EFFECT OF INULIN ON ORAL BIOAVAILABILITY OF MINERALS AND DRUGS

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EFFECT OF INULIN ON ORAL BIOAVAILABILITY OF MINERALS AND DRUGS

by

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To my beloved parents and family members

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ABSTRAK

Kajian ini dijalankan untuk menyiasat potensi inulin dan kesan dos-dos inulin yang berbeza dalam peningkatan penyerapan oral zink dan magnesium. Kesan dos-dos inulin yang berbeza dalam biokeperolehan kedua-dua model drug oral, acyclovir dan atenolol, juga telah disiasat. Sebelum kajian tersebut, dua kaedah spektrometer *nyala* serapan *atom* yang ringkas, pantas dan sensitive telah berjaya dibangunkan untuk menentukan kepekatan zink dan magnesium dalam plasma tikus. Ketepatan nilainya adalah di antara 91-107% untuk zink dan 92-105% untuk magnesium. Manakala semua kejituan nilainya adalah kurang daripada 12.5% untuk kedua-dua zink dan magnesium. Penyerapan zink dan magnesium adalah tidak lengkap selepas diberikan secara oral. Ini mungkin disebabkan oleh sistem homeostatis dalam badan untuk mengimbangkan kandungan mineral di dalam lingkungan yang sempit. Satu dos tunggal inulin sebanyak 45 mg diberikan bersama dengan zink dan magnesium dapat meningkatkan oral biokeperolehan kedua-dua mineral tersebut. Walaubagaimanapun, oral biokeperolehan untuk zink dan magnesium tidak ditingkatkan secara signifikan jikalau dos inulin bertambah daripada 45 mg sehingga 135 mg. Keadaan ini mungkin disebabkan oleh mekanisme peningkatan dalam penyerapan zink dan magnesium adalah melalui pengaktifan calmodulin yang berkaitan dengan jalur paracellular. Dalam kajian selanjutnya, satu dos tunggal 45 mg inulin juga didapati dapat meningkatkan biokeperolehan acyclovir dan atenolol sebanyak 1.37 dan 1.24 kali, di mana drug adalah dikelaskan dalam BCS kelas III dengan ketelapan usus yang kurang berkesan. Adalah juga dipercayai inulin dapat

membuka persimpangan ketat dan seterusnya meningkatkan ketelapan usus acyclovir dan atenolol. Peningkatan dalam dos inulin daripada 45 mg kepada 135 mg tidak dapat meningkatkan penyerapan kedua-dua drug model secara signifikan.

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ABSTRACT

The present study was conducted to evaluate the potential and effect of different doses of inulin in enhancing the oral absorption of zinc and magnesium. The effect of different doses of inulin in the oral bioavailability of the 2 model drugs, acyclovir and atenolol, was also assessed. Prior to the study, two simple, rapid and sensitive flame atomic absorption spectrophotometer methods were successfully developed for the determination of zinc and magnesium in rat plasma, respectively. The accuracy values were between 91 – 107% for zinc and 92 – 105% for magnesium, while the coefficient of variation values were all less than 12.5% for both zinc and magnesium. The absorption of zinc and magnesium was incomplete upon oral administration. This could be due to the existing homeostasis system in the body to regulate mineral content within a narrow range. A single dose of inulin with 45 mg co-administration was capable of increasing the extent of oral absorption of zinc and magnesium. However, the oral absorption was not significantly increased with the increasing dose of inulin from 45 mg until 135 mg. The possible underlying mechanism for the enhancement of zinc and magnesium absorption was via the activation of calmodulin associated paracellular pathway by inulin. In the subsequent study, a single dose of 45 mg inulin was also able to increase the bioavailability of acyclovir and atenolol by 1.37 and 1.24-fold, which belong to BCS class III drugs with poor intestinal permeability. It was postulated that the inulin could open up the tight junction and thus increase the intestinal permeability of acyclovir and atenolol. An increase in the dose of inulin from 45 mg to 135 mg did not increase the absorption of the 2 model drugs significantly.

CHAPTER 1

INTRODUCTION

1.2 DRUG DELIVERY

INTRODUCTION

In order to optimize the desired therapeutic response of a drug, the correct amount should be delivered to the site of action at the correct rate, timing and duration. Drugs can be delievered by enteral, transdermal, parenteral, inhalation, or intranasal administration. Each route may serve a different purpose and has its own advantages and disadvantages. For example, intravenous injection can be given to obtain immediate effect and maximal bioavailability, but the potential for adverse reaction to occur also increases. Inhalation may be used for local or systemic effects, however, it may stimulate cough reflex (Shargel *et al.*, 2005). Among the different modes of drug administration, the oral route is still the most preferred due to its safety, efficiency and accessibility with minimal discomfort to the patient in comparison with the other routes (Pefri & Lennernäs, 2003).

1.1.2 MEMBRANE TRANSPORT MECHANISM

For drugs to be absorbed from the gastrointestinal tract after oral administration, they must be protected from enzyme degradation and traverse through the barrier membrane into the blood stream (Semalty et al., 2007). Transmembrane movement of drugs is influenced by the composition and structure of the cell membrane.

The cell membrane is a thin layer of approximately 70 to 100 Å in thickness. It is composed of 2 layers of phospholipids, amphipathic molecules, proteins and

carbohydrates. The phospholipid layers are arranged so that the hydrophilic "head" groups will face the extracellular water environment, while the hydrophobic "tail" groups are aligned to the interior. This explains the observation that hydrophobic drugs tend to penetrate barrier membrane more easily (Shargel *et al.*, 2005). There are also pores present in cell membranes and they provide channels for the movement of water, urea and certain charged ions. Figure 1.1 shows the structure of a plasma membrane. The different proteins present on the surface of cell membranes also function as cell surface receptors and transporters for various drugs and molecules. Transport of drugs across the membrane barrier can occur transcellularly or paracellularly.

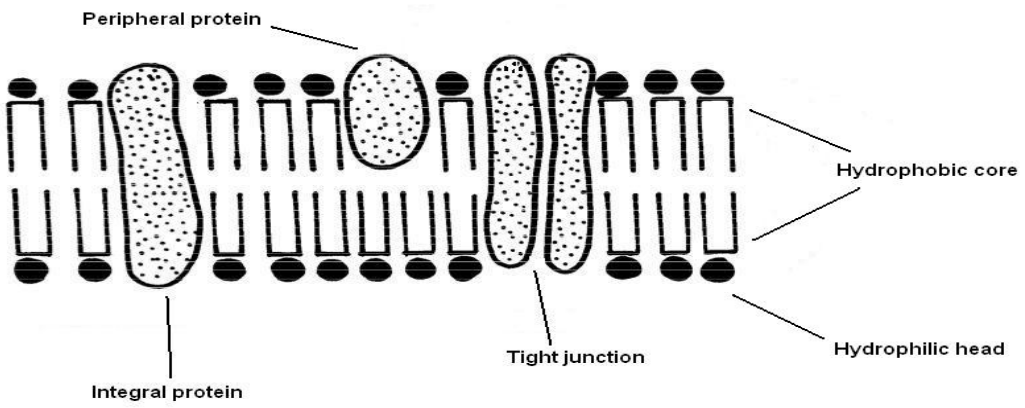

Figure 1.1 Structure of a cell membrane

1.1.2.1 TRANSCELLULAR

Transcellular transport is thought to be the main route of absorption of many drugs and is related to the hydrophobicity of the molecule (Pefri & Lennernäs, 2003). It involves transfer of drugs and macromolecules across the epithelial cellular membrane (Hsieh, 1993). Various mechanisms are involved in this transcellular transport of drugs.

1.1.2.1(a) SIMPLE PASSIVE DIFFUSION

Passive diffusion is the process by which molecules spontaneously diffuse from a region of higher concentration to a region of lower concentration without the assistance of a transport protein and expenditure of energy (Shargel *et al.*, 2005). It is the preferred route of transport for small molecules and lipophilic molecules. The rate of penetration of a drug across the membrane follows Fick's law as shown below:

$$\text{rate of penetration} = (DK/\delta)A \cdot \Delta C \qquad (1.1)$$

in which A = surface area

ΔC = concentration difference across the membrane

D = diffusivity of the solute

K = partition coefficient between membrane and water

δ = membrane thickness

The partition coefficient, K, is an important determinant of the rate of transfer across membranes. Many lipid-soluble molecules such as oxygen, nitrogen gas, carbon dioxide and benzopyrene are transported by simple diffusion (Oh & Amidon, 1999).

1.1.2.1(b) CARRIER-MEDIATED TRANSPORT

Carrier-mediated transport is the transport system in which substances can be transferred through the lipid phase of biological membrane using a carrier or specialized system (Pefri & Lennernäs, 2003). Many of the compounds transported via this mechanism are polar, with low lipid solubility. This transport system is characterized by (1) being saturable and (2) subjected to inhibition by competitive inhibitors. There are

two types of carrier-mediated transport: active transport and facilitated diffusion. Active transport results in an accumulation of a solute on one side of membrane and often against the electrochemical gradient. During this process, energy is required to move ions or molecules against the electrochemical potential gradients (Oh & Amidon, 1999). The transportation involves binding of the drug to a carrier to form a carrier-drug complex that shuttles the drug across the membrane. The drug is then dissociated from the carrier once it has crossed the membrane. Examples of active transport include intestinal absorption of some amino-β-lactam antibiotics and angiotensin-converting enzyme inhibitors via the dipeptide transport system (Rowland & Tozer, 1995).

In contrast to active transport, energy source is not needed for facilitated diffusion. Molecules enter and leave cell by following their electrochemical potential gradient (Oh & Amidon, 1999). However, similar to active transport, it involves a carrier, is saturable, selective for the drug and shows competition kinetics for drugs of similar structures. The binding site alternates between 2 states: exposure to one side first, and then to the other side of the membrane (Oh & Amidon, 1999). Only a few drugs undergo passive facilitated diffusion. An example is the transport of vitamin B_{12} across the gastrointestinal epithelium (Rowland & Tozer, 1995).

1.1.2.1(c) ENDOCYTOSIS

Most of the transportation of macromolecules is mediated by endocytosis. During the process, inimize cules are engulfed by the cell membrane, migrate to the perinuclear region within membrane-bound vehicles, where the vehicles coalesce with lysosomes

and release the macromolecules (Hsieh, 1993). Endocytosis process includes pinocytosis, receptor-mediated endocytosis, phagocytosis and transcytosis (Boer, 1994).

1.1.2.2 PARACELULAR

In paracellular transport, the drug is transferred through the intercellular spaces of the cell membrane. This pathway is governed by tight junctions. The complexity of the tight junction fibril branching pattern is thought to influence the permeability of tight junctions (Boer, 1994). Pauletti *et al*. (1997) reported that the transportation of drug through the tight junctions is dependent on the size and charge of the drug. Generally, small hydrophilic solutes traverse the membrane barrier predominantly via this paracellular route (Pauletti *et al*., 1997). A study conducted by Saitoh *et al.* (2004) demonstrated that hydrophilic drugs like ketoprofen, imipramine, zidovudine are transported using the paracellular route. As reviewed by Itallie & Anderson (2004), paracellular transport differs from transcellular transport in two ways. First, it is exclusively passive, driven by electroosmotic gradients. Second, it shows identical selectivity and conductance in both the mucosal and serosal sides. There is a growing interest in employing the paracellular pathway to promote the transport of metabolically labile peptide drugs, with the expectation that the paracellular space is deficient in proteolytic enzymes (Boer, 1994).

1.1.3 DELIVERY VEHICLES

Although there exists different transport mechanisms to facilitate drug transportation, limited absorption still exists for certain drugs due to the presence of physiological, biochemical and chemical barriers. Hence, researchers have come out with various ways

and means to increase the absorption of these drugs. These include co-administration of enzyme inhibitiors, use of suitable carriers, bioadhesion and permeation enhancers as well as converting them into prodrugs.

1.1.3(a) CO-ADMINISTRATION OF ENZYME INHIBITORS

Degradation by enzyme in the gastrointestinal tract is one of the main obstacles in achieving good bioavailability for some drugs, especially biopharmaceuticals. To circumvent the degradation, co-administration of enzyme inhibitors have been explored (Semalty *et al.*, 2007). Esterases, secreted by the pancrease, play a crucial role in the presystemic metabolism of many drugs which bear ester moieties. Consequently, specific inhibition of the responsible esterase may be useful in enhancing the oral bioavailability of a susceptible drug. Li *et al.* (2007) demonstrated that co-administration of grapefruit juice could inhibit the esterase activity and increase the absorption of lovastatin and enalapril in rats. Although enzyme inhibitors can enhance drug absorption, there are concerns on the toxicity of the enzyme inhibitors. Moreover, the dilution effect during gastrointestinal passage necessitates the administration of high concentration of inhibitors in order to exert the inhibitory effect.

1.1.3(b) CARRIERS

In order to prevent degradation from GI enzymes as well as to improve the absorption of drugs, carriers like liposomes, nanoparticles, glycoproteins, synthetic polymers and lipoproteins have been employed to improve the oral delivery into the systemic circulation. Drugs can either be covalently attached to the carrier molecules or entrapped within the carrier matrix (Banker & Rhodes, 2002). One of the advantages of using

carriers likes liposomes or nanoparticles is that drug absorption can be enhanced through transport via the Peyer's patches or other lipid absorption mechanisms (Lieberman *et al.*, 1996). Absorption by Peyer's patches can help to circumvent presystemic metabolism in the liver. In certain cases, physicochemical properties of the carriers such as size, shape, specific surface area and surface charge can affect the absorption of the drugs. Sakuma *et al.* (2002) showed that the enhancement in absorption of salmon calcitonin could be achieved through the optimization of the chemical structure of nanoparticles by introducing cationic poly-vinylamine (PVAm) groups to the surface of the poly-*N*-isopropylacrylamide (PNIPAAm) nanoparticles.

1.1.3(c) PRODRUG

A prodrug is a pharmacologically inert form of an active drug that must undergo transformation to the active parent compound in vivo by either a chemical or an enzymatic reaction to exert its therapeutic effects (Banker & Rhodes, 2002). Some authors designed prodrugs by chemical attachment to the parent molecules of a variety of pro-moieties to functional groups such as carboxyl, hydroxyl, amine, imine, phosphate and CH-acidic (Pefri & Lennernäs, 2003). Prodrugs can be used to increase oral bioavailability of drug by improving solubility and membrane permeability as well as by reducing the influence of by efflux transporters in the gastrointestinal tract (Stella, 2007). Chae *et al.* (2007) has demonstrated that VP-0502AL, a prodrug of an anti-human immunodeficiency virus drug, VP-0502, had better oral bioavailability than the parent compound VP-0502 itself in rats. The improvement in absorption might be due to the enhancement of drug solubility by attaching an amino acid, alanine to the drug.

1.1.3(d) BIOADHESION

Bioadhesives are materials that bind to biological substrates such as membrane surface or mucin layers. They are capable of being retained on biological membranes for an extended period of time and serve to increase the dosage form residence time in the gastrointestinal tract. Moreover, they can be used to improve intimacy of contact with various absorptive membranes of the biological system (Gupta *et al.*, 1990). Prolonged contact time of a drug with a body tissue, through the use of a bioadhesive polymer, can significantly improve the absorption of many drugs. This has been shown by Tur *et al.* (1997) that a bioadhesive polymer, poly (acrylic acid) crosslinked with 2,5-dimethyl-1,5-hexadiene, was able to improve the absorption of griseofulvin. They suggested that the increment in absorption might be due to an increase in the gastrointestinal transit time and the close contact of griseofulvin with the absorbing membrane brought about by the bioadhesive polymer.

1.1.3(e) PERMEATION ENHANCER

In recent years, permeation enhancers have been increasingly used to improve the oral absorption of large hydrophilic drugs. Permeation enhancers can disrupt the integrity of the membrane barrier in the gastrointestinal tract in a number of ways: increasing the fluidity of cell membrane, extracting inter- and intracellular lipids, disrupting lipid structure, altering cellular proteins, increasing the thermodynamic activity of a drug, overcoming enzymatic barriers and altering surface mucin rheology (Touitou & Barry, 2006). As reviewed by Ward *et al.* (2000), a variety of exogenous compounds have been identified to have the ability to increase the paracellular permeability. They include calcium chelators, surfactants, medium chain fatty acids and cationic polymers. The

effectiveness of permeation enhancers can be influenced by many factors including the physiochemical properties of the drugs, administration site of the permeation enhancers and species differences (Knäblein, 2005). An ideal enhancer should be pharmacologically inert, not toxic and provides immediate onset of action. Moreover, normal barrier properties of the gastrointestinal tract should recover immediately when the enhancer is removed (Hsieh, 1993).

1.2 INULIN

1.2.1 INTRODUCTION

Inulin is a type of plant storage carbohydrate that can be found in several fruits and vegetables including banana, onion, leek, chicory, garlic and wheat. It can also be found in some bacteria and fungi. The role of inulin in plants is mostly documented as a long-term reserve carbohydrate stored in underground overwintering organs of plants. It is also referred as a cryoprotectant and osmotic regulator which allows the plants to survive and grow under conditions of water shortage (Stephen *et al.*, 2006).

Inulin is a polydisperse fructan (Phelps, 1965) which consists mainly of ß(2→1) fructosyl-fructose linkages. A glucose moiety can link to the starting point of inulin by α(1→2) bond (Niness, 1999). The chemical structure of inulin is shown in Figure 1.2. Functionality of inulin can be influenced by the degree of polymerization (DP) and presence of branches. DP and presence of branches can vary according to plant species, weather conditions and physiological ages of the plant (Franck & Leenheer 2005).

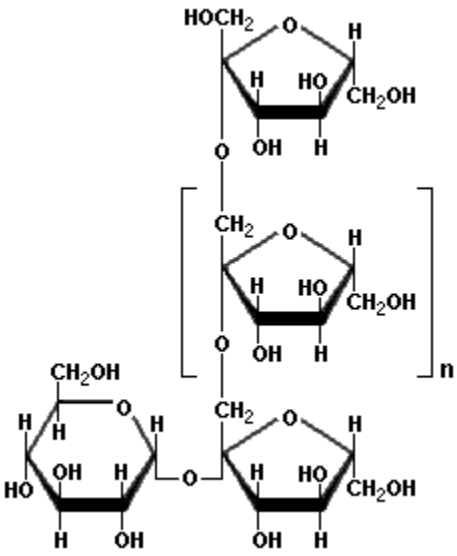

Figure 1.2 Chemical structure of inulin

Today, chicory is the only plant that has been used industrially for the extraction of inulin. Two phases are required for the production of inulin. The first phase involves extraction with hot water in a countercurrent diffuser and a primary purification step is applied to the extraction juice by liming and carbonation at high pH, yielding an impure syrup. The second phase is a refining phase using cationic and anionic ion exchange resins for demineralization and active carbon for decolorization. Pure inulin can be obtained after the juice is passed over a 0.2 μm filter to be sterilized, evaporated and spray-dried (Stephen *et al.*, 2006). The production process is shown in Figure 1.3.

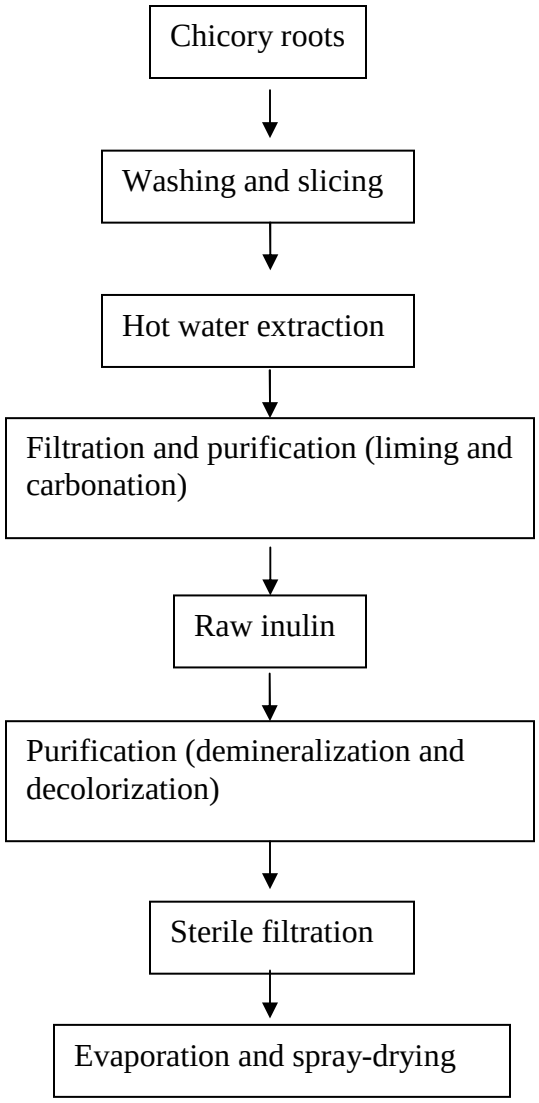

Figure 1.3 Production process of inulin (Stephen *et al.*, 2006)

Chicory inulin can be derivatized into different forms through different chemical and physical processes. Chicory inulin varies from 2 to 60 units (DP_{av}=12), can undergo partial enzymatic hydrolysis using endo-inulinase to produce oligofructose, in which the DP varies from 2 to 7 (DP_{av}=4). Long-chain inulin or inulin HP with DP of 10 to 60 (DP_{av}=25), is produced by applying physical separation techniques to eliminate all oligomers with a DP<10. Mixtures of oligofructose and

inulin HP are available commercially, such as Synergy® 1(Roberfroid, 2005, Roberfroid, 2004). The term inulin-type fructans covers all ß(2→1) linear fructans including native inulin, oligofructose, inulin HP and Synergy® 1(Roberfroid, 2007).

1.2.2 POTENTIAL THERAPEUTIC BENEFITS OF INULIN

1.2.2(a) PREBIOTIC

Gibson and Roberfroid (2008) defined prebiotic as a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth or activity of one or a limited number of bacteria in the colon, and thus improves host health.

Inulin resists hydrolysis and absorption in the upper intestine as the pancreas, gastric and small intestine are not able to produce enzyme which can split ß(2→1) fructosyl-fructose linkages of inulin (Cherbut, 2002). This has been further proven by Ellegård *et al.* (1997) who performed a double blind crossover study with inulin and oligofructose in 10 patients with conventional ileostomy. The percentages of Inulin and oligofructose recovered in the ileostomy effluent were 88% and 89%, respectively. As inulin which escaped digestion in the upper gastrointestinal tract was not recovered in the stools, indicated that the portion reaching the colon was completely fermented by colonic flora (Molis *et al*., 1996).

In a study reported by Propst *et al.*, (2003), the end product of microflora fermentation, namely short chain fatty acid (SCFA), was increased in the faeces of healthy adult dogs after being fed inulin and oligofructose as compared with control. Inulin and oligosaccharides have been shown to increase *Bifidobacterium* and *Lactobacillus* population that are perceived to have health-promoting properties (Gibson and

Roberfroid, 2008; Blay *et a*l., 1999). Rada *et al.* (2001) also demonstrated in a study that caecal bifidobacterial concentration was increased for more than 3-fold in inulin-treated laying hens. Moreover, *Bifidobacterium* and *Lactobacillus* can help to maintain the health of host by suppressing pathogenic bacteria such as *E. coli* and *Salmonella typhimurium*.

1.2.2(b) PREVENTION OF COLORECTAL CANCER (CRC)

Family history, dietary pattern, lifestyle exposure patterns and physical activities are factors which can increase CRC risks (Potter, 1999). Healthy diet intake is one of the ways for CRC prevention.

Genetic mutations in the gut can lead to aberrant crypt foci (ACF) and grow into polyps or adenomas, which are recognized as early key determinants of CRC. Verghese *et al.*, (2002) has shown that long-chain inulin was able to suppress azoxymethane-induced ACF formation in rats in a dose dependent manner. The suppression effect was even more significant when the rats were treated with both inulin and probiotics together (Femia *et al.*, 2002).

Apoptosis is also an important regulatory process in the protection against the development of cancer. In a study conducted by Leu *et al*., (2005), inulin together with probiotic, *Bifidobacterium lactis*, could elevate acute apoptotic response to a genotoxic carcinogen in distal colon of rats. It was speculated that inulin could decrease the colonic pH, produce SCFA or change the balance of microfloral species which enabled *B.lactis* to exert proapototic effect.

1.2.2(c) PREVENTION OF ATHEROSCLEROSIS

Many studies have demonstrated the importance of plasma triglyceride levels as a risk factor for atherosclerosis (Hokanson & Austin, 1996; Austin *et al.*, 1998). Klag *et al.* (1993) also reported that there was a strong correlation between plasma cholesterol and atherosclerosis.

In a study conducted in male golden Syrian hamsters, which were randomly assigned to four diet groups containing 0 (control), 8, 12, and 16% inulin respectively, it was observed that dietary inulin managed to decrease very low-density lipoprotein (VLDL) cholesterol, plasma triacylglycerol and hepatic total cholesterol. Daily fecal bile acid excretion also increased when compared with controls (Trautwein *et al.*, 1998). Letexier et al., (2003) showed that inulin was able to reduce plasma triacylglycerol concentrations and hepatic lipogenesis in humans. The effect of inulin in attenuating atherosclerosis was also supported by the study of Rault-Nania et al., (2006) which showed that plasma cholesterol concentration, plasma triacylglycerol concentration, hepatic cholesterol concentration as well as hepatic triacylgycerol concentration were reduced in apolipoprotein E-deficient mice which fed with inulin. Mice fed with inulin also had less atherosclerotic lesion area compared with control.

There are a few speculations for the underlying mechanisms. They include altered hepatic triacylglycerol synthesis and VLDL secretion, impaired reabsorption of circulating bile acids and changes in lipid metabolism (Trautwein *et al.*, 1998; Beylot, 2005; Rault-Nania *et al.*, 2006).

1.2.2(d) ENHANCEMENT OF CALCIUM ABSORPTION

Calcium and phosphorus ions are required for normal bone formation. Calcium deficiency can impair mineralization and increase bone resorption. This can result in bone fractures and osteoporosis. Hence, calcium intake is one of the ways for the prevention of osteoporosis.

Inulin-type fructans have been shown to increase calcium transport *in vitro* via the paracellular route (Mineo *et al.*, 2001; Suzuki and Hara, 2004). In rat studies, bone mineral density, bone mineral content and calcium absorption were significant increased in inulin-fed groups (Kruger *et al.*, 2003; Zafar *et al.*, 2004, Coudray *et al.*, 2005). Several hypotheses have been put forward to explain the mechanisms involved. They include the production of SCFA that would acidify the luminal contents, enhancing minerals solubility and hence absorption, or enhancement of another mechanism via calbindinD9k (Kruger *et al.*, 2003). Positive results in calcium absorption were also observed in adolescents (Heuvel *et al.*, 1999, Griffin, 2003).

According to Parhami *et al.* (2000), atherosclerosis and bone loss are interwoven. Lipid-lowering agents are able to reduce atherosclerosis, vascular calcification as well as osteoporotic fractures. Hence inulin, which is able to help in attenuating atherosclerosis as mentioned earlier, may protect the bone indirectly.

1.2.2(e) IMPROVEMENT OF IMMUNE SYSTEM

Cebra (1999) reported the important roles of microflora on intestinal immune system development. Bifidobacteria and lactobacilli are two probiotics which can activate host immune system and affect goblet cell dynamics (Deplancke & Gaskins, 2001), suppress

pathogenic bacteria (Kolida *et al.*, 2002) and treat intestinal inflammation (Bai & Quyang, 2006). Consequently inulin, which is able to stimulate the growth of bifidobacteria and lactobacillus (Buddington *et al.*, 1996; Kleessen *et al.*, 1997; Niness, 1999; Blay *et al.*, 1999; Watzl *et al.*, 2005) is able to enhance the immune system indirectly.

Other than the benefits mentioned above, inulin showed a better laxative effect in elderly. Thus helping to prevent constipation (Kleessen *et al.*, 1997). It could also decrease ß-glucuronidase and glycocholic acid hydroxylase activities which were associated with the conversion of procarcinogens to carcinogens in humans (Buddington *et al.*, 1996). It has been shown to reduce the incidences of ACF in the distal colon, lower the densities of *C. albicans*, and increased the resistance to *L. monocytogenes* and *S. typhimurium* in inulin-fed mice (Buddington *et al.*, 2002). In a recent study, Benyacoub *et al.*, (2008) reported that inulin was able to stimulate mucosal immunity and improve the efficacy of a vaccine upon challenge with virulent *Salmonella* by increasing the activity of immunoglobin G, fecal immunoglobin A as well as the phagocytic activity of the peritoneal macrophage.

1.3 ZINC

1.3.1 INTRODUCTION

Zinc is an essential trace element in human nutrition. Adult contains approximately 2 to 3 grams of zinc throughout the body with the highest concentration in the muscle and bone. Zinc plays catalytic, structural or regulatory roles in more than 200 metalloenzymes and metalloproteins which are involved in protein and nucleic acid

metabolism and energy production. For example, zinc plays an important catalytic role in protein farnesyltransferase that catalyzes the addition of farnesyl isoprenoid to conserve cysteine in peptide or protein substrates (Huang *et al.*, 1997). Zinc also occurs naturally in carbonic anhydrase, carboxypeptidase A, alcohol dehydrogenase and metallothionein (Parisi & Vallee, 1969).

Physiologically, zinc is necessary for healthy immune system, normal growth and development, dark vision adaptation, fertility as well as renewal of skin cell. It has been shown that abnormalities of taste and sexual function in uremic patients could be improved significantly with the intake of zinc supplements (Mahajan *et al.*, 1982). Besides, oral zinc supplementation also helped in the improvement of height velocity of short males (Kaji *et al.*, 1998) and potentiated the effect of vitamin A in restoring night vision among night-blind pregnant women (Christian *et al.*, 2001). Zinc possesses antioxidant properties which can retard oxidative processes (Powell, 2000). Zinc also affords beneficial effects to the barrier of the skin, gene regulation within lymphocytes, normal development and functioning of cells mediating the nonspecific immunity and acquired immunity (Shanker & Prasad, 1998). Consequently, zinc-deficient persons could experience increased susceptibility to a variety of pathogens.

Diet is the main source of zinc. It can be found in meat based diet as well as milk, yogurt, beans, nuts, seeds and wholegrain cereals. The dose required by pregnant and lactating women would be higher than those under normal conditions (Tsalev & Zaprianov, 1983).

1.3.2 PHYSICOCHEMICAL AND BIOLOGICAL PROPERTIES

Zinc is the 23rd most abundant element in the earth crust. It is a transition metal in periodic table with atomic number 30. The atomic weight is 65.39 g/mol with valence of 2. It has a high boiling and melting point of 907.0℃ and 419.5℃, respectively. Five stable isotopes have been discovered in nature.

Zinc can be manufactured commercially into different types of salt form including zinc chloride, zinc sulphate, zinc gluconate and zinc lactate. The physicochemical properties of zinc vary for different salt forms. For example, zinc chloride and zinc gluconate are white, odourless powder while zinc sulphate is odourless but transparent powder (Parfitt, 1999). In general, zinc salts can dissolve easily in water with the exception of zinc carbonate and zinc oxide.

Zinc is often referred as a "closed shell", good Lewis acid which is unable to undergo redox reactions. At neutral pH, all acid-base catalysis in biological systems are catalysed by zinc (Kendrick *et al.*, 1992).

1.3.3 BIOAVAILABILITY AND PHARMACOKINETICS

The gastrointestinal tract plays a vital role in zinc absorption, excretion and homeostatic control mechanism. In a perfusion study in human, Lee *et al.* (1989) revealed that zinc absorption occurred throughout the small intestine with the highest rate of absorption in jejunum. Zinc is also absorbed in the cecum and colon as shown in a rat study (Hara *et al.*, 2000). Zinc is incompletely absorbed from the gastrointestinal tract. Its oral bioavailability is approximately 20 to 30% (Parfitt, 1999).

Zinc is absorbed through the transcellular and paracellular pathways (Steel & Cousins, 1985). Hempe & Cousins (1991) suggested that cysteine-rich intestinal proteins that conferred metal-binding properties are important for zinc transport. After zinc passes through the barrier membrane of the intestinal mucosal cells, it will be taken up by albumin or globulin in the portal circulation to the liver (Fell & Lyon, 1994). A portion of zinc is extracted by hepatocytes. The remaining zinc will undergo tissue and cellular redistribution to maintain zinc homeostasis (King *et al.*, 2000).

An increase in zinc intake will increase the excretion of zinc in the stool but the urinary excretion remains unchanged (McCance & Widdowson, 1942). Other minor losses also occur in hair, nails, and epithelial skin cells (Tsalev & Zaprianov, 1983).

1.4 MAGNESIUM

1.4.1 INTRODUCTION

Magnesium is the fourth most abundant cation the in human body, only exceeded by calcium, sodium and potassium (Herring *et al.*, 1960). An adult body contains approximately 24 grams of magnesium, which are distributed in the muscles, tissues, extracellular fluids and bones (Gerstner, 2007). Magnesium is a cofactor in more than 300 enzymatic reactions in human body. It is involved in various biological processes including enzymatic reactions catalyzed by creatine kinase, pyruvate kinase, leucine, aminopeptidase, RNA and DNA polymerase, deoxynucleotidyl transferase and phosphoglucomutase (Kendrick *et al.*, 1992). Besides, it also plays a vital role in membrane function, energy metabolism and neuromuscular excitability. Toba *et al.* (2000) demonstrated that magnesium could promote bone formation, prevent

bone resorption, and increase the dynamic strength of bone in ovariectomized rats.

Deficiency of magnesium is common among the populations in developed countries such as the United States and France due to the changes in lifestyle and increased consumption of processed food (Ma *et al.*, 1995, Rayssiguier *et al.*, 2000). In addition, magnesium absorption can also be inhibited by certain dietary factors. Bohn et al. (2004) showed that fractional magnesium absorption could be impaired by phytic acid in a dose-dependent manner. Consequences of magnesium deficiency include cardiac arrhythmias, dysphagia, tremors, depression and eclampsia (Seelig, 1989).

It is estimated that the daily requirement of magnesium is not more than 250 mg in normal adults, 150 mg in infants and 400 mg in pregnanct women (Herring *et al.*, 1960). Food that is rich in magnesium includes legumes, whole grains, green leafy vegetables, nuts, coffee, chocolate and milk (Bernstein, 2002).

1.4.2 PHYSICOCHEMICAL AND BIOLOGICAL PROPERTIES

Magnesium is an alkali earth metal situated in Group II in periodic table with an atomic number of 12 and an atomic weight of 24.31 g/mol. It is the seventh most abundant element in the earth's crust by mass and it exists in 3 stable isotopes: ^{24}Mg, ^{25}Mg, ^{26}Mg. In nature, it is silvery white and very light. Thus, it has been used in the structural metal industry for a long time.

Many types of magnesium salts such as magnesium lactate, magnesium oxide, magnesium pidolate and magnesium chloride have been used for the manufacturing of magnesium supplements. Similar with zinc, different magnesium salts have their

own distinctive physicochemical properties. Magnesium gluconate is freely soluble in water but magnesium phosphate is practically insoluble in water (Parfitt, 1999). The rate at which magnesium is absorbed from the gastrointestinal tract also depends to a large extent on the form in which it is ingested (Tansy, 1971).

In plants, magnesium plays a pivotal role in photosynthesis process. The structure of chlorophyll is consisted of a porphyrin ligand system and a central magnesium ion (Kendrick *et al.*, 1992). The chemical structure of chlorophyll a is shown in Figure 1.4. Magnesium must be incorporated into the chlorophyll molecule before it can gather light for photosynthetic carbon reduction reactions (Sigel H. & Sigel A., 1990).

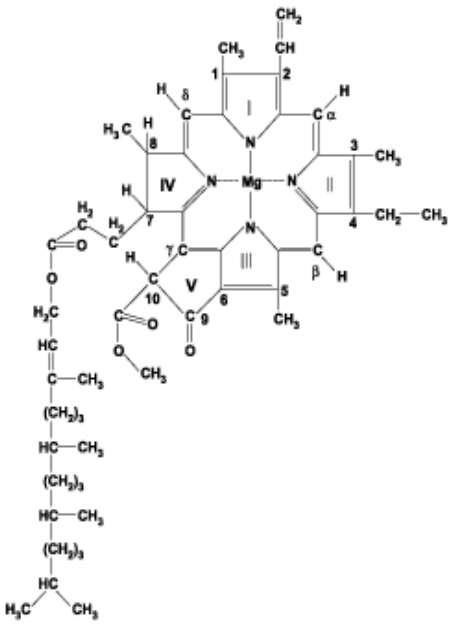

Figure 1.4 Chemical structure of chlorophyll a (Kendrick *et al.*, 1992)

1.4.3 BIOAVAIABILITY AND PHARMACOKINETICS

Approximately one third to one half of ingested magnesium is absorbed following oral administration and even soluble magnesium salts are generally very slowly absorbed (Parfitt, 1999). Magnesium absorption is dependent on the amount of endogenous magnesium ions present in rats (Tansy,1971). The principal site of magnesium absorption is the small intestine, with smaller amounts being absorbed in the colon (Konrad *et al.*, 2003). It has been reported that magnesium is transported through the intestinal barrier via both transcellular and paracellular pathways (Schweigel & Martens, 2000, Sigel H. & Sigel A., 1990). Vitamin D may also play a major role in the transportation as magnesium absorption has been shown to be reduced in patients who had a deficiency of the active metabolite of vitamin D (Brannan et al., 1976).

After magnesium passes through the barrier membrane of the gastrointestinal tract, about 35% of serum magnesium is bound to albumin and globulin while the rest remain ionized (Herring *et al.*, 1960). It is then distributed to bone, muscle, soft tissue and extracellular fluid. Approximately 30% of the magnesium present in the bone is exchangeable and functions as a reservoir to stabilize the serum concentration (Swaminathan, 2003).

Magnesium homeostasis is not regulated by hormone. Instead, it depends on the balance between intestinal absorption and renal excretion. Under normal circumstances in human, the renal excretion is about 120-140 mg/ 24hr (Sigel H. & Sigel A., 1990) and 95% of the excreted magnesium is reabsorbed from the proximal tubular segments, thick ascending limb of Henle’s loop and distal segments. During magnesium deficiency,

the kidney will reduce magnesium loss in urine. This was further confirmed by Dengel *et al*. (1994) that lactating women would compensate magnesium losses in breast milk by reducing urinary magnesium losses.

1.5 SUMMARY AND SCOPE OF STUDY

As mentioned earlier, inulin is a plant carbohydrate which can benefit human health in many ways. One of the benefits is the enhancement of mineral absorption from the gastrointestinal tract. In recent studies, inulin is shown to be able to promote mineral absorption through paracellular pathway *in vitro*. However, this has yet to be borne out in *in vivo* studies.

Thus, the present study was conducted to investigate the potential of co-administered inulin in enhancing zinc and magnesium absorption. Besides, acyclovir and atenolol, which are amenable to be transported paracellularly, were also used as model drugs to investigate the enhancement effect of inulin. The study was conducted in various stages with the following objectives:

1. To develop a simple flame atomic absorption spectrophotometry method for the determination of zinc and magnesium in rat plasma;
2. To determine the absolute bioavailability of zinc gluconate and magnesium lactate in male Sprague-Dawley rats;
3. To study the effect of different doses of inulin on the oral bioavailability of zinc gluconate and magnesium lactate using a rat model;
4. To assess the effect of different dose of inulin on the oral bioavailability of 2 model drugs, acyclovir and atenolol using a rat model.

CHAPTER 2

DEVELOPMENT OF ATOMIC ABSORPTION SPECTROPHOTOMETRY METHODS FOR THE ANALYSIS OF ZINC AND MAGNESIUM IN HUMAN PLASMA

2.1 INTRODUCTION

Different techniques for the analysis of zinc and magnesium concentration in plasma have been reported in the literature. They included ion chromatography (Lane *et al.*, 1999), capillary zone electrophoresis (Nemutlu and Özaltın, 2005), flame photometric method (Willis, 1959, Alcock *et al.*, 1960), colorimetric method (Garner, 1946), flame emission (Pruden *et al.*, 1966) and flame atomic absorption spectrophotometer (Pruden *et al.*, 1966)

In earlier times, colorimetric method was commonly used for determination of trace elements. The sample preparation procedures involved protein precipitation by trichloroacetic acid, addition of either Titan yellow or Magon sulfate reagents to the resulting water-clear filtrate followed by element precipitation (Garner, 1946). The associated problems with this method were low stability of the elements and high blank absorbance of the reagents (Loannou & Konstantianos, 1989). Using another method in analyzing elements, Lane *et al*. (1999) showed that ion chromatography had the advantage of determining multiple elements in one sample of 25 µl volume and offered a more cost effective choice. However, the sample required laborious microwave digestion in the sample pre-treatment. Fluorometric method has been used to determine plasma magnesium concentration. In spite of the high sensitivity of the fluorometric method, it

has a few disadvantages such as low reagent stability and low stability of magnesium complexes. These were overcome by using 2-hydroxy-1-naphthaldehyde salicyloylhydrazone (HNASH) as the fluorometric reagent which is stable in alkaline media. Sodium chloride was also added to stabilize HNASH-magnesium complexes (Loannou & Konstantianos,1989).

An alternative technique for the quantification of zinc and magnesium in plasma is atomic absorption spectrophotometer (AAS). Flameless AAS with graphite furnace atomizers has high sensitivity and requires only a small volume of sample for analysis (Vieira & Hansen, 1981, Haese *et al.*, 1992). However, it requires an expensive special attachment for the AAS (Makino & Takahara, 1981). On the other hand, flame AAS technique is more commonly employed in laboratories due to its simplicity, specificity and lower cost. However, bulk-matrix effects can cause different uptake rate and affect the accuracy of analysis (Smith & Butrimovitz, 1979). Other drawbacks of flame AAS include clogging of the burner head or nebulizer by the proteins and salts in the plasma and the presence of chemical interferences (Haese *et al.*, 1992). Thus, certain sample pre-treatments are needed. Some workers suggested the addition of a solution containing 0.444 mM potassium dihydrogen phosphate and 3.33% (v/v) perchloric acid or trichloroacetic acid for the deproteinization of the plasma (Pruden *et al.*, 1966, Gimblet *et al.*, 1967). Standard addition calibration and preconcentration techniques were also used to eliminate the bulk-matrix effects of the plasma samples with the aqueous working standards (Tsalev, 1984). Simple dilution, consisting of 1 part plasma to 1 part water or 1 part plasma with 4 part water, was also often practiced so that the dilution did not cause low sensitivity to the method (Tsalev, 1984). However, direct dilution consisting of