002). It has been suggested that changes in membrane fluidity result in conformation changes in membrane bound transporters; Rege *et al.*, (2002) reported P-gp inhibition in the presence of Tween 80, Cremophor EL (non-ionic surfactants) and vitamin E TPGS (α- tocopheryl polyethylene glycol 800 succinate), and their findings support the suggestion that excipients alter membrane fluidity because different patterns of P-gp inhibition were observed for vitamin E TPGS compared to Tween 80 and Cremophor EL. A possible explanation for the difference in P-gp inhibition observed by these agents is that vitamin E TPGS is known to increase rigidity of lipid bilayers; in contrast Tween 80 and Cremophor EL are known to increase membrane fluidity (Rege *et al.*, 2002). Increases in absorption in the presence of excipients may be brought about following disruption of cellular membrane lipid arrangement caused by interaction between the surfactants and the polar head groups of the lipid bilayers, resulting in changes in membrane fluidity or following insertion of the surfactants between the lipophilic tails of the bilayer. Hydrogen bonding and ionic forces may be modified following interactions between surfactants and polar heads groups of the lipid bilayer causing an increase membrane fluidity, enhancing absorption (Dudeja *et al.*, 1995; Sakai *et al.*, 1997; Ferte, 2000; Lo, 2003).

8.1.4 Objective

The objective of this chapter was to determine the effect of liposomal formulations on efflux using digoxin as the chosen probe. Digoxin uptake and transport across Caco-2 cell monolayers was measured.

8.2 Material and Methods

General material and methods were previously described in Chapter 2. Thin-layer chromatography (TLC) was used to ensure the purity of [^{3}H(G)]-Digoxin, details of the methodology can be found in Section 2.3.12 To measure [^{3}H(G)]-Digoxin (24 nM) uptake, Caco-2 cells were seeded on 24-well plates (Section 2.3.3.1 and Section 2.3.8) or seeded on Transwell inserts to measure [^{3}H(G)]-Digoxin (24 nM) transport (Section 2.3.3.2 and Section 2.3.9). Uptake was measured over 30 minutes at 37°C unless otherwise stated; transport was measured over 60-minutes. Inhibitors of digoxin uptake were prepared using transport media – sodium azide (15 mM); Triton X-100 (0.05 % v/v); cyclosporin A (CsA, 50 μM); verapamil (100 μM); testosterone (100 μM); terfenadine (100 μM) and nifedipine (100 μM). Ethanol was used as a co-solvent for CsA due to low solubility; ethanol was used at ≤ 5 % which has been shown not to be detrimental to Caco-2 cells (Biggs 2003).

8.3 Results and Discussion

8.3.1 Thin-Layer Chromatography (TLC)

Thin-layer chromatography (TLC) is a technique used to determine purity of a compound; if the compound is pure a single peak is observed. The purity of digoxin and [^{3}H(G)]-Digoxin were determined using TLC to ensure it had not degraded.

From Figure 8.2, it can be concluded that the TLC for [^{3}H(G)]-Digoxin produced a single peak with a calculated Rf value of 67, compared to the text book Rf value of 62 (British Pharmacopoeia, 1998). The Rf value for digoxin was 60.6; therefore, it can be concluded that digoxin and [^{3}H(G)]-Digoxin had not degraded and could be used for experimental use.

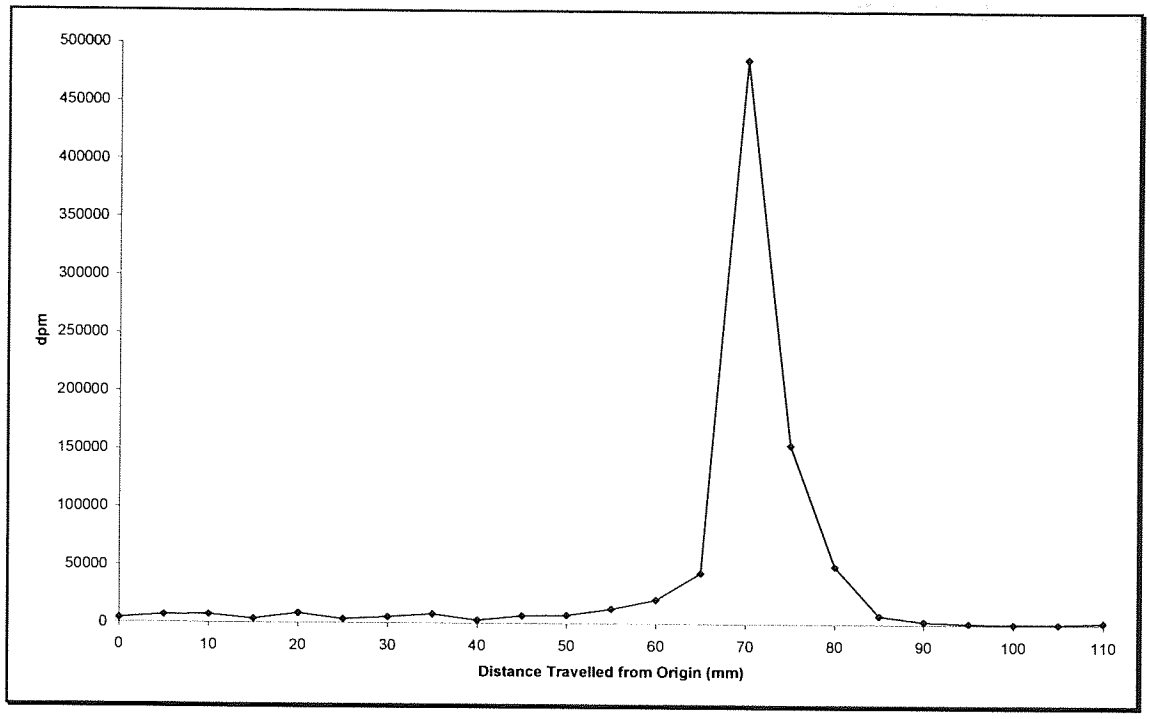

Figure 8.2 – Purity of [^{3}H(G)]-Digoxin was analysed using thin layer chromatography (TLC).

8.3.2 Uptake of Digoxin

Uptake of digoxin is achieved *via* the transcellular route (Katoh *et al.*, 2001). As described before the transcellular route comprises of both passive transcellular and carrier-mediated (active) components, and digoxin utilises both routes. Digoxin enters the Caco-2 cells *via* the transcellular route; however, once inside the plasma membrane digoxin is a substrate for the efflux pump P-gp. Therefore, during the uptake experiments digoxin will enter and leave the Caco-2 cells. Uptake of digoxin was measured over time (15, 30, 45 and 60-minutes, and 2, 3, 4-hours) (Figure 8.3). Uptake of digoxin was time-dependent (Figure 8.3). Uptake of digoxin was linear over the first 60-minutes of the experiment, followed by a decreased rate of digoxin uptake between 1 and 4 hours. During the first hour of the experiment digoxin was accumulating within the Caco-2 cells. However, as the uptake experiment progressed the concentration of digoxin within the Caco-2 cells would have increased; therefore an increased amount of digoxin would have been available to the efflux pump P-gp. During the latter stages of the uptake experiment uptake < efflux resulting in the observed decrease in the rate of digoxin uptake (Figure 8.3).

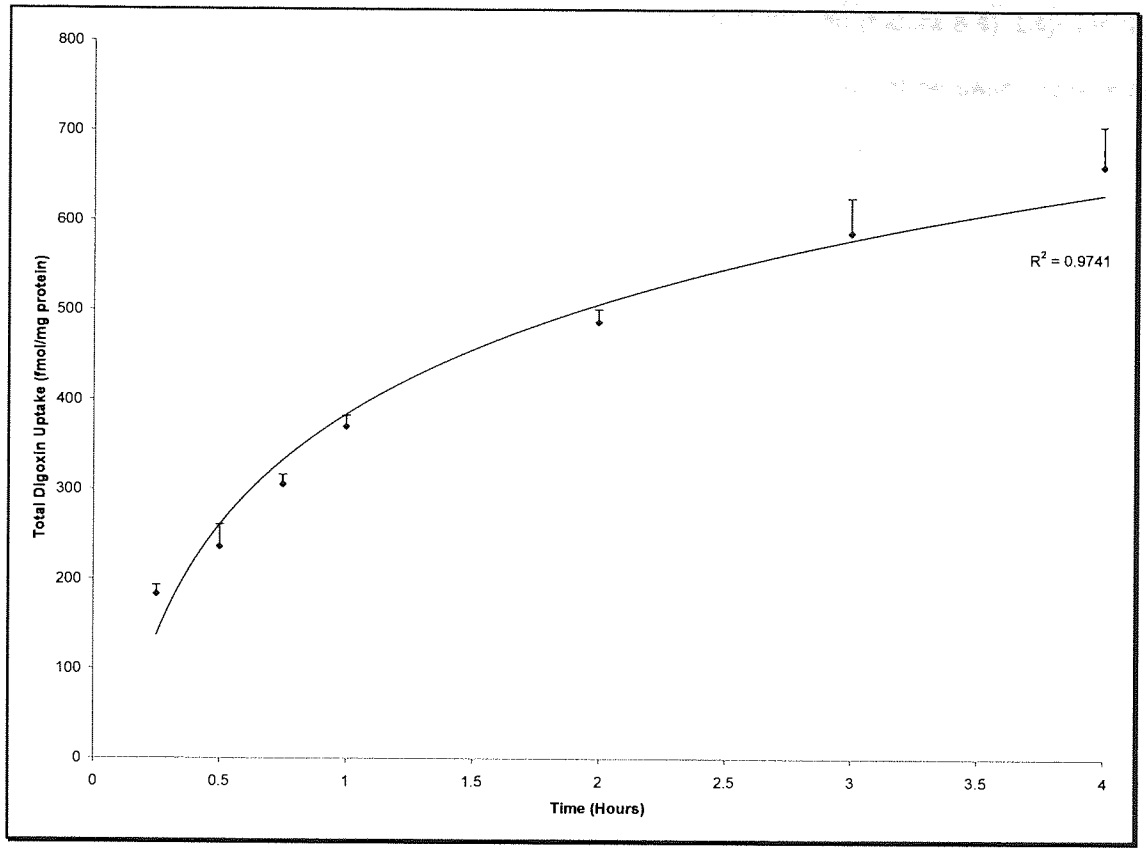

Figure 8.3 – Uptake of [^{3}H(G)]-Digoxin (24 nM) was measured over 4-hours using Caco-2 cell monolayers.

Caco-2 cells were seeded onto plastic 24-well plates between passage 26 and 40, monolayers were used 7-days post seeding. Uptake was measured over 30 minutes at 37°C. Data are mean and standard deviation of results from four wells.

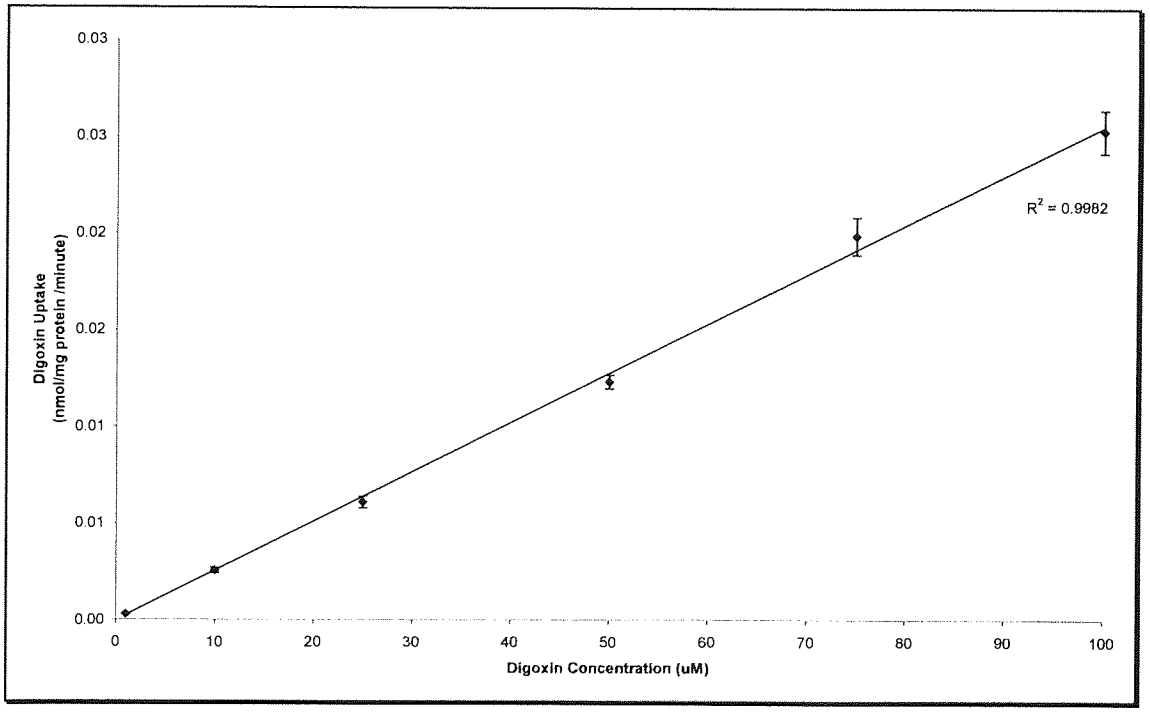

Figure 8.4 – Uptake of [^{3}H(G)]-Digoxin (24 nM) into Caco-2 cell monolayers was measured at increasing concentrations of digoxin.

Caco-2 cells were seeded onto plastic 24-well plates between passage 26 and 40, monolayers were used 7-days post seeding. Uptake was measured over 30 minutes at 37°C. Data are mean and standard deviation of results from four wells.

Digoxin uptake was measured at increasing concentrations of digoxin (Figure 8.4). Digoxin has a limited solubility; consequently, concentrations above 100 μM could not be used. Increasing the concentration resulted in a linear increase in digoxin uptake (R^2 = 0.9982). A linear increase in digoxin uptake with increasing concentration is expected from a compound that typically utilises the passive transcellular route, thus the data is in agreement with digoxin using the passive uptake route. The increase in digoxin uptake with increasing digoxin concentration will increase the concentration gradient across the Caco-2 cell membrane, thus increasing the passive component of digoxin uptake. Digoxin also uses the carrier-mediated route, this route is saturable; therefore, at higher digoxin concentrations a decrease in the rate of digoxin uptake would be expected; this was not observed in Figure 8.4. There are three possible explanations for the decrease in the rate of uptake not being observed; firstly, due to the solubility issues associated with digoxin, the transporters may not have been saturated at the digoxin concentrations used; or secondly the passive uptake of digoxin is greater, therefore, a linear uptake of digoxin is observed; or thirdly P-gp is not expressed or functional in the Caco-2 cell line used. Further experiments must be performed to verify the presence and activity of P-gp (Section 8.3.2.1 and Section 8.3.2.2).

Additionally, increasing the concentration of digoxin will decrease the effects of P-glycoprotein efflux because P-glycoprotein is a saturable transporter so once saturation has been reached the rate of digoxin efflux will become constant and will not have a diminished effect on uptake resulting in the increased digoxin uptake that is observed in Figure 8.4.

8.3.2.1 Effect of Temperature on Digoxin Uptake

Uptake of digoxin was measured at 4°C, 20°C and 37°C (Figure 8.5). Temperature will decrease the active uptake of digoxin but should not influence the passive uptake of digoxin. Digoxin uptake showed a temperature-dependent uptake (Figure 8.5). Digoxin uptake was significantly ($p < 0.01$) reduced as temperature decreased, indicating that digoxin uptake has an active uptake component (Figure 8.5). Although the reduction in digoxin uptake was significant a greater reduction was expected because the reduction was comparable to the reduction (2-

fold) in mannitol P_{app} (Section 5.3.1). The active component of digoxin uptake would have been decreased following the reduction in temperature and the passive component would remain unaffected. Additionally, the reduction in mannitol was not expected because mannitol transport is passive (Section 5.3.1). Digoxin uptake has previously been shown to be partly carrier-mediated and temperature-dependent (Okudaira *et al.*, 1988; Okudaira *et al.*, 1989; Olinga *et al.*, 2001). The activation energy for digoxin, calculated using an Arrhenius plot is 17.46 kJ/mol, further indicating digoxin uptake has an active component as the activation energy is higher than expected for a passive process – less than 16.38 kJ/mol.

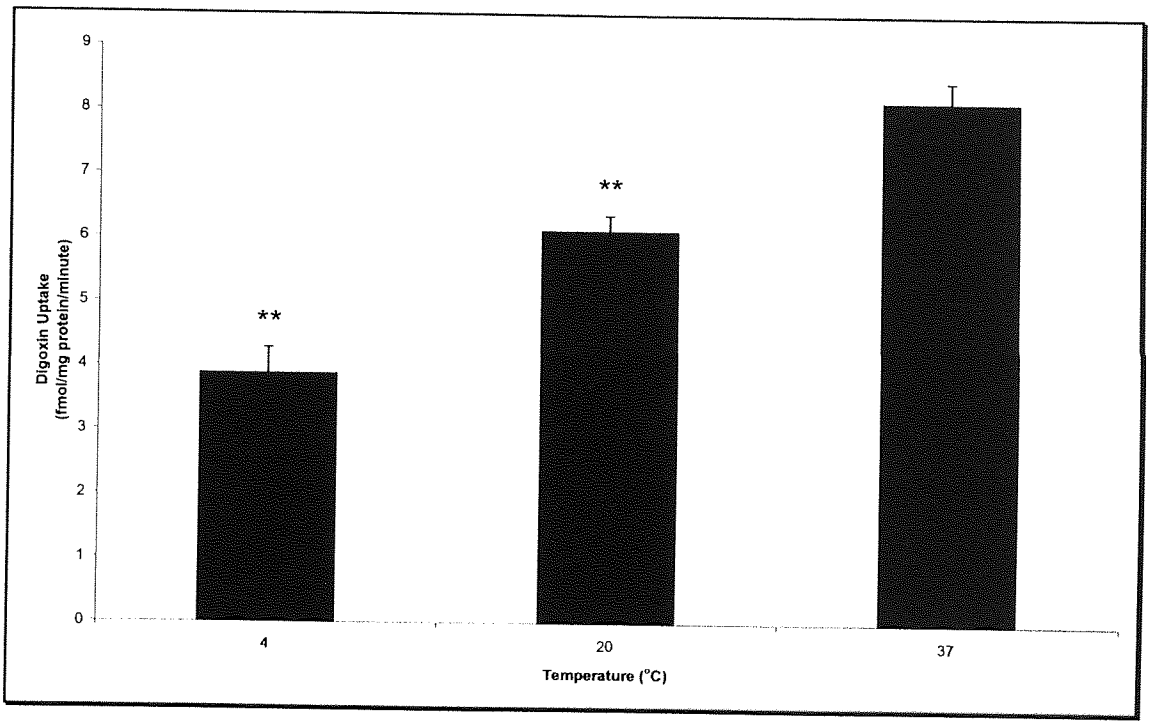

Figure 8.5 – Uptake of [^{3}H(G)]-Digoxin (24 nM) into Caco-2 cell monolayers was measured at different temperatures (4°C, 20°C and 37°C).

Caco-2 cells, passage 26-40, were seeded onto plastic 24-well plates, monolayers were used 7-days post seeding. Uptake was measured over 30 minutes. Data are mean and standard deviation of results from four wells. ** denotes a very significant difference ($p < 0.01$) compared to 37°C (ANOVA).

8.3.2.2 Effect of Inhibitors and Metabolic Inhibitors on Digoxin Uptake

Digoxin uptake was measured in the presence of sodium azide (a metabolic inhibitor) and Triton X-100 (Figure 8.6). Sodium azide was used to deplete ATP thereby reducing active processes that require ATP as a source of energy. Digoxin uptake was significantly ($p < 0.01$) reduced by 11.5 per cent in the presence of sodium azide (Figure 8.6). Digoxin uptake is achieved *via* both

active and passive processes and it is likely that the observed decrease in digoxin uptake was because ATP was decreased in the presence of sodium azide, thus reducing the active uptake component of digoxin uptake. Digoxin efflux *via* P-glycoprotein will also have been reduced in the presence of sodium azide; however, an overall decrease in digoxin uptake was observed because the active component of digoxin uptake has also been decreased and the observed digoxin uptake is only the passive component of uptake. The decrease in digoxin uptake provides further evidence of an active component for digoxin uptake, in agreement with previous studies that have shown that digoxin uptake is energy-dependent (Okudaira *et al.*, 1988).

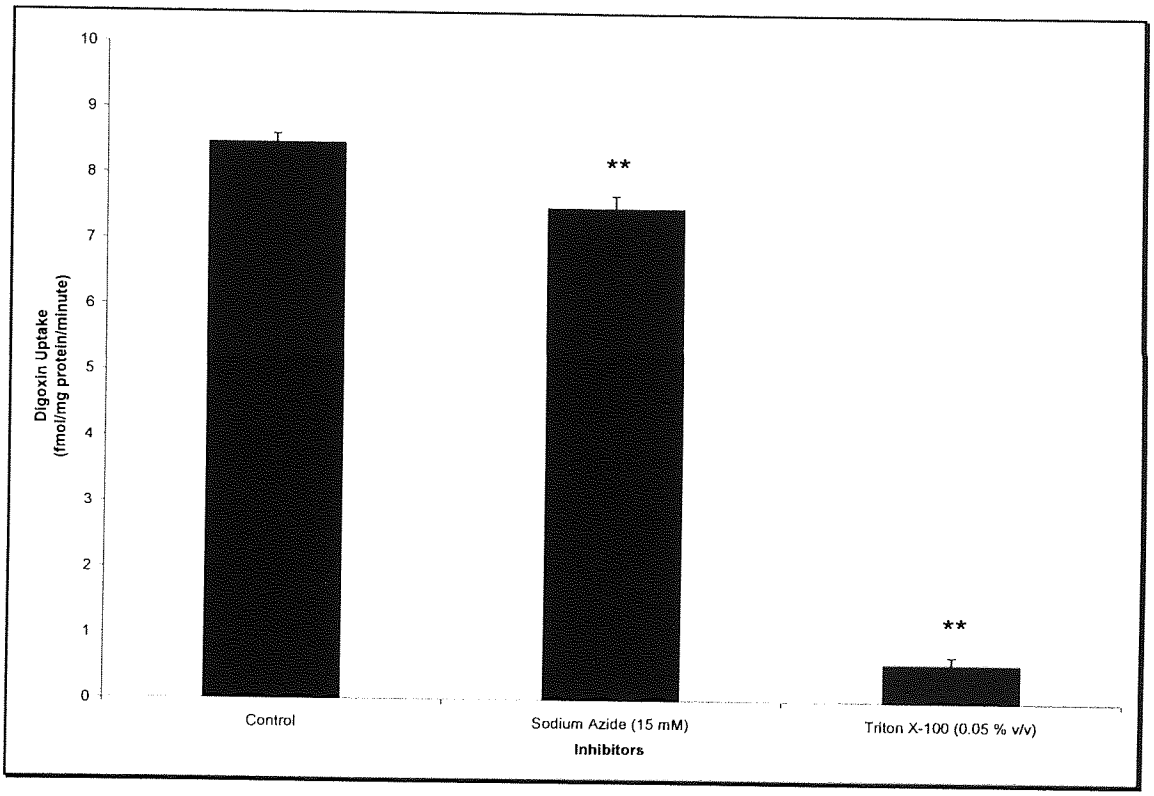

Figure 8.6 – The effect of sodium azide (15 mM) and Triton X-100 (0.05 % v/v) on [^{3}H(G)]-Digoxin (24 nM) uptake into Caco-2 monolayers was measured.

Caco-2 cells, passage 26-40, were seeded onto plastic 24-well plates, monolayers were used 7-days post seeding. Uptake was measured over 30 minutes at 37°C. Data are mean and standard deviation of results from four wells. ** denotes a very significant ($p < 0.01$) difference compared to the control (ANOVA).

The effect of Triton X-100 (0.05 % v/v) on digoxin uptake was determined because Triton X-100 is toxic to Caco-2 cells (Section 4.3.3), therefore, uptake of digoxin should be reduced because in the presence of Triton X-100 the Caco-2 cells will either be non-viable or in the process of becoming non-viable cells; non-viable cells will lift off the base of the well. As can be seen from

Figure 8.6, Triton X-100 significantly reduced ($p < 0.01$) uptake of digoxin because it is toxic towards Caco-2 cells (Section 4.3.3).

Inhibitors of P-glycoprotein will inhibit digoxin efflux, thus digoxin uptake will increase. P-glycoprotein substrates will compete for the transporters thus increasing digoxin uptake. Cyclosporin A (CsA) is a known P-glycoprotein inhibitor and substrate (Matheny *et al.*, 2001; Spatzenegger and Jaeger, 1995; Hollo *et al.*, 1996; Ambudkar *et al.*, 1999). Therefore digoxin uptake was measured overtime (5, 10, 20, 30, 45 and 60-minutes) in the presence of CsA (50 μM) (Figure 8.7). Lowes *et al.*, (2003) showed cellular uptake of digoxin increased in the presence of CsA.

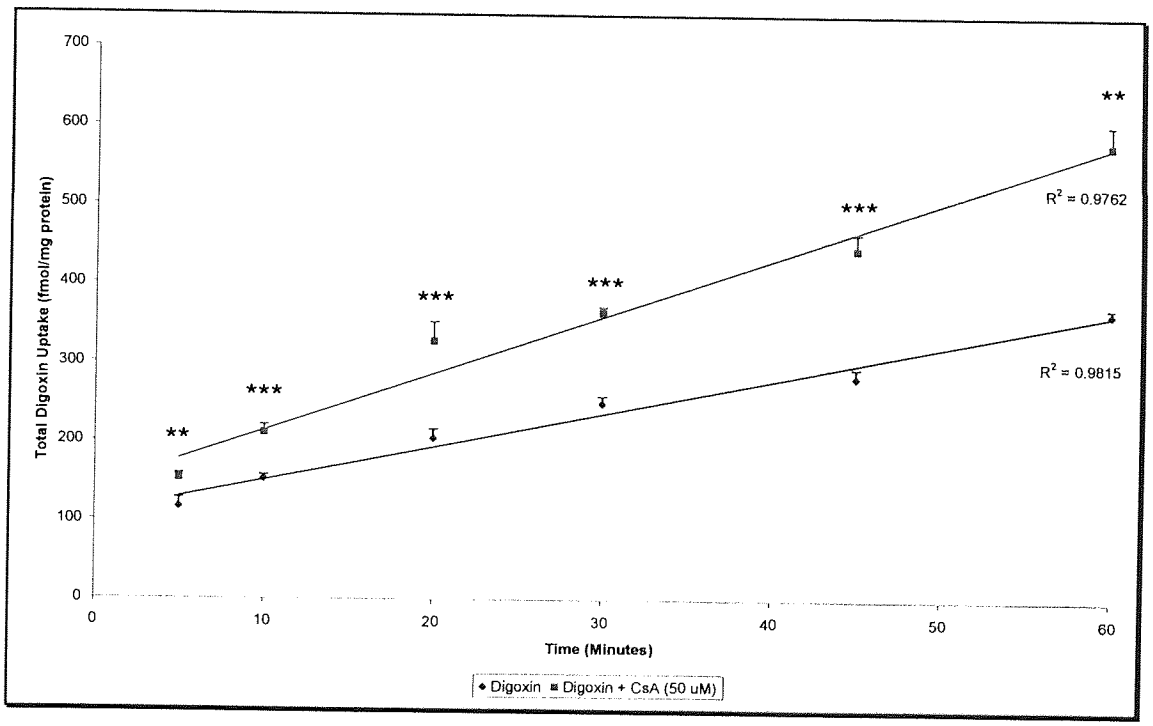

Figure 8.7 – Uptake of [^{3}H(G)]-Digoxin (24 nM) into Caco-2 cell monolayers was measured over time and in the presence of Cyclosporin A (50 μM).

Caco-2 cells, passage 26-40, were seeded onto plastic 24-well plates, monolayers were used 7-days post seeding. Uptake was measured over 30 minutes at 37°C. Data are mean and standard deviation of results from four wells. ** denotes a very significant ($p = 0.0014$) difference compared to the control; *** denotes an extremely significant ($p < 0.0001$) difference compared to the control (t-test).

Digoxin uptake was significantly increased in the presence of CsA (Figure 8.7), probably because CsA decreased digoxin efflux thereby increasing digoxin measured intracellularly

(Figure 8.7). CsA is both a substrate and inhibitor of P-glycoprotein, therefore, digoxin uptake may have increased following a decrease in digoxin efflux by CsA competing with digoxin for the P-glycoprotein efflux pump and by CsA inhibition of P-glycoprotein. Digoxin uptake was then measured in the presence of additional P-glycoprotein substrates and inhibitors. As mentioned previously there are many inhibitors and substrates of P-gp (Appendix 4), therefore, experimental undertakings were limited to five inhibitors / substrates that have previously been shown to modulate P-gp – verapamil (substrate / inhibitor; 100 μM), testosterone (substrate / inhibitor; 100 μM), terfenadine (substrate / inhibitor; 100 μM), nifedipine (inhibitor; 100 μM), and cyclosporin A (CsA; substrate / inhibitor; 50 μM) (Matheny *et al.*, 2001; Spatzenegger and Jaeger, 1995; Pascaud *et al.*, 1998; Hollo *et al.*, 1996; Ambudkar *et al.*, 1999). CsA has limited solubility; consequently, CsA was used at a lower concentration (50 μM) compared to the other inhibitors / substrates.

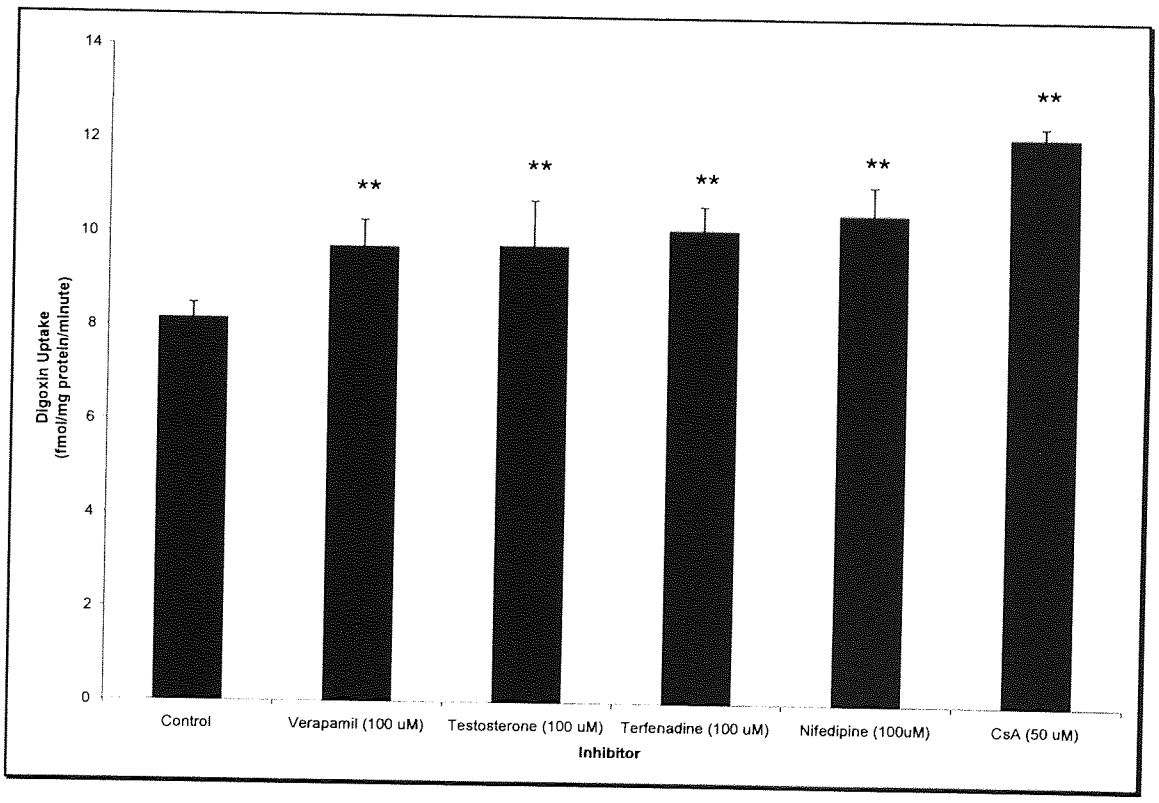

Figure 8.8 – Uptake of [^{3}H(G)]-Digoxin (24 nM) into Caco-2 cells was measured in the presence of P-glycoprotein inhibitors.

Caco-2 cells, passage 26-40, were seeded onto plastic 24-well plates, monolayers were used 7-days post seeding. Uptake was measured over 30 minutes at 37°C. Data are mean and standard deviation of results from four wells. ** denotes a very significant ($p < 0.01$) difference compared to the control (ANOVA).

Digoxin uptake was significantly ($p < 0.01$) enhanced in the presence of verapamil, testosterone, terfenadine, nifedipine and CsA (Figure 8.8). The presence of the P-glycoprotein inhibitors / substrates probably decreased digoxin efflux, thus increasing uptake (Figure 8.8). Co-administration of amprenavir (a HIV protease inhibitor) with P-gp inhibitors has enhanced the CNS penetration of amprenavir (Polli *et al.*, 1999).

8.3.2.3 Effect of Liposome Formulations on Digoxin Uptake

Digoxin uptake was measured in the presence of PC, PC:Cholesterol (16:4), DSPC, and DSPC:Cholesterol (16:4), two lipid amounts were used, 500 and 1000 μg. Neither PC nor PC:Cholesterol (500 and 1000 μg) increased digoxin uptake (Figure 8.9).

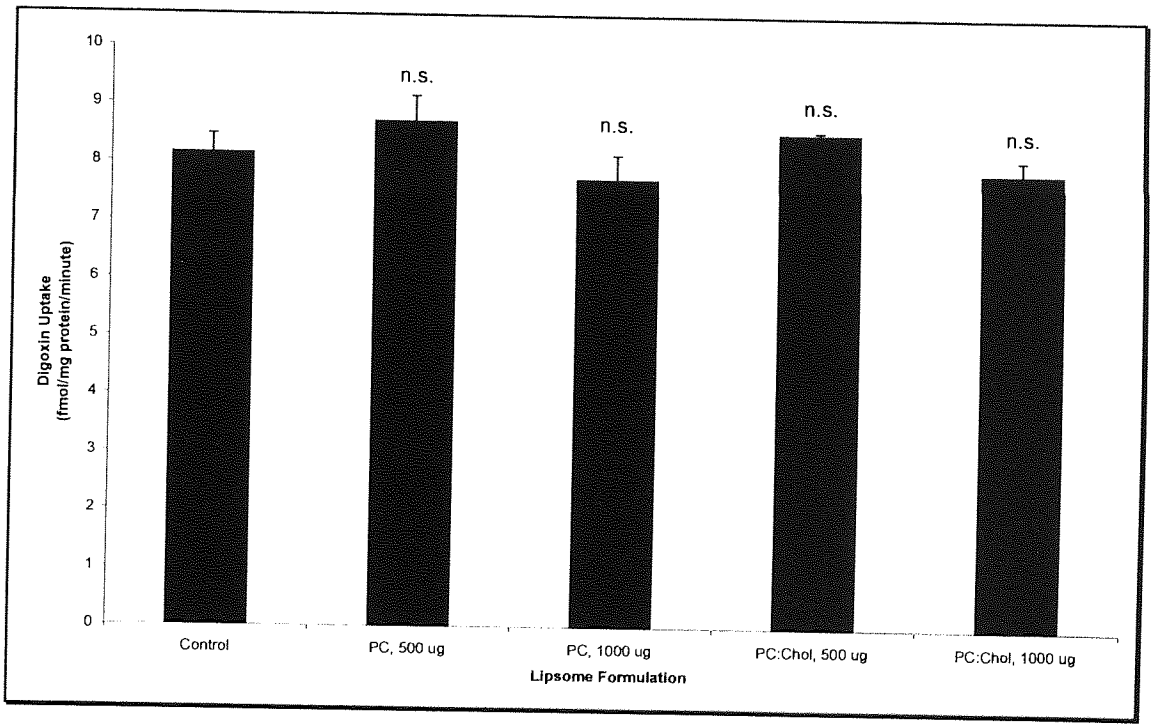

Figure 8.9 – Uptake of [^{3}H(G)]-Digoxin (24 nM) into Caco-2 cell monolayers was measured in the presence of PC and PC:Cholesterol (16:4) liposomes (500 and 1000 μg).

Caco-2 cells, passage 26-40, were seeded onto plastic 24-well plates, monolayers were used 7-days post seeding. Uptake was measured over 30 minutes at 37°C. Data are mean and standard deviation of results from four wells. n.s. denotes no significant ($p > 0.05$) difference compared to the control (ANOVA).

The inclusion of cholesterol in the liposome formulation did not increase digoxin uptake (Figure 8.9). Although the liposome formulations did not enhance digoxin uptake, digoxin uptake was not significantly reduced either.

PC was replaced with the higher transition temperature lipid DSPC and the uptake experiments repeated (Figure 8.10). DSPC significantly ($p < 0.01$) increased digoxin uptake and increasing the total lipid amount from 500 μg to 1000 μg resulted in a further increase in digoxin uptake (Figure 8.10). Inclusion of cholesterol into the formulation showed no effect on digoxin uptake.

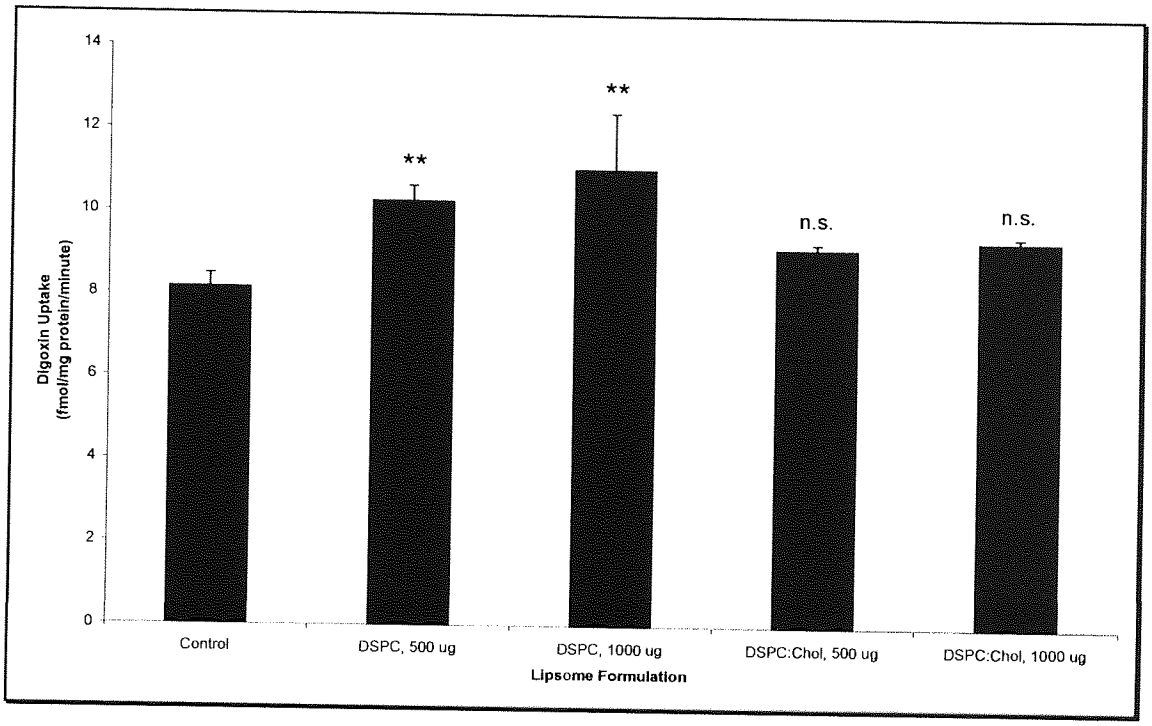

Figure 8.10 – Uptake of [^{3}H(G)]-Digoxin (24 nM) into Caco-2 cell monolayers was measured in the presence of DSPC and DSPC:Cholesterol (16:4) liposomes (500 and 1000 μg).

Caco-2 cells, passage 26-40, were seeded onto plastic 24-well plates, monolayers were used 7-days post seeding. Uptake was measured over 30 minutes at 37°C. Data are mean and standard deviation of results from four wells. ** denotes a significant ($p < 0.01$) difference compared to the control; n.s. denotes no significant ($p > 0.05$) difference compared to the control (ANOVA).

The presence of DSPC and DSPC:Cholesterol may have induced changes in membrane fluidity causing alterations in membrane transporter conformation. P-glycoprotein is a membrane transporter thus will be influenced by any changes in membrane fluidity and if the conformation of the transporter is altered P-glycoprotein may not be able to recognise substrates as efficiently, thus decreasing efflux and increasing uptake. Since the work presented here began it has been postulated that non-ionic surfactants reduced P-gp by altering membrane fluidity (Rege *et al.*, 2002). PC and PC:Cholesterol did not have the same effect on digoxin uptake this may be due to the fact that PC has a lower transition temperature compared to DSPC;

therefore, PC and DSPC may alter membrane fluidity to different extents resulting in the difference in digoxin uptake observed.

In the presence of P-gp inhibitors digoxin uptake was significantly increased (Figure 8.8) the same increase in digoxin uptake was not observed in the presence of the liposome formulations (Figure 8.9 and Figure 8.10). Digoxin uptake was significantly increased only in the presence of DSPC, 500 μg and 1000 μg (Figure 8.10), digoxin uptake increased 1.4-fold compared to a 1.3-fold increase in the presence of nifedipine and 1.5-fold increase in the presence of CsA (Figure 8.8). Verapamil, testosterone, terfenadine and CsA are both P-gp inhibitors and substrates (Appendix 4) hence implying that they function as competitive inhibitors. The mechanism of interaction between the liposome formulations and changes in cell membrane dynamics were beyond the scope the thesis; however, insight into these areas might present a possible explanation of the data, for example changes in membrane fluidity can alter transporter function.

8.3.3 Digoxin Transport Studies

8.3.3.1 Effect of Temperature on Digoxin Transport

Digoxin transport was measured using Transwell inserts, in both directions (A – B and B – A) at 4°C and 37°C. Active transport processes are sensitive to changes in temperature, therefore active processes will be reduced at the lower temperature of 4°C. Digoxin transport decreased in both directions (Figure 8.11). Digoxin transport in the apical-to-basolateral direction was reduced 2.1-fold compared to a 5.1 fold decrease in the basolateral-to-apical direction (Figure 8.11).

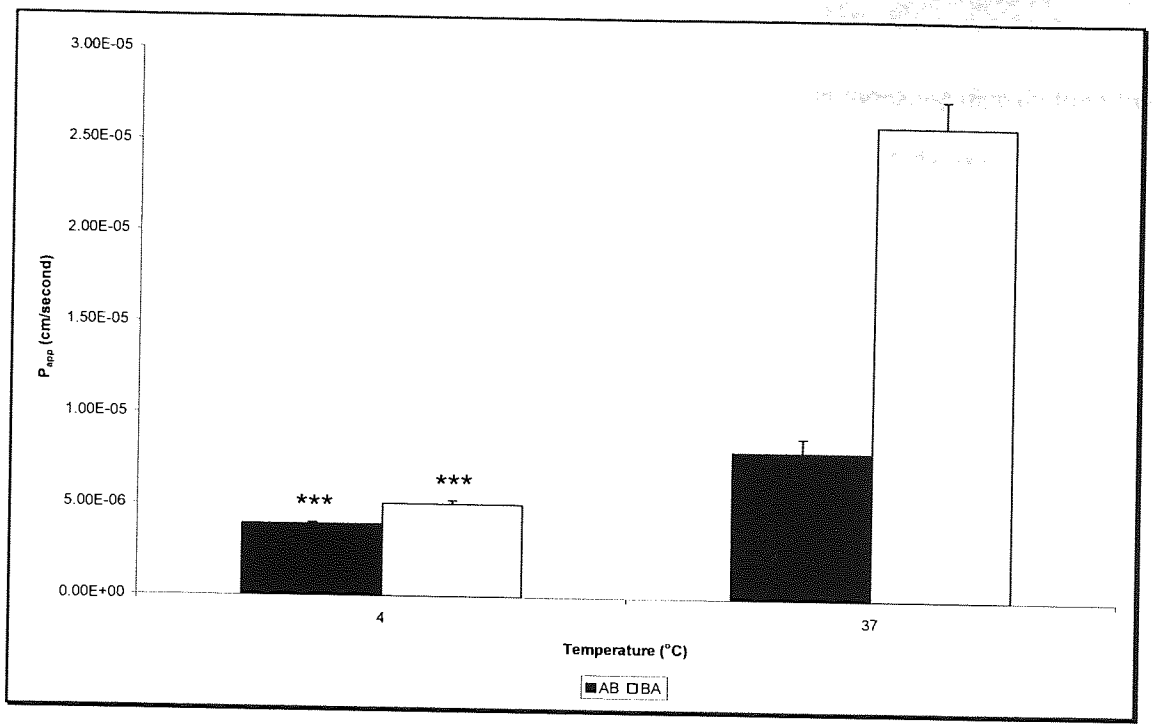

Figure 8.11 – The effect of temperature (4°C and 37°C) on [^{3}H(G)]-Digoxin (24 nM) transport (A – B and B – A) was measured using Transwell inserts.

Caco-2 cells were seeded on Transwell inserts, passage 26-40 and used 14-21 days post-seeding, transport was measured over 60-minutes, n = 4. *** denotes an extremely significant (A – B: $p = 0.0007$; B – A: $p < 0.0001$) difference compared to 37°C (t-test).

The decrease in temperature affected digoxin transport to a lesser extent in the A – B direction because digoxin is transport by both passive and active process; only the active transport will be affected by temperature. In contrast, digoxin transport in the B – A direction *i.e.* efflux is mediated by the active efflux transporter P-glycoprotein.

Digoxin transport is higher in the B – A direction compared to the A – B direction at both 4°C and 37°C. This indicates that digoxin efflux (B – A) exceeds A – B transport of digoxin resulting in a net secretion of digoxin. Efflux of digoxin is 3.2-fold greater than A – B transport at 37°C. Transcellular transport of digoxin has been shown to be temperature-dependent and B – A transport greater than A – B transport (Takara *et al.*, 2002). The apparent permeability of digoxin in the B – A direction was $20.0 \times 10^{-6} \pm 1.5 \times 10^{-6}$ cm/second which is similar to reported apparent permeability for digoxin *e.g.* $14.80 \times 10^{-6} \pm 2.06 \times 10^{-6}$ cm/second (Ingels *et al.*, 2004).

8.3.3.2 Effect of Inhibitors on Digoxin Transport

P-glycoprotein transports digoxin across the apical membrane – thus removing digoxin from the cells. Consequently, P-gp reduces digoxin permeability by promoting B – A digoxin transport. P-glycoprotein inhibitors and substrates should reduce B – A transport of digoxin, thus leading to an overall increase in digoxin absorption (A – B). CsA and nifedipine were chosen as P-glycoprotein inhibitors, digoxin transport was then measured in both direction of transport (Figure 8.12).

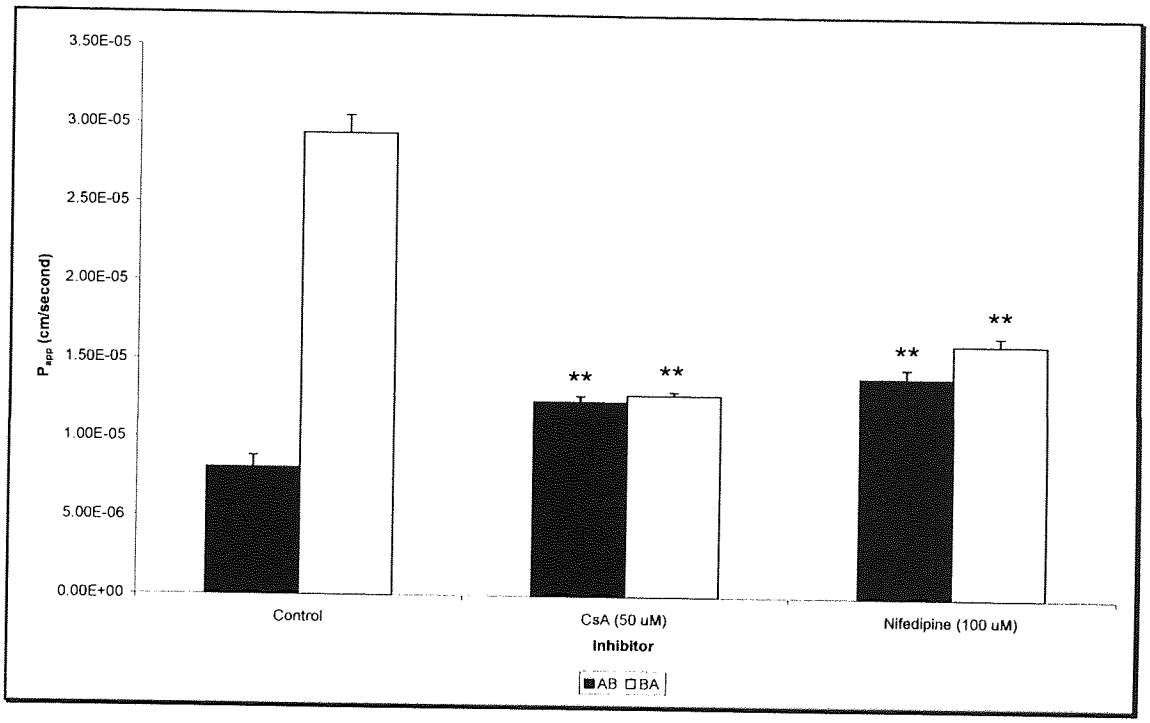

Figure 8.12 – Effect of the P-glycoprotein inhibitors CsA (50 μM) and nifedipine (100 μM) on [^{3}H(G)]-Digoxin (24 nM) transport (A – B and B – A).

Caco-2 cells, passage 26-40 were seeded on Transwell inserts and used 14-21 days post-seeding. Transport was measured over 60 minutes at 37°C, n = 4. ** denotes a very significant ($p < 0.01$) difference compared to the control (ANOVA).

In the absence of P-glycoprotein inhibitors B – A transport of digoxin was significantly (p 0.0001; t-test) greater than in the A – B direction (Figure 8.12). This indicates that P-glycoprotein contributes in the formation of an effective transport barrier to digoxin influx. The presence of CsA significantly ($p < 0.01$) increased A – B transport of digoxin; whereas the presence of CsA significantly ($p < 0.01$) reduced B – A transport of digoxin (Figure 8.12). Digoxin transport significantly ($p < 0.01$) increased in the A – B direction and was significantly ($p < 0.01$) reduced

in the B – A direction in the presence of nifedipine (Figure 8.12). Digoxin A – B permeability increased in the presence of CsA and nifedipine because P-gp efflux is reduced in the presence of inhibitors thus A – B permeability is not reduced by efflux of digoxin. B – A permeability of digoxin is decreased in the presence of CsA and nifedipine because of P-glycoprotein inhibition. The results are in agreement with a study that found nifedipine inhibited the basolateral-to-apical transport of digoxin in Caco-2 cells *via* inhibition of P-glycoprotein (Cavet *et al.*, 1996). Digoxin transport in the presence of CsA and nifedipine indicates that P-glycoprotein is important barrier to digoxin transport across Caco-2 cell monolayers.

8.3.3.3 Effect of Liposome Formulations on Digoxin Transport

TEER values were measured at the beginning and end of all transport experiments to determine if the Caco-2 cell monolayer had been detrimentally affected by the liposome formulations. TEER values were significantly reduced in the presence of all liposome formulations (Table 8.2). The decrease in TEER measurements infers that monolayer integrity was reduced in the presence of the liposome formulations. The decrease in monolayer integrity may enhance digoxin transport *via* the paracellular route resulting in increased transport.

	TEER (% of Initial Value)	Significance (ANOVA)	
PC (1000 μg)	88.2 ± 2.5	Very Significant	$p < 0.01$
PC:Cholesterol (1000 μg; 16:4)	87.6 ± 4.6	Very Significant	$p < 0.01$
DSPC (1000 μg)	86.5 ± 3.5	Very Significant	$p < 0.01$
DSPC Cholesterol (1000 μg; 16:4)	79.2 ± 6.4	Very Significant	$p < 0.01$

Table 8.2 – Percentage reduction in TEER value at the end of the experiment, compared to the control at the end of the experiment.

The effect liposome formulations (PC, PC:Cholesterol, DSPC and DSPC:Cholesterol, 1000 μg) had on TEER measurements (ANOVA with Dunnett's post-test, Instat 3).

Digoxin transport was significantly ($p < 0.01$) increased in the A – B direction in the presence of both PC and PC:Cholesterol, 1000 μg (Figure 8.13). The increase in cellular permeability of digoxin was expected from the reduction in TEER data (Table 8.2). Digoxin transport was increased 4.4-fold in the presence of PC, 1000 μg compared to a 2.1-fold increase in the presence of PC:Cholesterol, 1000 μg.

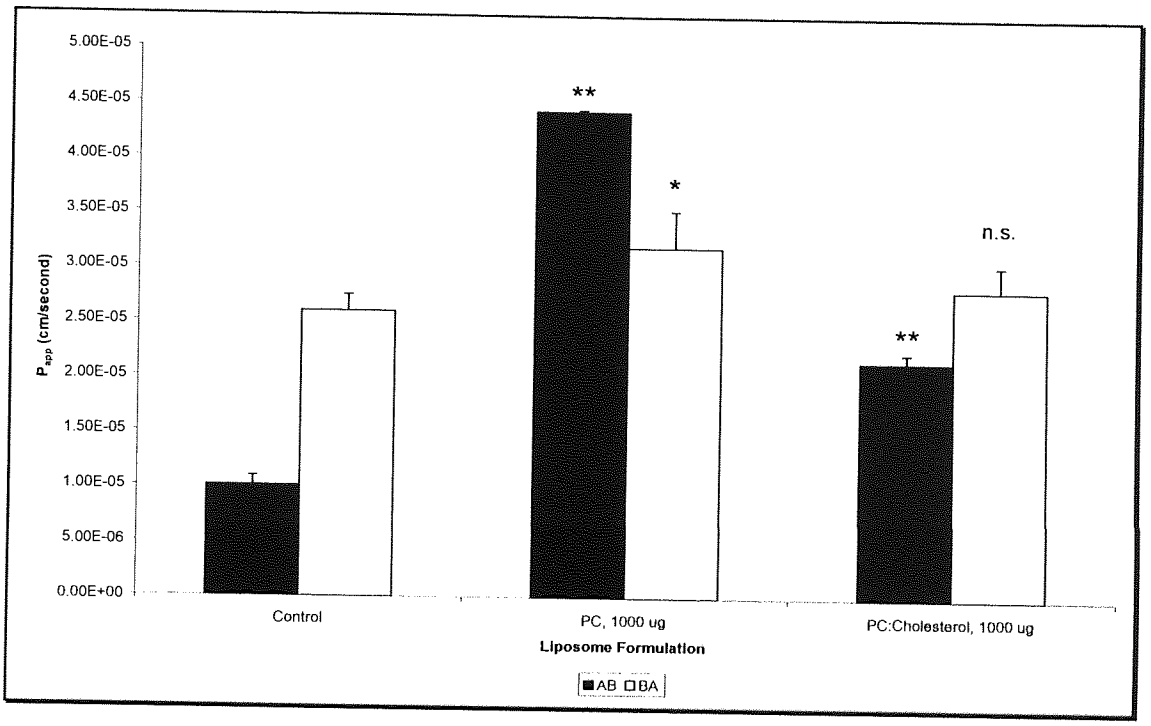

Figure 8.13 – [^{3}H(G)]-Digoxin (24 nM) transport in the apical-to-basolateral and basolateral-to-apical direction was measured in the presence of PC (1000 μg) and PC:Cholesterol (1000 μg; 16:4) liposomes.

Transport was measured at 37°C over 60 minutes, n = 4. Caco-2 cells were seeded onto Transwell inserts, passage 26-40 and used 14-21 days post seeding. ** denotes a very significant difference ($p < 0.01$); * denotes a significant ($p < 0.05$) difference compared to the control; n.s. denotes not a significant ($p > 0.05$) difference compared to the control (ANOVA).

Replacement of PC with DSPC also significantly ($p < 0.01$) increased A – B transport of digoxin (Figure 8.14). However, the DSPC-only liposomes increased digoxin transport 1.9-fold compared to 4.4-fold in the presence of PC. DSPC:Cholesterol and PC:Cholesterol both increased digoxin transport at a comparable rate 1.8-fold and 2.1-fold respectively. TEER values were reduced in the presence of DSPC and DSPC:Cholesterol (Table 8.2) therefore, the increased cellular permeability towards digoxin was expected. The increase in cellular permeability was probably due to increased passive transport of digoxin as the monolayer

integrity was reduced. Membrane fluidity may have been altered in the presence of the liposome formulations causing increased digoxin A – B transport.

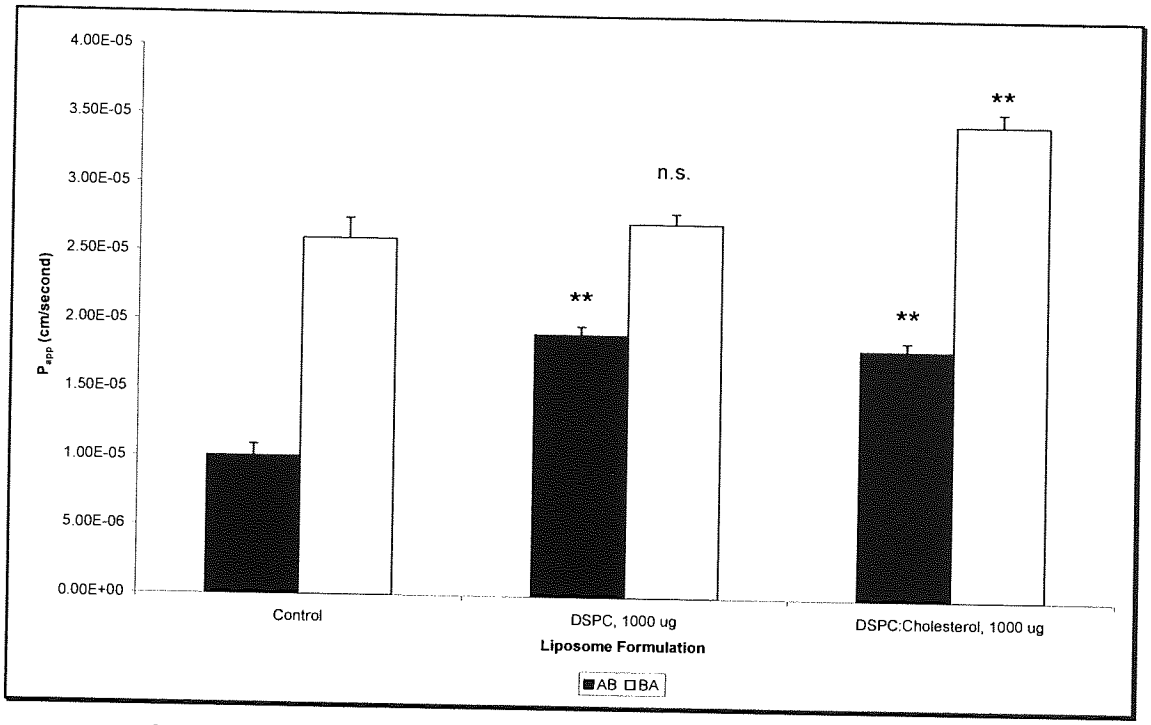

Figure 8.14 – [^{3}H(G)]-Digoxin transport (24 nM) in the apical-to-basolateral and basolateral-to-apical direction was measured in the presence of DSPC (1000 μg) and DSPC:Cholesterol (1000 μg; 16:4) liposomes.

Transport was measured at 37°C over 60 minutes, n = 4. Caco-2 cells were seeded onto Transwell inserts, passage 26-40 and used 14-21 days post seeding. ** denotes a very significant ($p < 0.01$) difference compared to the control; n.s. denotes not a significant ($p > 0.05$) difference compared to the control (ANOVA).

As an additional comparison, B – A digoxin transport was measured in the presence of the liposome formulations (Figure 8.13 and Figure 8.14). Mixed results were obtained from the B – A transport experiments; digoxin transport was significantly increased in the presence of PC (Figure 8.13) and DSPC:Cholesterol (Figure 8.14), 1000 μg and was not affected by the presence of PC:Cholesterol (Figure 8.13) and DSPC (Figure 8.14), 1000 μg. Digoxin transport was not reduced in the presence of PC:Cholesterol or DSPC. The increase in digoxin transport (B – A) was not as pronounced as in the A – B direction, PC, increased digoxin transport 1.2-fold (Figure 8.13) and DSPC:Cholesterol increased digoxin transport 1.3-fold (Figure 8.14). Membrane fluidity was most probably altered in the presence of the liposome formulations resulting in modified digoxin B – A transport, the differences observed between the formulations are probably due to differences in the transition temperature of PC and DSPC.

Dendrimers are potential drug carriers and in agreement with the PC:Cholesterol and DSPC data, dendrimers had no significant effect on B – A transport of ^{14}C-Pacilitaxel, a P-gp substrate (El-Sayed *et al.*, 2003).

The modulation of membrane transporters by several excipients has been investigated with P-gp a main focus (Woodcock *et al.*, 1992; Nerurkar *et al.*, 1996; Miller *et al.*, 1999; Koga *et al.*, 2000; Rege *et al.*, 2001). Numerous non ionic surfactants including sodium lauryl sulphate, Tween 80 and Cremophor EL have been shown to inhibit efflux by P-gp (Woodcock *et al.*, 1982; Sokol *et al.*, 1991; Badary *et al.*, 1998; Mountfield *et al.*, 2000; Rege *et al.*, 2001; Rege *et al.*, 2002). The mechanism behind the changes in permeability caused by excipients is not fully understood; however, changes in membrane fluidity is one explanation offered for changes in transporter function and consequently changes in movement of compounds across cell membranes (Rege *et al.*, 2002; Nano *et al.*, 2003).

In addition to modulation of membrane fluidity by excipients other possible explanations for alteration in transporter activity include changes in protein kinase C activity (Zhao *et al.*, 1989) and disruption of enzyme activity, for example Tween 80 and oleic acid inhibit CYP3A (Mountfield *et al.*, 2000).

CsA is a commonly used immunosuppressive agent however there are serious side effects *in vivo*. SDZ PCS 833 [(3'keto-Bmt^1)-(Val^2)-cyclosporin] (PCS 833) is more potent and less toxic analogue of CsA; however, PSC 833 interacts with anticancer drugs resulting in increased toxicity. Consequently, PSC 833 was entrapped in liposomes and markedly increased the intracellular accumulation of epirubicin in Caco-2 cells and increased the A – B absorption, thus liposomal preparations of PSC 833 may provide an alternative dosage form (Lo *et al.*, 2001a).

Ingels *et al.*, (2004) investigated the effect simulated intestinal fluid (FaSSIF) has on transport of a number of drugs; they showed that FaSSIF reduced passive transport of propranolol (Chapter

6). However, the reduction in A – B transport was not observed for P-gp substrates, this maybe due to inhibition of B – A transport (Ingels *et al.*, 2004).

8.4 Conclusion

From the uptake studies, it can be concluded PC, 500 μg, PC, 1000 μg, PC:Chol, (16:4), 500 μg and PC:Chol, (16:4) 1000 μg had no significant impact on digoxin uptake across Caco-2 cell monolayers (Figure 8.9). In contrast, DSPC, 500 μg, DSPC, 1000 μg DSPC:Chol, (16:4), 500 μg and DSPC:Chol, (16:4), 1000 μg all had a positive effect on digoxin uptake (Figure 8.10).

From the transport studies, it can be concluded that all four liposome formulations significantly enhanced digoxin transport in the apical-to-basolateral direction, (Figure 8.13 and Figure 8.14). The presence of the liposome formulations affected the B – A transport of digoxin to a lesser extent; only PC and DSPC:Cholesterol significantly affected digoxin transport (Figure 8.13 and Figure 8.14). The presence of the liposome formulations probably altered membrane fluidity increasing A – B transport of digoxin. Vitamin E TPGS, Cremophor EL (Rege *et al.*, 2002), Tween 80 (Rege *et al.*, 2002; Lo, 2003) Tween 20, Myri 52, and Brij 30 (Lo, 2003) have all been shown to modify P-glycoprotein activity and thus the transport of P-gp substrates: rhodamine 123 and epirubicin. These excipients had different affects on P-glycoprotein – Cremophor EL (Rege *et al.*, 2002) Tween 20, Tween 80 Myri 52, and Brij 30 (Lo, 2003) increased A – B permeability and decreased B – A permeability thus inhibiting P-glycoprotein activity (Rege *et al.*, 2002; Lo, 2003). Vitamin E TPGS inhibited P-glycoprotein activity by reducing B – A permeability, whilst A – B permeability remained unaffected (Rege, *et al.*, 2002). P-glycoprotein activity was decreased possibly due to the modulation of membrane fluidity. However, the effect was brought about by two different mechanisms; membrane fluidity was either increased (*e.g.* Tween 80 or Cremophor EL) or decreased (*e.g.* vitamin E TPGS) in the presence of the excipients (Rege *et al.*, 2002; Lo, 2003). Although numerous excipients have been identified to inhibit P-gp, the mechanism remains unclear. However, a number of excipients have been shown to modulate membrane fluidity. The physical state of the cell membrane is important in the regulation of membrane bound transporters, consequently it is has been postulated that

changes in membrane fluidity cause changes in the conformation of membrane bound transporters; thus, reducing the activity of the transporter (Rege *et al.*, 2002). The influence the liposome formulations used here have on membrane fluidity of Caco-2 cells needs to be assessed in order to verify if the liposome formulations have the same or different influences on membrane fluidity and thus provide an explanation for the differences observed.

From the uptake studies and transport studies either DPSC or DSPC:Cholesterol are the more favourable liposome formulations for further investigations because digoxin uptake (Figure 8.10) was increased and digoxin A – B permeability was increased (Figure 8.14). PC or PC:Cholesterol should not be excluded as potential excipients as digoxin A – B permeability was significantly increased in their presence (Figure 8.13). The differences observed between the formulations may result from differential effects of liposomes on Caco-2 cell membrane fluidity.

It can be concluded that all four liposome formulations tested are potentially suitable candidates for use as delivery vehicles for drugs that are P-gp substrates or drugs that require P-gp activity to be modified; however, DSPC or DSPC:Chol, (16:4) are liposome formulations that are most likely to deliver the greatest benefits. The mechanism by which the liposome formulations influence digoxin uptake and transport is likely to involve altered membrane fluidity of the Caco-2 cell membrane; however this requires further investigation, by utilising fluorescence anisotropy techniques (Section 7.3.2.3). Changes in membrane fluidity may result in changes in membrane transporter activity. Liposome interactions with the Caco-2 cell membrane need to be investigated, for example, liposome fusion, phospholipid exchange and liposome uptake in order to determine if liposome formulations are capable of delivering drugs into Caco-2 cells (Section 5.3.2).

Chapter 9

Conclusions

9. Conclusions

The aim of this thesis was to study the potential use of liposomes as drug delivery vehicles for compounds with intermediate LogP values and their potential as modulators of drug absorption using the Caco-2 cell line. The first part of the thesis investigated the influence of LogP values on incorporation, size, release and transport of hydroxybenzoates. The second part investigated the toxicity of liposome formulations towards the Caco-2 cell line using the MTT assay and crystal violet assay and their influence on compounds absorbed *via* both passive and active routes of absorption.

Liposomes were chosen because both hydrophilic and lipophilic drugs can be incorporated and liposome size, composition, charge and lamellarity can all be manipulated with ease (Section 1.8). Liposome stability in the GI tract can be problematic due to the presence of bile salts and degradation by intestinal lipases; however, component lipids can be chosen to reduce these effects (Section 1.8.6.1). PC, DSPC and cholesterol were chosen as component lipids for this study because they have previously been used in research to improve oral delivery. PC and cholesterol have been used in formulations to improve oral delivery of insulin (Patel and Ryman, 1976) (Section 1.8.5.2 and Section 1.8.5.7). DSPC and cholesterol have previously been shown to be stable in simulated GI conditions and retain incorporated drugs (Rowland and Woodley, 1980; Regnault *et al.*, 1996; Minato *et al.*, 2003) (Section 1.5.8.1, Section 1.8.5.3 and Section 1.8.5.7).

LogP values determine where a compound will reside within a liposome; hydrophilic (low LogP value, < -0.3) reside in the aqueous compartments of the liposomes whereas lipophilic compounds (high LogP value, > 5) reside within the lipid bilayer. Compounds with intermediate LogP values (1.7 < 4) are able to partition between phases (Gulati *et al.*, 1998). The four hydroxybenzoates chosen as model drugs have similar molecular weights (152.1 – 194.2), with intermediate LogP values (1.85 – 3.44). MP has the lowest LogP value, the smallest critical

volume and was most successfully incorporated within all liposome formulations tested. In contrast, BP with the highest LogP value and largest critical volume was the least successfully incorporated into similar formulations. Key factors noted to influence bilayer drug loading included cholesterol bilayer concentration: increasing the cholesterol content from 20% (16:4) to 50% (16:16) (total lipid) was seen to reduce incorporation values of all of the hydroxybenzoates tested; indeed, attempts to incorporate BP within liposomes composed of PC:Chol (16:16 μmoles) were unsuccessful. Secondly, phospholipid structure was shown to play a role. Replacement of PC with the long alkyl chain length lipid DSPC significantly improved incorporation of MP (1 mg) into most of the liposome formulations tested.

Studying the release rates of the model drugs from both PC:Cholesterol and DSPC:Cholesterol liposomes were similar, the release profiles were noted to be similar: MP had the slowest release compared to the larger molecule BP that had the highest release. The dissolution media also had an effect on drug release rates with release of MP being enhanced when FaSSIF was used as the receiver medium compared to both water and SGF. Subsequent transport studies confirmed the findings of the release study; that BP was preferentially transported compared to MP even though the opposite would be predicted from their LogP values. Free MP (not incorporated into liposomes) and free BP were also used in the transport studies and as predicted from the LogP values, free MP was transported at a faster rate than BP. Transport of both MP and BP was slower from the liposomes compared to the free solutions. The reversal of the transport rates when the drug was incorporated within liposomes provided further evidence that not only are LogP values important but the structure of the solute and the strain the solute puts on the bilayer is an important determinant on how a solute is released and transported from liposome formulations.

The results indicate that although LogP values are an important determinant, the size and structure of a solute are also very important. As molecular weight increases MP < EP < PP < BP; in accordance with increasing R-group size ($CH_3 - C_4H_9$), the increase in critical volume will increase the strain on the bilayer and if the strain is too great, the bilayer will collapse. In this

study, as the R-group size increased from MP – BP the increased strain on the bilayer is more likely to cause the bilayer to collapse. BP has the largest critical volume and the longest R-group and was the least successfully incorporated into the liposome formulations. It was released at the fastest rate and the total amount released was greatest for BP from both PC:Cholesterol (16:4) and DSPC:Cholesterol (16:4). This infers that the increased size of BP had a detrimental affect on bilayer stability especially as BP was expected to release the least amount as it is the most lipophilic of the hydroxybenzoates used. Moreover, incorporation was most successful in liposomes composed of PC:Cholesterol and DSPC:Cholesterol (16:4) and were therefore employed in further studies involving the Caco-2 cell line.

TEER measurements over 21-days showed Caco-2 cells grew well and tight junction formation were well developed rendering the Caco-2 cells suitable for experimental use. Mannitol was used as a paracellular marker and mannitol transport was measured in the presence and absence of a developed Caco-2 cell monolayer to determine that the Transwell membrane provided no resistance to transport. In addition, it provided additional evidence that the Caco-2 cell monolayer and tight junctions had formed. Apparent permeability (P_{app}) was reduced from $7.91 \times 10^{-4} \pm 9.94 \times 10^{-5}$ cm/second in the absence of cells to $9.88 \times 10^{-7} \pm 7.48 \times 10^{-8}$ cm/second in the presence of cells, supporting the TEER values as a measure of a well-developed monolayer. The MTT assay and crystal violet assay were employed as toxicity assays. The controls used included cell culture media, EDTA, Triton-X, FaSSIF and SGF. The cell culture media and FaSSIF proved not to be toxic, in contrast ETDA, Triton-X and SGF were found to be toxic towards the Caco-2 cell line; the data obtained for SGF and FaSSIF was in agreement with Ingels *et al.*, 2002. The toxicity of the liposome formulations was then evaluated. None of the liposome formulations (PC, PC:Cholesterol, DSPC, DSPC:Cholesterol) proved to be toxic and therefore could be used in with the Caco-2 cell line for further experiments.

The effect PC, PC:Cholesterol (16:4), DSPC, and DSPC:Cholesterol (16:4) liposomal formulations had on paracellular, passive transcellular, carrier-mediated and efflux were investigated using mannitol, propranolol, glutamine and digoxin as probes.

Mannitol transport was shown to be a passive process; A – B and B – A transport were similar (AB/BA P_{app} ratio = 0.96 at 37°C) and the P_{app} was very low, in line with paracellular transport and similar to those found within the literature (Artursson and Karlsson, 1991; Rao *et al.*, 1999; Lentz *et al.*, 2000). Propranolol uptake and transport was also shown to be a passive process (temperature-independent uptake and bidirectional transport of propanolol was similar). Glutamine uptake and transport was shown to be temperature-dependent, concentration-dependent and was inhibited in the presence of metabolic inhibitors and other amino acids, and dipeptides did not alter glutamine uptake, as expected. Digoxin transport was greater in the B – A direction compared to the A – B direction as predicted from a compound that is subjected to efflux *via* the efflux pump, P-glycoprotein. P-glycoprotein inhibitors and substrates increased A – B transport and decreased B – A transport as expected. Excipients are currently being actively researched because many new drugs being developed are lipophilic presenting both delivery and solubility problems. The action of excipients varies, some increase the solubility of the compound as a drug must be dissolved before it can be absorbed. Some excipients modulate the tight junctions, thus increasing absorption through the paracellular route, and some excipients alter membrane fluidity. The work presented here investigated the potential use of liposomes as excipients to improve mannitol, propranolol, glutamine and digoxin uptake and transport across Caco-2 cell monolayers (Table 9.1).

PC was chosen because it has a low transition temperature (< 0°C), therefore, at physiological temperature (37°C), PC will be in the liquid crystal phase. In contrast DSPC has a higher transition temperature (57°C) and will be in the gel phase at 37°C. Cholesterol has been shown to alter membrane fluidity (Rege *et al.*, 2002) and was shown to be beneficial from the hydroxybenzoate studies; consequently, cholesterol was included into the formulation at 4 μM.

The data obtained is summarised in Table 9.1. Varied data was obtained, in some circumstances the liposomes had no effect on the uptake or transport of the probe, in others they significantly increased or decreased the uptake and transport. The increase in mannitol transport A – B indicates that the tight junctions have been affected, potentially increasing absorption *via* the paracellular route. The data obtained from the study using propranolol as the probe raised serious questions because propranolol was used to study the passive transcellular route, which is a major route of absorption; even those compounds that are transported *via* carrier-mediated routes have a passive transcellular component. The presence of the liposome formulations significantly decreased both uptake and transport (Table 9.1), which translates directly as a significant decrease in absorption – the opposite effect was intended. Propranolol is a lipophilic compound, therefore, propranolol may have partitioned into the liposome formulations and not across into the Caco-2 cell monolayer. Propranolol transport has also been shown to be reduced by possible encapsulation or interaction with sodium taurocholate/lecithin mixed micelles (Ingels *et al.*, 2004).

The carrier-mediated route was significantly aided by the presence of the liposome formulations, as was A – B transport of digoxin. The mechanism of action was outside the scope of the project presented here, however, from other studies exploring the use of excipients potential mechanisms include alteration of the tight junctions and / or membrane fluidity (Dudeja *et al.*, 1995; Sakai *et al.*, 1997; Ferte, 2000; Rege *et al.*, 2002; Lo, 2003). Changes in tight junctions and membrane fluidity have improved the permeability of drugs including fluorescein, doxorubicin and rhodamine-123; however, the improvement is not restricted to either an increase or decrease in membrane fluidity. It is postulated that changes in membrane fluidity alter transporter conformation and consequently activity. Knowledge of how the liposome formulations influence the membrane fluidity of the Caco-2 cell monolayer would aid interpretation of the data obtained. The mechanism(s) of action for liposomes needs to be ascertained. Two potential mechanisms are plausible; the first being a change in membrane fluidity and the second being loss of monolayer integrity resulting in an increase in paracellular permeability.

Probe	PC (16 μM)	PC:Cholesterol (16 μM : 4 μM)	DSPC (16 μM)	DSPC:Cholesterol (16 μM : 4 μM)
Mannitol Transport A – B	n.s. ($p > 0.05$)	Increase ($p < 0.01$)	Increase ($p < 0.01$)	Increase ($p < 0.05$)
Mannitol Transport B – A	n.s. ($p > 0.05$)	n.s. ($p > 0.05$)	n.s. ($p > 0.05$)	Increase ($p < 0.05$)
Propranolol Uptake	Decrease ($p < 0.01$)	Decrease ($p < 0.01$)	n.s. ($p > 0.05$)	n.s. ($p > 0.05$)
Propranolol Transport A – B	Decrease ($p < 0.01$)	Decrease ($p < 0.01$)	Decrease ($p < 0.05$)	Decrease ($p < 0.05$)
Propranolol Transport B – A	Decrease ($p < 0.01$)	Decrease ($p < 0.01$)	Decrease ($p < 0.01$)	Decrease ($p < 0.01$)
Glutamine Uptake	Increase ($p < 0.01$)	Increase ($p < 0.01$)	Increase ($p < 0.01$)	Increase ($p < 0.01$)
Glutamine Transport A – B	Increase ($p < 0.01$)	Increase ($p < 0.01$)	Increase ($p < 0.05$)	Increase ($p < 0.01$)
Glutamine Transport B – A	Increase ($p < 0.01$)	Increase ($p < 0.01$)	Increase ($p < 0.01$)	Increase ($p < 0.01$)
Digoxin Uptake	n.s. ($p > 0.05$)	n.s. ($p > 0.05$)	Increase ($p < 0.01$)	n.s. ($p > 0.05$)
Digoxin Transport A – B	Increase ($p < 0.01$)	Increase ($p < 0.01$)	Increase ($p < 0.01$)	Increase ($p < 0.01$)
Digoxin Transport B – A	Increase ($p < 0.05$)	n.s. ($p > 0.05$)	n.s. ($p > 0.05$)	Increase ($p < 0.01$)

Table 9.1 – Summary of the effects of liposome formulations on the absorption probes used.

The work presented within this thesis has indicated the importance of both solute structure and LogP values on incorporation, release and transport. The importance of including cholesterol within the formulation was also identified for incorporation. The effect PC, PC:Cholesterol (16:4), DSPC, and DSPC:Cholesterol (16:4) had on absorption was determined and diverse data was

obtained. However, the liposome formulations tested did show potential to enhance absorption of drugs that utilise the paracellular route; carrier-mediated route and those that are subject to efflux *via* P-gp.

In conclusion, the hydroxybenzoate model drug series were successfully incorporated into PC:Cholesterol liposomes, however, efficiency of this is dependent on the structure of the solute. Both PC:Cholesterol and DSPC:Cholesterol (16:4) both proved to be appropriate formulations for the controlled release and transport of MP and BP. The liposome formulations are non-toxic towards Caco-2 cell monolayers. In addition, liposome formulations increased paracellular transport of mannitol, and carrier-mediated uptake and transport of glutamine. Liposomes have the potential to be used as excipients to improve drug absorption, if the drug is also loaded into the liposome, the liposome will also act as a drug delivery carrier.

9.1 Future Work

Further work must be carried out before a final assessment of the use of liposomes as excipients can be made. Changes in membrane fluidity need to be determined using fluorescence anisotropy techniques and recovery studies performed to determine if decreases in TEER measurements, thus indicating loss of monolayer integrity, are reversible or permanent. Interactions between the liposome formulations and the Caco-2 cell monolayer that potentially alter membrane dynamics need to be ascertained, for example, occurrence of liposome fusion or phospholipid exchange.

The experiments need to be repeated using micelles, LUVs and SUVs to ascertain if the data obtained here is specific to MLVs or if micelles, LUVs and SUVs have the same or different affects on uptake and transport across Caco-2 cell monolayers. To fully investigate the potential use of liposomes to increase and enhance the delivery of drugs, additional formulations need to be investigated *e.g.* use of other lipids in replacement of / or in addition to PC and DSPC. To further investigate the use of liposomes as both a drug delivery system and potential absorption and permeation enhancer the total amount of lipid used needs to be increased whilst monitoring

toxicity levels. To verify that the effect of absorption is not unique to the probes used, the experiments must be repeated using alternative probes for each of the absorption routes, and repeated using loaded liposomes. Drug(s) of interest must be incorporated into the liposome formulation and their release and transport across Caco-2 cell monolayers measured. This is particularly important for propanolol and the passive transcellular route in order to verify if the lipophilicity of propranolol caused partitioning into the liposome bilayer, thus decreasing uptake and transport.

Many drugs are subject to efflux *via* P-glycoprotein, therefore, the ultimate goal would be to have a liposome formulation that increases absorption, delivers the drug of interest and also offers a P-glycoprotein inhibitor within the same formulation. For this to be possible one of the drugs must be lipophilic so it will reside in the bilayer and the other to be hydrophilic so the drug can reside in the aqueous spaces. The presence of the P-gp inhibitor would thus reduce the effects of P-gp and improve absorption. Drug absorption would be further improved if the liposome formulation modulates membrane fluidity causing an improvement in absorption *via* paracellular and / or transcellular absorption and by reducing P-gp activity.

The most convenient aspect of oral drug delivery is that capsules can be administered at anytime and in a relatively discrete manner, requiring only fluid to aid swallowing. Following oral delivery the stomach must be navigated before reaching the small intestine. The environment of the stomach is very harsh; one option to overcome the environment of the stomach is to use gelatin capsules (Gursoy and Benita, 2004) because the capsules are insoluble at low pH (gastric fluid) and dissolve at a higher pH (intestinal fluid) (Pearnchob *et al.*, 2004). The capsule would provide protection for the liposome formulation as it moves along the oesophagus and through the stomach until reaching the small intestine, where the capsule would disintegrate leaving the liposome formulation to bring about the required effect; the liposome formulation acting as delivery vehicle releasing the drug for absorption and the components of the liposome acting as excipients enhancing absorption.

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World Wide Web Sources

http://www.mhc.com/PGP/PgpTable.HTML

Appendix 1

Michaelis-Menten Kinetics

A1. Michaelis-Menten Kinetics

A1.1 Calculation of Kinetic Parameters

The binding of a substrate to its transport protein resembles that of an enzyme-substrate reaction. The rate of uptake will be maximal when the transporter protein is saturated; this rate is referred to as V_{max}. The transporter has a characteristic binding constant for the substrate that is known as the Michaelis constant (K_m). K_m is equivalent to the concentration of substrate for half maximal uptake. The relationship of the reaction is shown in Equation A1.1.

$$V = \frac{(V_{max} x[S])}{(K_m + [S])}$$

Equation A1.1 – The Michaelis-Menten equation for active uptake.

Where:

V	=	Uptake (moles/mg protein/minute)
V_{max}	=	Maximum uptake (moles/mg protein/minute)
$[S]$	=	Initial substrate concentration (mM)
K_m	=	Michaelis-Menten constant (mM)

Measured uptake includes a passive component and the Michaelis-Menten equation can be adapted to include a new constant related to passive uptake, K_d. The adapted equation is shown below (Equation A1.2).

$$V = \left(\frac{(V_{max} x[S])}{(K_m) + [S]}\right) + (K_d x[S])$$

Equation A1.2 – The adapted Michaelis-Menten equation for total uptake.

Where:

V	=	Uptake (moles/mg protein/minute)
V_{max}	=	Maximum uptake (moles/mg protein/minute)

$[S]$ = Initial substrate concentration (mM)

K_m = Michaelis constant (mM)

K_d = Passive uptake constant (nmol/mg protein/minute/mM)

The passive component (K_d) can be calculated using computer software. Once known the passive component of uptake can be removed leaving only active uptake. Plotting active uptake at different concentrations (V against [S]) produces a curved line that flattens off. It is not easy to calculate V_{max} or K_m from this curve so different plots are used. Inverting Equation A1.1 produces the following equation (Equation A1.3).

$$\left(\frac{1}{V}\right) = \left(\left(\frac{K_m}{V_m}\right) x \left(\frac{1}{[S]}\right)\right) + \left(\frac{1}{V_{max}}\right)$$

Equation A1.3 – Inverting the Michaelis-Menten equation.

Where:

V = Uptake (moles/mg protein/minute)

V_{max} = Maximum uptake (moles/mg protein/minute)

$[S]$ = Initial substrate concentration (mM)

K_m = Michaelis-Menten constant (mM)

Plotting $\left(\frac{1}{V}\right)$ against $\left(\frac{1}{[S]}\right)$ will produce a straight line with a gradient of $\left(\frac{K_m}{V_{max}}\right)$ and a y-intercept of $\left(\frac{1}{V_{max}}\right)$. This is known as a Lineweaver-Burk plot.

The Lineweaver-Burk plot derived from Equation A1.3 above can be used to calculate K_m and V_{max} although it is not very accurate. Inverting results at low concentrations produces large numbers so any errors are amplified while results are high concentrations are less emphasised. A more accurate plot is an Eadie-Hofstee plot derived from a rearranged Michaelis-Menten equation as shown in Equation A1.4. This plot produces a line with a gradient of $-K_m$ and a Y-

intercept of V_{max}. Both kinetic parameters can be calculated and, as there is no inverting, errors are constant along the line.

$$V = \frac{(V_{max} x[S])}{(K_m + [S])}$$

Multiply by	$(K_m + [S])$	$V(K_m + [S]) = (V_{max} x[S])$
Subtract	(VxK_m)	$(Vx[S]) = (V_{max} x[S]) - (VxK_m)$
Divide by	$[S]$	$V = V_{max} - \left(K_m x \left(\frac{V}{[S]}\right)\right)$

Equation A1.4 – Rearranging the Michaelis-Menten equation.

Where:

V	=	Uptake (moles/mg protein/minute)
V_{max}	=	Maximum uptake (moles/mg protein/minute)
$[S]$	=	Initial substrate concentration (mM)
K_m	=	Michaelis-Menten constant (mM)

Plotting V against $\left(\frac{V}{[S]}\right)$ will produce a straight line with a gradient of $(-K_m)$ and a y-intercept of V_{max}. This is known as an Eadie Hofstee Plot.

Appendix 2

Hydroxybenzoate Data

A2. Hydroxybenzoate Data

A2.1 Hydroxybenzoate Structures and Calculated Parameters

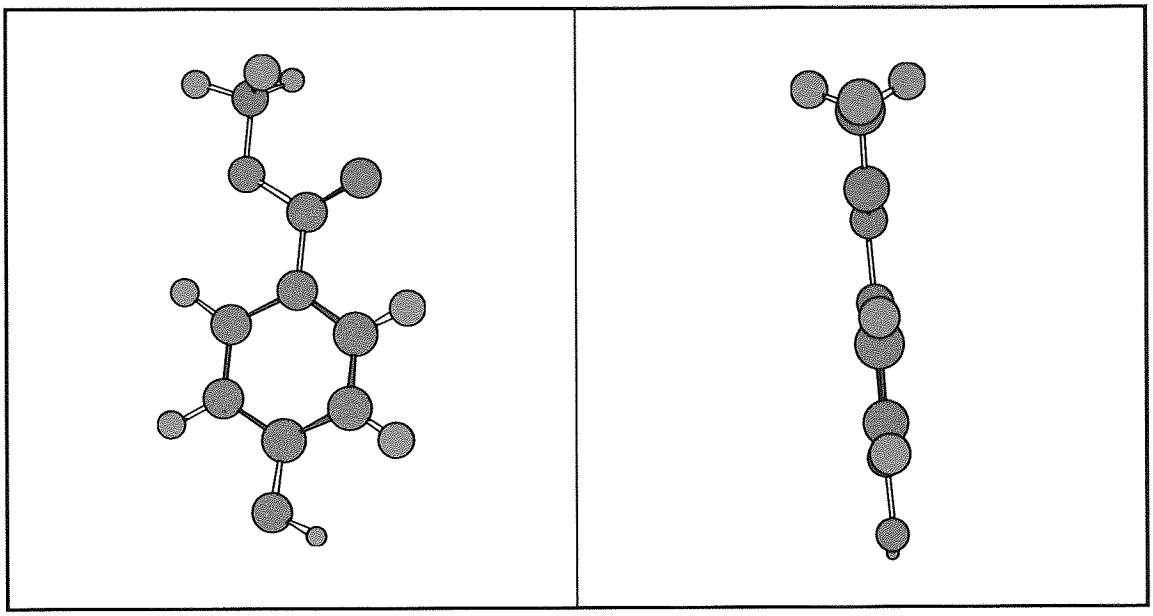

Figure A2.1 – Structure of methyl hydroxybenzoate.

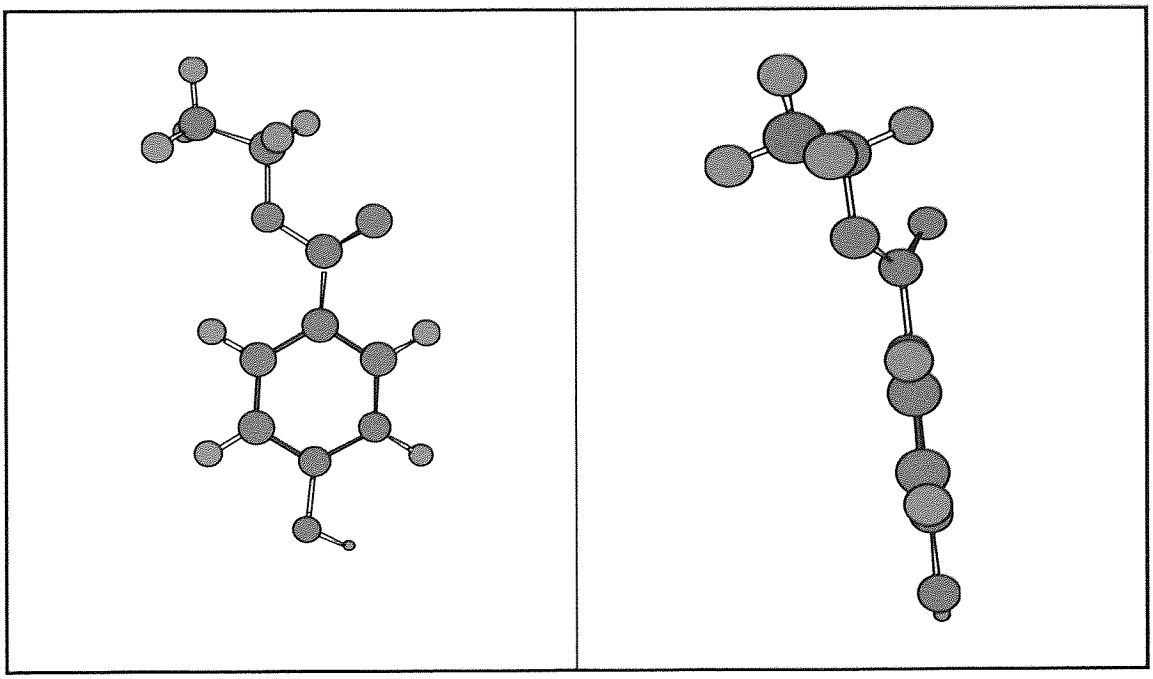

Figure A2.2 – Structure of ethyl hydroxybenzoate.

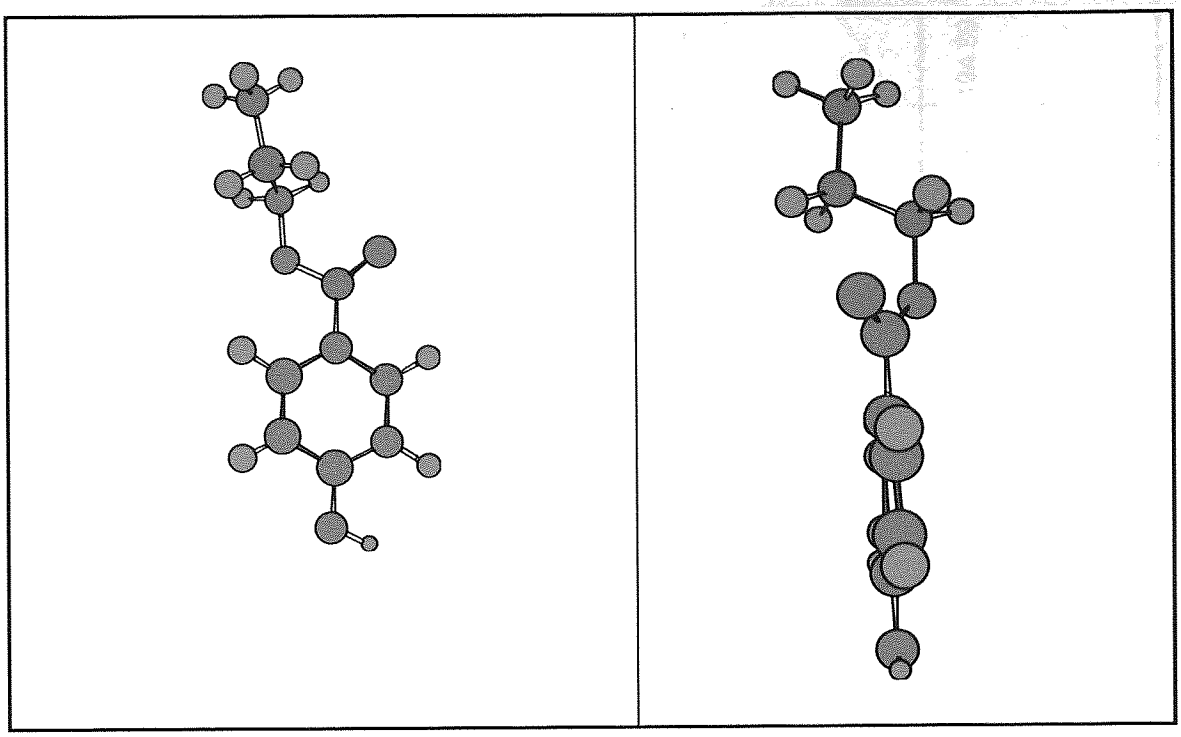

Figure A2.3 – Structure of propyl hydroxybenzoate.

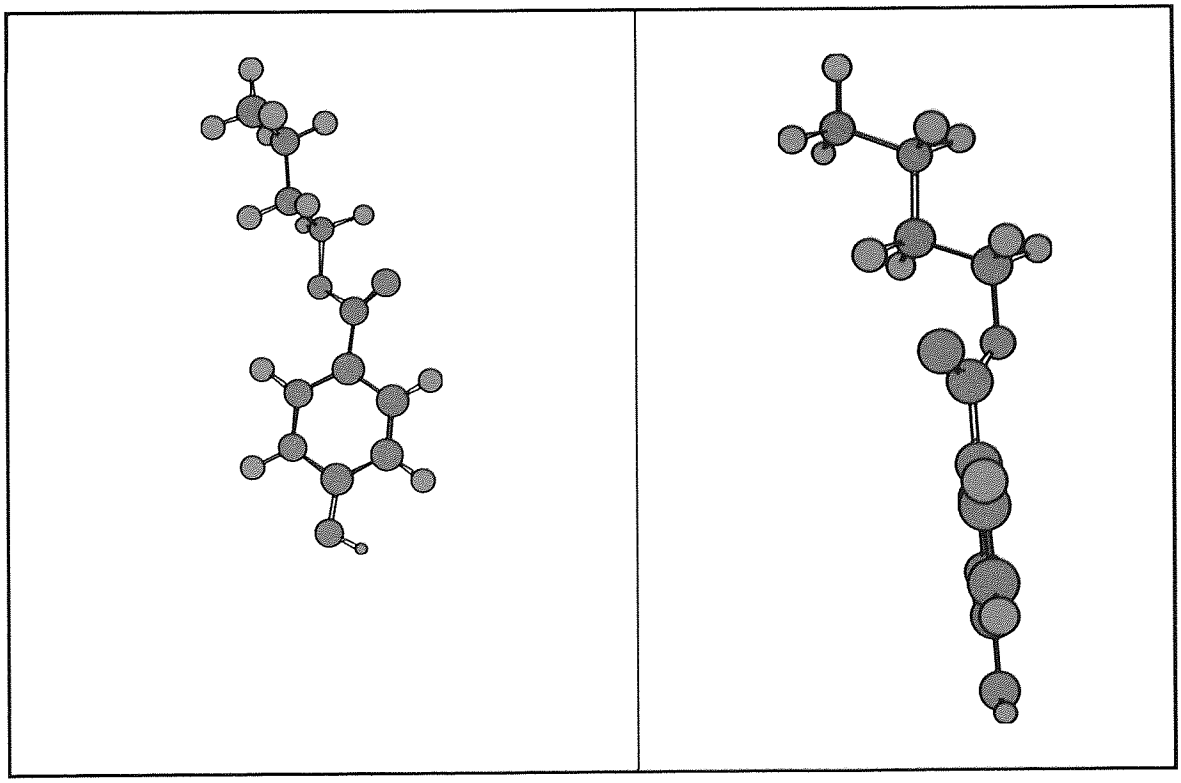

Figure A2.4 – Structure of butyl hydroxybenzoate.

Hydroxybenzoate	Structure	Conformation	Heat of Formation	Dipole (D)	Connolly Area (Δ^2)	Connolly Volume (Δ^3)	Ovality	Critical Volume ($cm^3\ mol^{-1}$)	Molar Refractivity ($cm^3\ mol^{-1}$)	LogP	Mass (Da)
Methyl			-102.707	4.387	317.545	117.174	1.2952	417.5	39.28	1.3	152.05
			-103.764	1.542	321.420	114.779	1.3236				
Ethyl			-107.401	4.124	349.679	131.692	1.3430	473.5	44.03	1.59	166.06
			-108.635	1.524	356.892	132.615	1.3704				
Propyl			-112.884	4.393	384.414	151.278	1.3760	529.5	48.55	2.05	180.08
			-114.403	1.379	383.547	150.427	1.3865				
Butyl			-118.268	4.387	415.028	168.490	1.4060	585.5	53.15	2.5	194.09
			-119.791	1.357	414.185	167.540	1.4167				

Table A2.1 – Calculated parameters for hydroxybenzoates.

Parameters have been calculated using MOPAC and the analysis module within CS Chem3D Pro 5.0 (CambridgeSoft, Ma) using structures drawn in CS Chemdraw 5.0. Minimisations were undertaken using the PM3 option to give the conformations shown.

A2.2 DSC Scans

The DSC was calibrated using indium and three scans were performed for each sample. Examples of DSC scans are shown below.

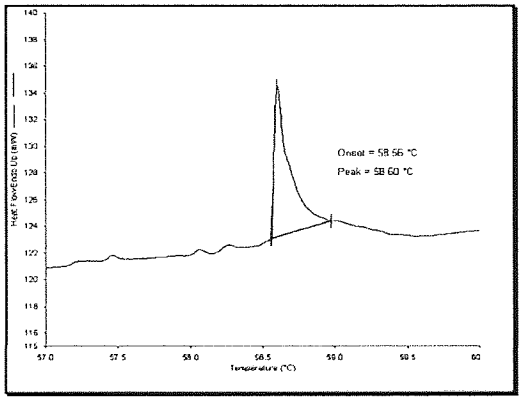

Figure A2.5 – DSC scan for DSPC liposomes.

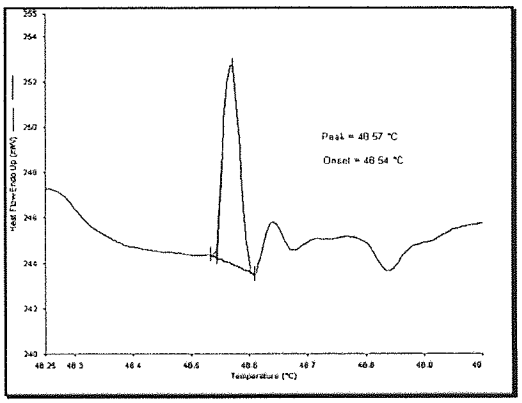

Figure A2.6 – DSC scan for DSPC:Chol, (16:4) liposomes.

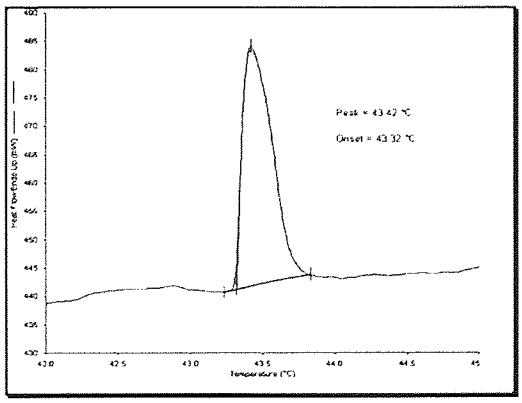

Figure A2.7 – DSC scan for DSPC:Chol. (16:8) liposomes.

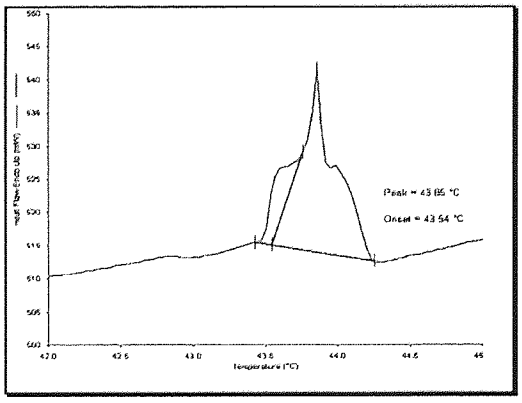

Figure A2.8 – DSC scan for DSPC:Chol, (16:16) liposomes.

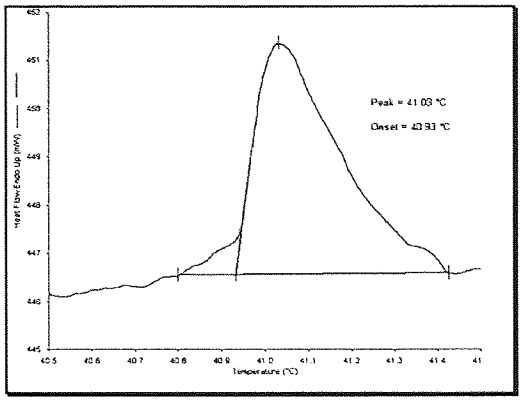

Figure A2.9 – DSC scan for DSPC:Chol, (16:4) liposomes with 1 mg MP incorporated.

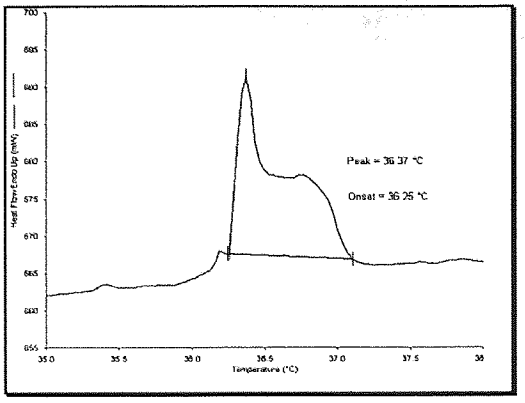

Figure A2.10 – DSC scan for DSPC:Chol, (16:4) liposomes with 1 mg EP incorporated.

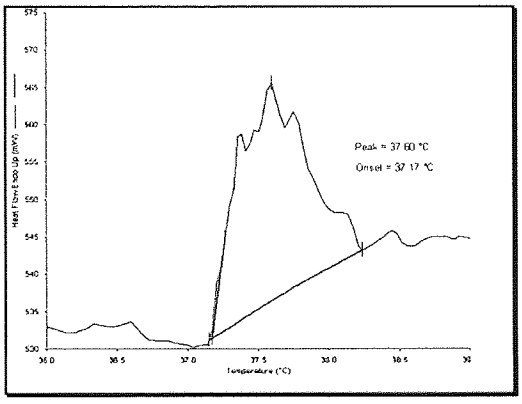

Figure A2.11 – DSC scan for DSPC:Chol, (16:4) liposomes with 1 mg PP incorporated.

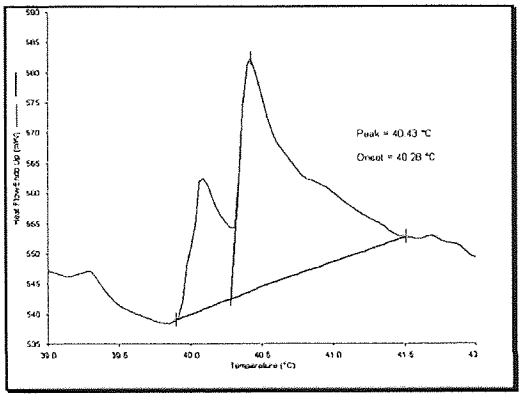

Figure A2.12 – DSC scan for DSPC:Chol, (16:4) liposomes with 1 mg BP incorporated.

Appendix 3

Method of Least Squares

A3. Method of Least Squares

The method of least squares assumes that the best-fit curve of a given type is the curve that has the minimal sum of the deviations squared (*least square error*) from a given set of data. Suppose that the data points are (x_1, y_1), (x_2, y_2),..., (x_n, y_n) where x is the independent variable and y is the dependent variable. The fitting curve $f(x)$ has the deviation (error) d from each data point, i.e., $d_1 = y_1 - f(x_1)$, $d_2 = y_2 - f(x_2)$,..., $d_n = y_n - f(x_n)$. According to the method of least squares, the best fitting curve has the property Π:

$$\Pi = {d_1}^2 + {d_2}^2 + \ldots + {d_n}^2 = \sum_{i=1}^{n} {d_i}^2 = \sum_{i=1}^{n} [y_i - f(x_i)]^2 = \text{a minimum} \qquad \ldots(\text{i})$$

However, $\Pi \equiv R^2$, can be simplified for linear lines of best fit, by following the work of Kenney and Keeping (1962)*.

$$R^2 = \frac{{SS_{xy}}^2}{SS_{xx} SS_{yy}} \qquad \ldots(\text{ii})$$

$$SS_{xx} = \sum_{i=1}^{n} (x_i - \bar{x})^2$$

$$SS_{xx} = \left(\sum_{i=1}^{n} {x_i}^2 \right) - n\bar{x}^2 \qquad \ldots(\text{iii})$$

$$SS_{yy} = \sum_{i=1}^{n} (y_i - \bar{y})^2$$

$$SS_{yy} = \left(\sum_{i=1}^{n} y_i^2\right) - n\bar{y}^2 \qquad \ldots(iv)$$

$$SS_{xy} = \sum_{i=1}^{n} (x_i - \bar{x})(y_i - \bar{y})$$

$$SS_{xy} = \left(\sum_{i=1}^{n} x_i^2 y_i^2\right) - n\bar{x}\bar{y} \qquad \ldots(v)$$

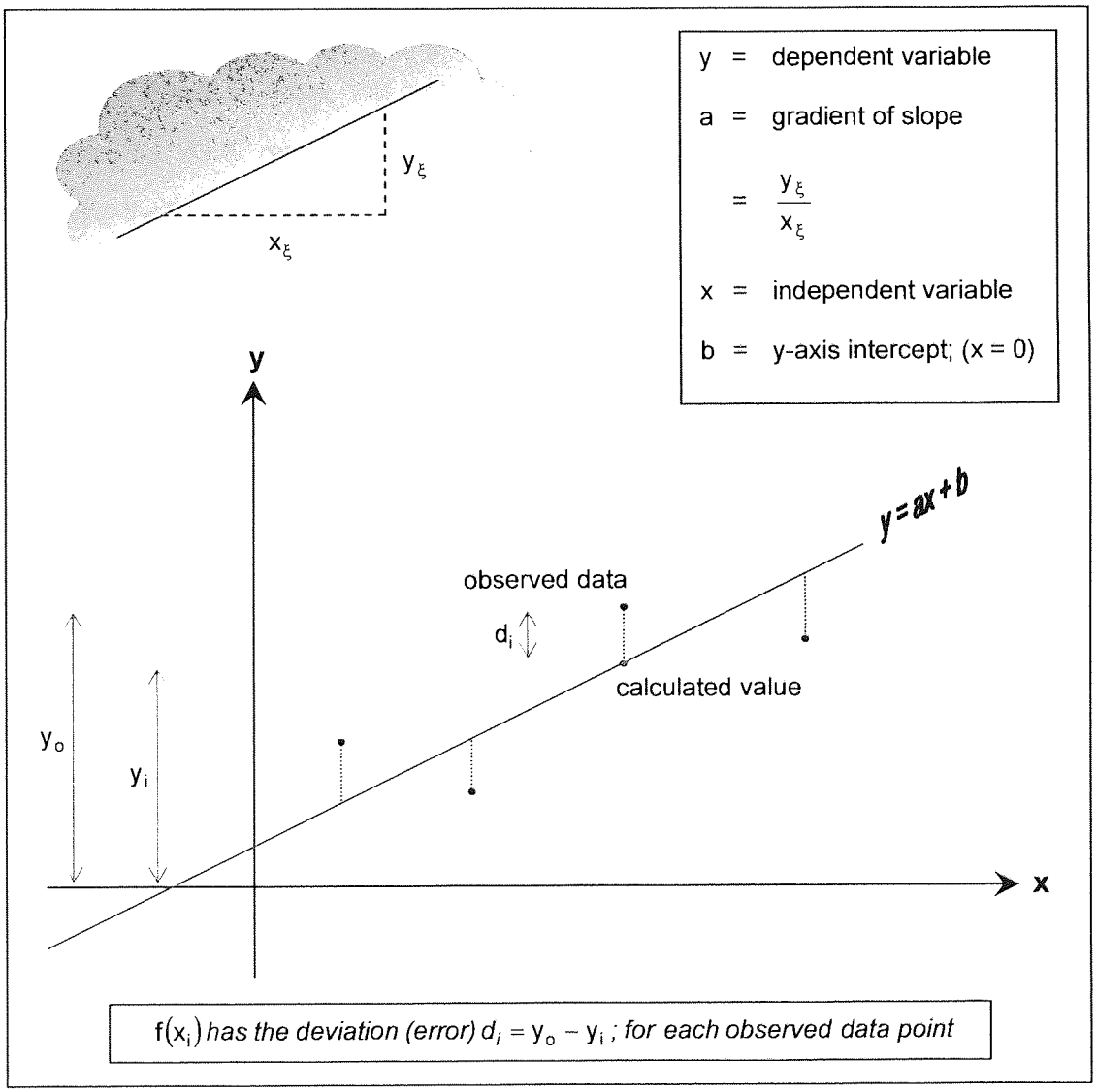

Figure A3.1 – Method of least squares.

With respect to obtaining the Method of Least Squares fitting a line to a set of logarithmic data points; we can assume and apply the Kenney and Keeping (1962) formula – so long as we approximate the logarithmic function to a linear function.

$$y = a \cdot \ln(x) + b \qquad \ldots(vi)$$

$$y = ax + b \qquad \ldots(vii)$$

Hence, if we find the natural log of both sets of (x_i, y_i) data points, we can pass a linear line that best fits the results. We can approximate R^2 by using Kenney and Keeping (1962). Moreover, it should be noted, that greater accuracy (line of better fit), can be achieved from a larger data set. As R^2 represents the fit of the line to the data – the closer R^2 tends to 1, the better fit of the line to the data set.

Appendix 4

P-Glycoprotein Substrates, Inducers and Inhibitors

A4. P-Glycoprotein Substrates, Inducers and Inhibitors

Name	Compound	P-gp Substrate	P-gp Inducer	P-gp Inhibitor
Propranolol	β-Adrenergic antagonist; 5-HT_1 antagonist	Yes	-	Yes
Cyproheptadine	5-HT_2/5-HT_{1c} antagonist; histamine H_1 antagonist	-	-	Yes
Ondansetron	$5HT_3$ antagonists	Yes	-	-
Dipyridamole	Adenosine transport inhibitor	Yes	-	Yes
Debrisoquine	Adrenergic neurone blocking	Yes	-	-
Yohimbine	Adrenergic receptor antagonist	No	Yes	-
Disulfiram	Alcohol dependence treatment	Yes	-	Yes
Doxazosin	Alpha blocker	-	-	Yes
Fentanyl	Analgesic	Yes/No	-	Yes
Sumatriptan	Analgesics / $5HT_1$ agonists	No	-	-
Testosterone	Androgen	Yes	-	Yes
Losartan	Angiotensin-II receptor antagonists	Yes	-	-
Daunorubicin	Anthracycline	Yes	Yes	-
Doxorubicin	Anthracycline	Yes	Yes	-
Epirubicin	Anthracycline	Yes	-	-
Ciprofloxacin	Antibacterial	Yes	-	-
Clarithromycin	Antibacterial	-	-	Yes
Fucidin	Antibacterial	-	-	Yes
Levofloxacin	Antibacterial	Yes	-	-
Ofloxacin	Antibacterial	No	-	Yes
Actinomycin D	Antibiotic	Yes	-	-
Amoxicillin	Antibiotic	Yes	-	-

Name	Compound	P-gp Substrate	P-gp Inducer	P-gp Inhibitor
Erythromycin	Antibiotic	Yes	Yes	Yes
Gramicidin D	Antibiotic	Yes	-	Yes
Mithramycin	Antibiotic	Yes	-	-
Mitomycin C	Antibiotic	Yes	-	Yes
Oligomycin	Antibiotic	-	-	Yes
Puromycin	Antibiotic	Yes	-	-
Rapamycin	Antibiotic	Yes	Yes	Yes
Rifampin	Antibiotic	Yes	Yes	-
Chlorambucil	Anti-cancer drug	Yes	Yes	Yes
Fluorouracil	Anti-cancer drug	Yes	Yes	-
Hydroxyurea	Anti-cancer drug	Yes	Yes	-
Paclitaxel / Taxol	Anti-cancer drug	Yes	Yes	-
Carbamazepine	Anti-convulsant	Yes	-	-
Phenobarbitone	Anti-convulsant	-	Yes	-
Phenytoin	Anti-convulsant	Yes	Yes	-
Citalopram	Antidepressant	No	-	-
Desipramine	Antidepressant	-	-	Yes
Fluoxetine	Antidepressant	No	-	Yes
Fluvoxamine	Antidepressant	-	-	Yes
Imipramine	Antidepressant	-	-	Yes
Nortriptyline	Antidepressant	Yes	-	-
Paroxetine	Antidepressant	-	-	Yes
Trimipramine	Antidepressant	-	-	Yes
Venlafaxine	Antidepressant	-	Yes?	Yes
Phenothiazine	Anti-emetic	-	Yes	Yes
Triflupromazine	Anti-emetic	-	-	Yes
Levetiracetam	Antiepileptic	-	No	-
Phenobarbital	Antiepileptic	-	Yes	-

Name	Compound	P-gp Substrate	P-gp Inducer	P-gp Inhibitor
Econazole	Antifungal	-	-	Yes
Fluconazole	Antifungal	No	No	-
Itraconazole	Antifungal	Yes/No	-	Yes
Ketoconazole	Antifungal	Yes/No	-	Yes
Fexofenadine	Antihistamine	Yes	-	-
Hydroxyzine	Antihistamine	-	-	Yes
Loratadine	Antihistamine	-	-	Yes
Azelastine	Anti-inflammatory	-	-	Yes
Hydrocortisone	Anti-inflammatory	Yes	-	Yes
Methylprednisolone	Anti-inflammatory	Yes	-	-
Prednisolone	Anti-inflammatory	Yes	-	-
Corticosteroids	Anti-inflammatory?	Yes	-	-
Chloroquine	Antimalarial	Yes	-	Yes
Mefloquine	Antimalarial	-	-	Yes
Colchicine	Anti-mitotic	Yes	Yes	-
Cyclosporin A	Anti-mitotic / immunosuppressant	Yes	Yes	Yes
Ivermectin	Anti-parasitic macrolide antibiotic	Yes	-	Yes
RU486 (mifepristone)	Antiprogestogenic steroid	-	-	Yes
Albendazole	Antiprotozoal	No	No	-
Atovaquone	Antiprotozoal			Yes
Chlorpromazine	Anti-psychotic	Yes/No	-	Yes
Fluphenazine	Anti-psychotic	-	-	Yes
Haloperidol	Anti-psychotic	No	-	Yes
Quetiapine	Anti-psychotic	Yes	-	-
Thioridazine	Anti-psychotic	-	-	Yes
Cisapride	Anti-psychotic?	No	-	Yes

Name	Compound	P-gp Substrate	P-gp Inducer	P-gp Inhibitor
Nevirapine	Antiviral - HIV	-	Yes	-
Lopinavir	Antiviral (Protease inhibitor – HIV)	-	Yes	Yes
Emitine	Apoptosis inducer	Yes	-	-
Midazolam	Benzodiazepine anxiolytic	No	Yes	Yes
Flunitrazepam	Benzodiazepines	No	-	-
Carveodilol	Beta Blocker	-	-	Yes
Celiprolol	Beta-Blocker	Yes	-	-
Amlodipine	Calcium channel blocker	-	-	Yes
Bepridil	Calcium channel blocker	-	-	Yes
Diltiazem	Calcium channel blocker	Yes	Yes	Yes
Felodipine	Calcium channel blocker	-	-	Yes
Loperamide	Calcium channel blocker	Yes	-	Yes
Nicardipine	Calcium channel blocker	Yes	Yes	Yes
Nifedipine	Calcium channel blocker	No	Yes	Yes?
Nimodipine	Calcium channel blocker	-	-	Yes
Nitrendipine	Calcium channel blocker	-	-	Yes
Verapamil	Calcium channel blocker	Yes	Yes	Yes
Norverapamil	Calcium channel blockers?	-	-	Yes
Digitoxin	Cardiac glycosides	Yes	-	-
Digoxin	Cardiac glycosides	Yes	-	-
Spironolactone	Competitive antagonist of aldosterone receptors	-	-	Yes
Indomethacin	Cyclooxygenase inhibitor	-	-	No
Docetaxel	Cytotoxic drugs / antineoplastic / Topoisomerase I inhibitors	Yes	-	-
Irinotecan	Cytotoxic drugs / antineoplastic / Topoisomerase I inhibitors	Yes	-	-

Name	Compound	P-gp Substrate	P-gp Inducer	P-gp Inhibitor
Topotecan	Cytotoxic drugs / antineoplastic / Topoisomerase I inhibitors	Yes	-	-
Risperidone	D_2 dopamine antagonist, anti-psychotic	Yes	-	-
Pimozide	D_2 receptor antagonist	-	-	Yes
Sodium Cholate	Detergent	-	-	No
Ethidium Bromide	DNA binder	Yes	-	-
Mitoxantrone	DNA intercalactor	Yes	Yes	-
Cytarabine	DNA synthesis inhibitor	Yes	-	-
Bromocriptine	Dopamine receptor agonist	-	Yes	Yes
Domperidone	Dopamine rector antagonist	Yes	-	-
Amantadine	Dopamine releaser	No	-	-
Rhodamine-123	Dye	Yes	-	-
Calcium-AM	Dye-Derivative	Yes	-	-
HIV Reverse Transcriptase	Enzyme	-	No	-
Etoposide	Epipodophyllotoxin	Yes	Yes	-
Teniposide	Epipodophyllotoxin	Yes	-	-
Naringenin	Flavone derivative	-	-	No
Naringin	Flavone derivative	-	-	Yes
Methotrexate	Folic acid antagonist	Yes/No	Yes	-
Garlic	Food / Drink	-	-	Yes
Grapefruit juice	Food / Drink	-	-	Yes
Green Tea (catechins)	Food / Drink	-	-	Yes
Orange juice	Food / Drink	-	-	Yes
St. John's Wort	Food / Drink	-	Yes	-
5',7'-dihydroxy bergamottin	Furanocoumarin	-	-	Yes
Bergamottin	Furanocoumarin	-	-	Yes/No

Name	Compound	P-gp Substrate	P-gp Inducer	P-gp Inhibitor
Bergapten	Furanocoumarin	-	-	Yes
Lansoprazole	Gastric proton pump inhibitor	Yes	-	Yes
Corticosterone	Glucocorticoid	Yes	-	Yes
Cortisol	Glucocorticoid	Yes	-	Yes
Dexamethasone	Glucocorticoid	Yes	Yes	Yes
Sulfinpyrazone	Gout	-	-	No
Chlorpheniramine	H_1 antagonist	No	-	-
Promethazine	H_1 antagonist; CNS; anti-cholinergic	-	-	Yes
Cimetidine	Histamine receptor antagonist	Yes	-	-
Ranitidine	Histamine receptor antagonist	Yes/No?	-	-
Terfenadine	Histamine receptor antagonist	Yes	-	Yes
Amprenavir	HIV protease inhibitor	Yes	-	-
Indinavir	HIV protease inhibitor	Yes	-	-
Nelfinavir	HIV protease inhibitor	Yes	-	Yes
Ritonavir	HIV protease inhibitor	Yes	Yes	Yes
Saquinavir	HIV protease inhibitor	Yes	-	Yes
Atorvastatin	HMG-CoA inhibitor	Yes	-	Yes
Lovastatin	HMG-CoA inhibitor	Yes	-	Yes
Pravastatin	HMG-CoA inhibitor	-	No	Yes
Simvastatin	HMG-CoA inhibitor	-	-	Yes
Estradiol	Hormone	Yes	-	-
Octreotide	Hormone antagonist	Yes	-	-
Zolpidem	Hypnotic	-	No	-
Tacrolimus	Immunosuppressant	Yes	Yes	Yes
Cisplatin	Immunotherapeutic	Yes	Yes	-
Quercetin	Inhibitor of mitochondrial ATPase and phosphodiesterase	-	-	Yes

Name	Compound	P-gp Substrate	P-gp Inducer	P-gp Inhibitor
Amiloride	Inhibitor of Na^+/H^+ antiport	Yes	-	Yes
Reserpine	Inhibits vesicular uptake of catecholamines and serotonin	-	Yes	Yes
Amiodarone	Ion channel blocker	-	Yes	Yes
Astemizole	Ion channel blocker?	-	-	Yes
Benzbromarone	Ion channel blocker?	-	-	No
Aldosterone	Mineralocorticoid	Yes	-	Yes
Methadone	Narcotic analgesic	Yes	-	Yes
Morphine	Narcotic analgesic	Yes	Yes	-
Efavirenz	Non-nucleoside reverse transcriptase inhibitors	-	Yes	-
Maprotiline	Norepinephrine uptake inhibitor	-	-	Yes
Alpha-methyldigoxln	-	Yes	-	-
ASA (aminosalicylic acid)	-	-	Yes	-
Azidopine	-	-	-	Yes
Beta-acetyldigoxin	-	Yes	-	-
Biricodari	-	-	-	Yes
Bisantrene	-	Yes	-	-
Bunitrolol	-	Yes	-	-
Cortexolone	-	No	-	-
Delavirdine	-	-	Yes	-
Desethylamiodarone	-	-	-	Yes
Dexniguldipine	-	-	-	Yes
Dexrazoxane	-	-	-	Yes
Emetine	-	Yes	-	Yes
Enoxacin	-	Yes	-	-
FK506	-	Yes	-	Yes
Flavonoids	-	-	-	Yes

Name	Compound	P-gp Substrate	P-gp Inducer	P-gp Inhibitor
Gallpamil	-	-	-	Yes
Grepafloxacin	-	Yes	-	-
Josamycin	-	-	-	Yes
Lanuquidar	-	-	-	Yes
Mibefradil	-	Yes	-	Yes
Nefazodone	-	-	Yes	Yes
Nobilitin	-	-	-	Yes
Piperine	-	-	-	Yes
Sparfloxacin	-	Yes	-	-
Sufentanil	-	No	-	Yes
Talinolol	-	Yes	-	-
Tariquidar	-	-	-	Yes
Trifluoperazine	-	-	-	Yes
Valspodar	-	-	-	Yes
Vanadate	-	-	-	Yes
Zosuquidar	-	-	-	Yes
Alfentanil	Opioid analgesics	No	-	Yes
Insulin	Peptide hormone	-	Yes	-
Clotrimazole	Potassium channel blocker	-	Yes	-
Propafenone	Potassium channel blocker	-	-	Yes
Quinidine	Potassium channel blocker	Yes	-	Yes
Quinine	Potassium channel blocker	-	-	Yes
Valinomycin	Potassium ionophore	Yes	-	Yes
L-dopa	Precursor of dopamine	Yes	-	-
Tamoxifen	Protein kinase C inhibitor	Yes	Yes	Yes
Genistein	Protein kinase inhibitor	-	-	No
Omeprazole	Proton pump inhibitor	-	-	Yes
Pantoprazole	Proton pump inhibitor	-	-	Yes

Name	Compound	P-gp Substrate	P-gp Inducer	P-gp Inhibitor
Vindesine	Semisynthetic derivative of vinblastine	Yes	-	-
Trazodone	Serotonin uptake inhibitor	-	Yes	-
Sertraline	Sertraline	-	-	Yes
Lidocaine	Sodium channel blocker	No	-	Yes
Progesterone	Steroid	No	-	Yes
Levothyroxin(e)?	Thyroid hormone?	-	-	Yes
Adriamycin	Trade name for doxorubcin	Yes	-	-
Amitriptyline	Tricyclic antidepressant	Yes	Yes?	Yes
Nortryptaline	Tricyclic antidepressant	Yes	-	Yes
Probenecid	Uricosuric drug (Gout)	-	Yes	Yes
Tetrabenazine	Used to control movement disorders in Huntington's chorea and related disorders	-	-	Yes
Prazosin	Vasodilator	-	Yes	-
Isosorbide	Vasodilators	-	No	-
Vinblastine	Vinca Alkaloid	Yes	Yes	Yes
Vincristine	Vinca Alkaloid	Yes	Yes	-
Vinorelbine	Vinca alkaloids and etoposide	Yes	-	-

Table A4.1 – Summary of P-glycoprotein (P-gp) inhibitors, inducers and substrates.

References: Katoh *et al.*, 2000; Matheny *et al.*, 2001; Spatzenegger & Jaeger, 1995; Pascaud *et al.*, 1998; Hollo *et al.*, 1996; Ambudkar *et al.*, 1999; Weber *et al.*, 2001; Wang *et al.*, 2001; Ueda *et al.*, 1997; Eagling *et al.*, 1999; Ho *et al.*, 2000; Abdel-Rahman *et al.*, 2000; http://www.mhc.com/PGP/PgpTable.HTML.

Appendix 5

List of Publications

A5. List of Publications

A5.1 Poster Presentations

Bradbury, E. L., Moore, V. A., Perrie, Y. and Irwin, W. J. (2001). The Influence of Drug Partition Coefficient on Incorporation and Transport of Hydroxybenzoates in Liposomes. AstraZeneca Ph.D. Review Day, 30th October 2001. AstraZeneca, Charnwood, Loughborough, UK.

Bradbury, E. L., Moore, V. A., Perrie, Y. and Irwin, W. J. (2001). Incorporation of Drugs into Liposomal Bilayers: The Influence of Drug Partition Coefficient. Fifth International Liposome Conference, Liposome Advances, Progress in Drug and Vaccine Delivery 17th-21st December 2001. London, UK.

Bradbury, E. L., Moore, V. A., Perrie, Y. and Irwin, W. J. (2002) Liposomes as Oral Drug Delivery Carriers: The Influence of Drug Partition Coefficient. 8th United Kingdom and Ireland Controlled Release Society (UKICRS), 17th January 2002. Belfast, Ireland.

Bradbury, E. L., Moore, V. A., Irwin, W. J. and Perrie, Y. (2002) Liposomes as Oral Drug Delivery Carriers: The Effect of Bilayer Composition. British Pharmaceutical Conference (BPC), 23rd-25th September 2002. Manchester, UK.

Bradbury, E. L., Moore, V. A., Irwin, W. J. and Perrie, Y. (2002) The Effect of Liposomal Formulations on Caco-2 Cell Viability and Uptake of ^{14}C-Glutamine. (28th November 2002) AstraZeneca, Charnwood, Loughborough, UK.

Bradbury, E. L., Moore, V. A., Murray, J. Irwin, W. J. and Perrie, Y. (2003) Liposomes as Oral Drug Carriers: Toxicity and Glutamine Uptake in Caco-2 Cells. American Association of Pharmaceutical Sciences (AAPS) Annual Meeting. 26th-30th October 2003. **5**(S1). Salt Lake City, Utah, USA.

A5.2 Oral Presentation

Bradbury, E. L. The Effect of Liposomal Formulations on Caco-2 Cell Viability and Uptake of ^{14}C-Glutamine. (28th November 2002) AstraZeneca, Charnwood, Loughborough, UK.