An investigation of the effects of fulvic and humic acids on the absorption of selected drugs, vitamins and minerals using the everted mouse gut model.

by

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Declaration by candidate

The experimental work contained in this dissertation was carried out by Kirsten Willis (author) from the Department of Pharmacology, Faculty of Health Sciences, University of Pretoria under the supervision of Prof A.D. Cromarty.

I declare that the work contained in this dissertation is my own work and has not been previously submitted for a degree at this, or any other tertiary institution.

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Abstract

Humic substances, such as the closely related humic and fulvic acids are ubiquitous, naturally occurring organic macromolecules of complex but undefined structure. These compounds are known complexing agents due to their supramolecular like structures and are capable of binding a wide variety of compounds.

Numerous studies have confirmed that humic and fulvic acids exhibit diverse medicinal and therapeutic properties. For this reason, alternative or "natural" medicinal preparations rich in these substances are being self-administered, often concomitantly with conventional drugs. The possibility exists that these humic substances, found in the alternative medicinal products, may result in drug-drug interactions and bind to simultaneously ingested drugs. Complex formation may affect absorption and alter overall bioavailability. Changes in these parameters may lead to reduced therapeutic effect or toxic side effects of prescribed drugs in patients.

Similarly, these humic substances may bind to and alter the uptake of ingested nutrients, such as vitamins and minerals, obtained from food sources as well as dietary supplements. Changes in absorption may result in a loss of proper physiological functioning in the body or in unwanted effects of overdose.

This study investigated the effect of fulvic and humic acids on the absorption of commonly administered classes of drugs, vitamins and minerals using the everted mouse gut model that was successfully used to assess the membrane transport of the test compounds. This model made use of everted segments of excised intestinal tissue placed in Krebs Ringer Buffer (pH7.4), where physiological functioning of the tissue is maintained for up to two hours after excision. The amount of test compound which crossed through the intestinal membrane without and in the

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presence of each humic substance was quantified using LC-MS/MS methods developed for each of the drugs and vitamins, and ICP-MS, in the case of the minerals.

The amount of test compound absorbed alone was compared to the amount absorbed when in the presence of each humic substance. Changes in the uptake, for each test compound was noted, the extent of the absorption increase or decrease was compound specific. The changes in absorption observed could be attributed to changes in compound solubility and mechanism of transport across the intestinal membrane once in complex.

Drugs and vitamins were seen to be more prone to decreases in absorption in the presence of the humic substances, whereas the majority of the minerals showed significantly increased absorption. Binding of the minerals to the humic substances through chelation, and not complex formation, could have a greater effect on compound solubility.

Health care professionals, as well as individuals ingesting these and other substances concurrently, should be aware of the potential effects on absorption that may occur due to drug-drug interactions in order to avoid a loss of therapeutic/physiological activity or negative toxic symptoms.

Key words: Humic substances, humic acid, carbohydrate-derived fulvic acid, complex, everted mouse gut model, drug absorption, bioavailability

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Glossary of abbreviations

AUCC	Animal Use and Care Committee
AT1	Angiotensin receptor
CHD-FA	Carbohydrate derived fulvic acid
cm	Centimetre
Da	Dalton
°C	Degrees Celsius
COX	Cyclo-oxygenase
CV	Coefficient of variation
ED50	The minimum dose at which half the population respond therapeutically
ESI	Electrospray ionisation
FA	Fulvic acid
GIT	Gastrointestinal tract
HA	Humic acid
HIV	Human immunodeficiency virus
HNO_3	Nitric acid
HPLC	High performance liquid chromatography
HS	Humic substances
ICP-MS	Inductively coupled plasma mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
IS	Internal standard
IUPAC	International Union of pure and applied chemistry
LOD	Limit of detection
LogD	Distribution coefficient
LogP	Partition coefficient
LOQ	Limit of quantitation
KRB	Krebs Ringer Buffer
NAD^+	Nicotinamide adenine dinucleotide
$NADP^+$	Nicotinamide adenine dinucleotide phosphate

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NaCl	Sodium chloride
NH_4OH	Ammonium hydroxide
NSAID	Non-steroidal anti-inflammatory
NRTI	Nucleoside reverse transcriptase inhibitor
NNRTI	Non-nucleoside reverse-transcriptase inhibitors
MS	Mass spectrometer
pH	Negative logarithm of the hydrogen ion concentration
pKa	Dissociation constant
PAMPA	Parallel artificial membrane permeability assay
Ppm	Parts per million
R^2	Coefficient of determination
RDI	Recommended daily intake
% RE	Percentage recovery
S/N	Signal to noise ratio
SPE	Solid phase extraction
TI	Therapeutic index
TD50	The dose at which half the population experience toxic side effect
UL	Upper intake level
µl	Microliter
UPBRC	University of Pretoria Biomedical Research Centre
USA	United States of America

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Chapter 1

1. Literature review

1.1 Background

An illness can be described as a disease or period of sickness affecting a person's body or mind. Illnesses may be classified as either acute or chronic. An acute illness begins suddenly, lasts for a short period of time and can usually be easily treated. Examples include headaches, colds and influenza. A chronic illness has a longer onset and lasts for an extended period, usually more than three months. Diabetes, arthritis, Alzheimer's and Parkinson's diseases are typical examples. Chronic illnesses are more difficult to treat than acute illnesses, and often require the use of prolonged treatment with multiple medications (Murrow & Oglesby, 1996). Many people worldwide suffer from one or more, acute or chronic, illness and the growing need to treat or prevent these illnesses has led to the increased use of many different types of medications.

Medications can be broadly classified into two main groups: conventional and alternative. Conventional medication is also referred to as "mainstream", "Western" or allopathic medicine and is recommended to patients by qualified healthcare professionals. Depending on the scheduling status, some conventional medications do not require a prescription and can be easily obtained over the counter at pharmacies. These are classified as non-prescribed conventional medications and are used to treat minor conditions which do not necessarily require a visit to a healthcare professional. Other conventional medications may require a prescription in order to be obtained. These prescribed conventional medications are registered and regulated and are recommended to patients to treat or prevent specific conditions. Prescriptions are necessary in order to ensure patient safety by specifying the drug and dose, especially when potent drugs or drugs with potential side effects are to be administered. Furthermore, proper prescribing can help to avoid drug-drug interactions and

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allow drugs with the potential for abuse and dependence to be more strictly regulated.

Alternative medications include all medications falling outside the range of conventional medication. The majority of alternative medications have been known since ancient times, long before conventional medication, and are comprised mostly of plant extracts and other naturally occurring substances. Alternative medications include traditional remedies, dietary and nutritional supplements including vitamins and minerals, herbal products, homeopathic and naturopathic remedies. Some of these medications are found to be as potent as conventional medications, possessing a wide variety of side effects as well as a high potential for drug-drug interactions (Izzo & Ernst, 2001). There has been an increase in the use of alternative medications due their lower costs, accessibility without the need for a prescription and due to the unfounded belief that because they are natural they are automatically "safe" with no adverse side effects. Cultural influences also play a role in the decision to use alternative medication (Astin, 1998; Hsiao *et al.,* 2003).

The use of both conventional and alternative medications are common the world over, but especially in South Africa, where it is a widespread occurrence for patients to use medications from each of these groups concurrently in order to treat or prevent the same illness or multiple illnesses (Haetzman *et al.*, 2003). The concurrent use of multiple medications whether conventional, alternative, or a combination of both, can often result in undesired effects due to drug interactions.

1.2. Drug interactions

Drug interactions occur when a drug is administered together with another substance, usually another drug, resulting in an altered or added drug effect. Other substances which have the potential to interact with drugs are food and beverages (Genser, 2008; Sulli & Ezzo, 2007). These drug interactions pose a risk

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to patients as they have the potential to alter the bioavailability of the drug, leading to increased or decreased drug concentrations in the body, which can result in changes in the therapeutic effect or toxicity profile. Drug interactions are potentially dangerous to patients concurrently using multiple medications. Both healthcare professionals and patients should be aware of which substances may exhibit drug interactions.

1.2.1 Types of drug interactions

There are many different types of drug interactions, these can be classified under two main headings: interactions affecting the pharmacodynamics and interactions affecting the pharmacokinetics of the drug (Dresser & Bailey, 2002). Pharmacodynamic interactions occur when there is a change in the effect that the drug exerts without there being any major change to the drug's concentration at the target site. These could be additive or antagonistic effects. Additive effects occur when drugs with a similar outcome are given together resulting in an enhanced effect, as seen in the case of the additive hypnotic and anaesthetic actions of propofol and ketamine when used together for the induction of anaesthesia (Hui *et al.*, 1995). Antagonistic effects occur when drugs possessing opposite actions are administered together, resulting in diminished effects of one or both drugs. Zidovudine and stavudine, both employed in the treatment of human immunodeficiency virus (HIV), should not be administered in combination due to their competitive affinity for thymidine kinase resulting in decreased therapeutic effect and the continuous decrease of $CD4^+$ cell count in HIV positive patients (Havlir *et al.,* 2000).

A pharmacokinetic interaction occurs when the effect of a drug is altered due to changes in the drug's concentration at the target site, caused by another drug or substance. These changes in drug concentration can occur during absorption, distribution, metabolism or excretion phases and can be brought on by indirect or direct factors. Indirect pharmacokinetic interactions arise when the bioavailability of one or both drugs is altered due to changes in physiological

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processes in various parts of the body. One of the most reported types of indirect pharmacokinetic interaction occurs in the liver and involves the cytochrome P450 (CYP) family of enzymes. These enzymes, responsible for the metabolism of exogenous compounds, including drugs, can be induced or inhibited by another drug or substance resulting in either reduced drug plasma concentration, leading to reduced drug effect, or in persistent elevated drug plasma concentration resulting in toxicity (Lynch & Price, 2007).

Direct pharmacokinetic interactions involve the actual physical interaction of the drug molecule with other molecules present. These interactions can take place at various sites in the body and are commonly seen in the gastrointestinal tract (GIT) when two orally administered compounds are ingested simultaneously. This interaction can affect the absorption of one or both of the drugs throughout the GIT. Direct pharmacokinetic interactions do not only occur between two drugs, but can also occur between a drug and another co-ingested substance like food, resulting in similar effects as the direct drug-drug interactions (Genser, 2008). There are two main direct interactions which can take place between a drug and another co-ingested drug or other substance: chelation and complex formation. Chelation is the process whereby a chelating agent forms a stable association through more than one coordination bond with a single metal ion resulting in heterocyclic compounds whereas complex formation involves the non-specific binding (through electrostatic bonds, hydrogen bonding and weak van der Waals forces) of a drug to another substance without bond formation.

1.2.2 The effects of direct pharmacokinetic interactions on the absorption and therapeutic activity of drugs

In order for an ingested substance to be used by the body, it must first be absorbed from the GIT and reach the systemic circulation. In the case of oral drugs, the amount and rate of absorption has a direct effect on the drug's bioavailability and in turn its therapeutic effect. If limited drug is absorbed, a decreased therapeutic response will be seen which may result in partial or total treatment failure. Rapid

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complete absorption could cause high systemic concentrations leading to toxicity or adverse side effects. It is therefore evident that any change in the absorption of a drug could alter the bioavailability and could therefore be harmful to patients.

There are many examples of how direct pharmacokinetic interactions through chelation and complex formation can cause alterations in drug absorption and bioavailability. Fluoroquinolones, a group of broad spectrum antibiotics, are reported to interact with multivalent cations, such as calcium, zinc and iron, contained in certain drugs, such as antacids, multivitamins as well as dairy products (Polk *et al.* 1989; Shiba *et al.*, 1992). This interaction takes place within the gut after concomitant ingestion and results in an insoluble drug chelation complex being formed that reduces the absorption of the fluoroquinolones (Fish, 2001). This diminished absorption raises the chances of therapeutic failure of the antibiotics. Similarly concurrent administration of tetracyclines with calcium, iron or zinc containing formulations results in decreased tetracycline absorption due to a chelation complex being formed. (Andersson *et al.*, 1976; Campbell & Hasinoff, 1991). Drug complex formation is also seen to have an effect on the absorption and bioavailability of drugs. Cholestyramine, a bile acid sequestrant, used for the treatment of hypercholesterolemia, easily binds and forms complexes with other drugs such as quinidine, valproic acid, digoxin and warfarin. These complexes render the drugs insoluble thus decreasing absorption and reducing the therapeutic effect (Brown *et al.* 1978; Gallo, *et al.*, 1965; Jahnchen *et al.*, 1978; Malloy *et al.*, 1996; Marino *et al.*, 1983; Toyoguchi *et al.*, 2005).

Direct pharmacokinetic interactions and their effect on absorption are often overlooked when compared to other types of drug interactions. Since the majority of drugs are taken orally, due to simplicity and convenience of self-administration, the ingestion of multiple concomitant medications is a common occurrence. Thus a large potential for direct pharmacokinetic interactions between two drugs to occur throughout the GIT exists. Furthermore, drugs are often taken at meal times, creating an environment where drug interactions can take place between any compounds contained in food or beverages.

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Due to the possible harmful effects brought about by this type of interaction, it is important that the absorption of specific compounds in the presence of other substances is more closely assessed and understood. Absorption studies would also be beneficial during the development stages of medications as this could influence the formulation of the drug.

1.3 Assessing intestinal absorption of substances

The absorption of an orally administered substance can occur throughout the GIT. The GIT is composed of the stomach, small intestine and large intestine. Some compounds are absorbed in the stomach, however, the major site of absorption is the intestines, specifically the small intestine, as it presents the largest surface area by far (Wilson, 1967).

The small intestine has a length of up to 6 metres and is further divided into 3 anatomically distinct regions: the duodenum, jejunum and ileum (Figure 1). The duodenum is the first region of the small intestine, directly following the stomach, and measuring approximately 20-30 cm. A mixture of chyme (partially digested food), bile and pancreatic juice is typically present in this region where further digestion takes place after the food leaves the stomach. The average pH of the duodenum ranges between pH 5 and 6. The jejunum follows the duodenum and measures approximately 2.5 m in length. This region is the primary site of nutrient absorption in the small intestine and has a pH range of between pH 7 and 8. The ileum is the last region of the small intestine leading into the large intestine. It is approximately 2 to 4 m in length and is responsible for the absorption of the remainder of the nutrients moving through the small intestine. The pH of this region ranges from pH 7 to 8.

Following the small intestine is the large intestine composed of the cecum, colon, rectum and anal canal. Unlike the small intestine, the large intestine does not play a significant role in the absorption of nutrients, however, the colon is

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responsible for the uptake of water and electrolytes. The pH of this portion of the intestine ranges between pH 6 and 7.

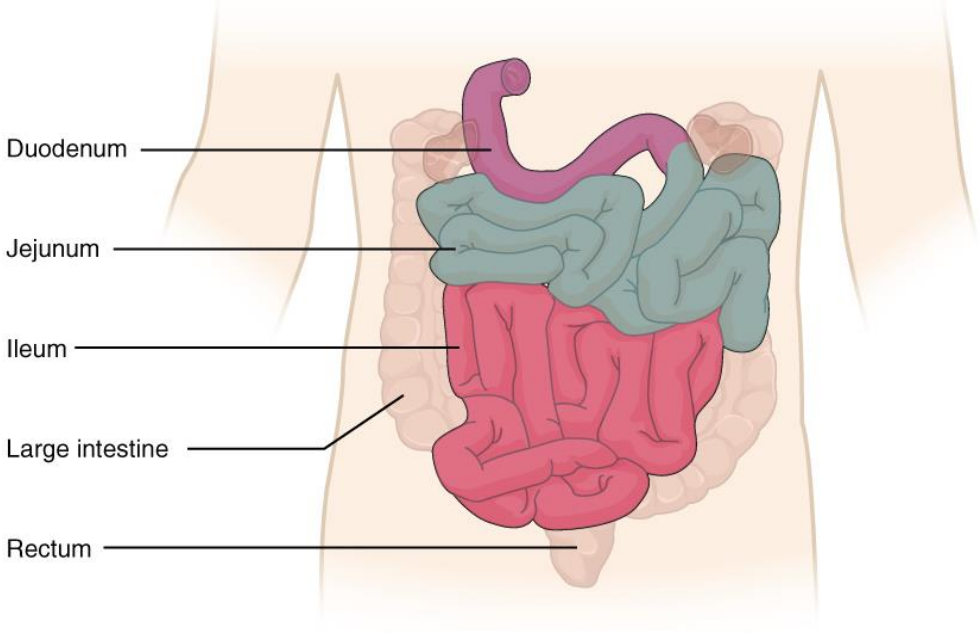

Figure 1. Diagram showing different regions of the intestines (with permission Openstax College, 2013)

The walls of the small intestine are composed of many finger-like projections known as villi, which significantly increase the surface area available for absorption. The border of the villi is composed of specialized epithelial cells responsible for absorption, known as enterocytes. Each enterocyte contains microvilli on their surface to further aid with surface area and absorption. The enterocytes make contact with adjacent cells through "tight junctions". These tight junctions can open to form small pores which are normally closed until absorption via the tight junction is required.

Substances can be transported across the intestinal membrane by different mechanisms depending largely on their physicochemical properties such as hydrophobicity, charge, molecular size and the presence of specific transporter proteins. There are four main transport mechanisms by which an orally administered drug can be absorbed: transcellular, paracellular, carrier mediated and transcytosis (Figure 2) (Artursson *et al.*, 2001; Barthe *et al.*, 1999).

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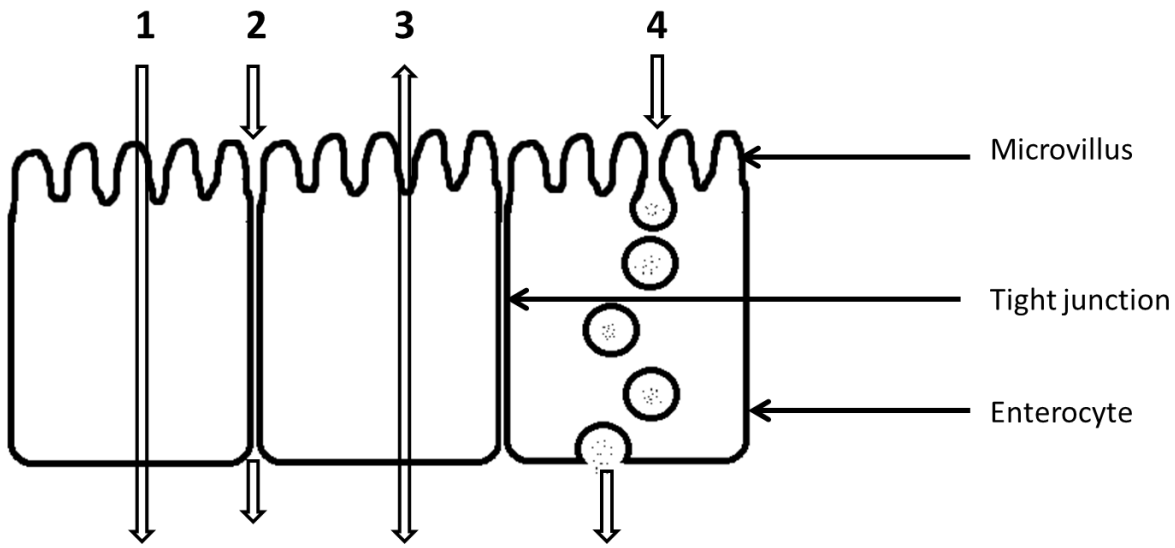

Figure 2. Schematic diagram of intestinal epithelium. Arrows indicate the different types of transport across the epithelium: 1, transcellular transport; 2, paracellular transport; 3, carrier mediated transport; 4, transcytosis.

1.3.1 Transcellular transport

Transcellular transport involves the movement of mainly low molecular weight lipophilic compounds across the intestinal membrane. (Barthe *et al.*, 1999). Lipophilic drugs absorbed via this route cross freely over the luminal cell membrane and distribute easily and rapidly into the intestinal cell due to concentration gradient driven diffusion. The majority of orally administered lipophilic drugs are absorbed via this mechanism (Stenberg *et al.*, 2000).

1.3.2 Paracellular transport

The majority of hydrophilic compounds make use of the paracellular route (Taylor, 1986). Paracellular transport involves the movement of substances through water-filled channels between adjacent cells, also known as tight junctions. These channels remain closed until the other mechanisms of transport are not possible. The movement through the tight junctions allows the substance to cross through the intestinal mucosa without entering cells and therefore cannot undergo any cellular metabolism. These water-filled pores make up approximately 0.01% to

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0.1% of the total surface area of the intestine, therefore paracellular transport usually plays a minor role in drug uptake (Stenberg *et al.*, 2000).

1.3.3 Carrier mediated transport

This mechanism involves the active or passive transport of small hydrophilic molecules across the intestinal cell membrane through the action of specific and highly selective trans-membrane protein transporters embedded in the phospholipid bilayer of the intestinal cells of the microvilli (Barthe *et al.*, 1999; Inui *et al.*, 1988; Steffansen *et al.*, 2004).

1.3.4 Transcytosis

Transcytosis involves the uptake of large peptides and other macromolecules into endocytotic vesicles formed from the invagination of the cell membrane (Laurence Barthe *et al.*, 1999; Heyman *et al.*, 1990). The cell membrane engulfs the drug molecule forming an intracellular drug-filled vesicle. Once in the vesicle, the macromolecule can be transported across the cell or be released into the cell interior. A drawback to this transport route lies in the fact that the vesicles may contain hydrolytic enzymes that results in partial or complete degradation of entrapped compounds during transport through the cell.

It has been observed that different substances show optimal absorption in certain anatomical regions of the intestine (Lacombe *et al.*, 2004; Quevedo & Briñón, 2009). These differences in absorption can be due to many different factors, including the differences in molecular receptors found on the surface of the microvilli in the different regions of the small intestine.

1.4 Factors affecting the absorption of orally administered drugs

There are many factors which can alter the absorption of orally administered drug other than direct chelation or complex formation with another substance. These factors can be drug-related, including all the factors pertaining to the physicochemical characteristics of the drug itself, or patient-related.

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1.4.1 Drug-related physicochemical factors

The physical and chemical properties (physicochemical properties) of a drug play an important role in determining drug solubility and permeability and are important factors when assessing drug absorption. Examples of common drug-related factors which can affect absorption are polarity and solubility (represented by the LogP and LogD: The LogP, the partition coefficient between octanol and water, is a measure of the polarity of a compound. The lower the LogP value, the more hydrophilic the compound. LogD, the distribution coefficient, represents a similar measure of the polarity of a compound but takes the specific pH into consideration.

The majority of drugs are weak acids or bases and exist in both un-ionized and ionized forms depending on the aqueous environment. The un-ionized form of a drug is generally lipophilic and can easily cross the cell membrane, whereas the ionized form of a drug is hydrophilic and has difficulty crossing the lipid plasma membrane. The pH of the environment as well as the pKa of the drug determines the ratio of un-ionized to ionized drug. When the environmental pH is lower than the pKa of weakly acidic drug, the un-ionized form predominates, this rule is conversely true for weakly basic drugs whereby the ionized form predominates. Thus weak acids are more easily absorbed at low pH and weak bases at high pH.

1.4.2 Systemic factors

Systemic factors are linked to the physiology of the patient. Gastric emptying, gastrointestinal motility, disease states, and demographic factors like gender and age also have the potential to affect drug absorption (Fujioka *et al.*, 1991; Liu, 2005).

1.5 Nutrient absorption in the GIT

Nutrients such as vitamins and minerals are essential to maintain proper physiological functioning of the body. Unlike plants, humans lack the natural

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ability to synthesize the large majority of these nutrients. Thus, adequate amounts are required to be administered at regular intervals through external sources, such as through a balanced diet or dietary supplements, in order to meet physiological needs.

The recommended daily intake (RDI) of nutrients, such as vitamins and minerals, describes the average amount which must be taken in on a daily basis in order to meet the physiological requirements of healthy individuals. These values vary depending on the nutrient and act as broad guidelines for dietary intake. They do not, however, adequately cater for differences in age, gender and level of activity, or for special population groups.

Taking in less than this recommended amount over a period of time will result in a nutrient deficiency, resulting in the disruption of various physiological processes leading to unwanted symptoms. The tolerable upper intake level (UL) describes the maximum amount of the nutrient which can be taken in daily, without resulting in negative toxic symptoms (National Institutes of Health, 2014).

Nutrient deficiencies can occur following limited dietary intake over prolonged periods. Deficiencies may be a common occurrence, especially in poorer regions or developing countries and are linked to many negative symptoms. It is, however, also possible to suffer from toxic symptoms from excessive nutrients intake exceeding the UL (DiPalma & Ritchie, 1977) (National Institutes of Health, 2014). These high levels are usually reached due to excessive supplementation rather than through dietary consumption.

Similarly to drugs, a specific amount of each nutrient is required to be absorbed to overcome deficiencies, ensure proper physiological functioning as well as to avoid toxic symptoms.

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1.6 Methods for assessing intestinal absorption of substances

Several models and techniques can be used in the evaluation of compound absorption in the GIT. Examples of commonly used *in-vitro* methods include the everted gut sack technique and the use of cultured cell monolayers (Acra & Ghishan, 1991; Barthe *et al.*, 1999). Other commonly used methods include the Ussing chamber (Clarke, 2009) and the parallel artificial membrane permeability assay (PAMPA) (Kansy *et al.*, 1998). These methods provide models for assessing compound diffusion across membranes and are often used as tools for predicting intestinal absorption in humans (Artursson & Karlsson, 1991; Rubas *et al.*, 1996).

1.6.1 The everted gut sack technique

The everted gut sack technique was first introduced by Wilson and Wiseman in 1954 (Wilson & Wiseman, 1954) and originally used to study the transport of sugars and amino acids. This technique involved the use of freshly excised intestinal tissue from a rat to predict the absorption of compounds across the intestinal mucosa. Once excised, the intestinal tissue is everted so that the luminal surface is facing outwards and the ends of the tissue are tied off resulting in a sack where the luminal surface is facing outwards. The intestinal tissue is able retain normal physiological functioning for up to two hours, if incubated in the correct solution, during the absorption study (Figure 3) (Barthe *et al.*, 1998).

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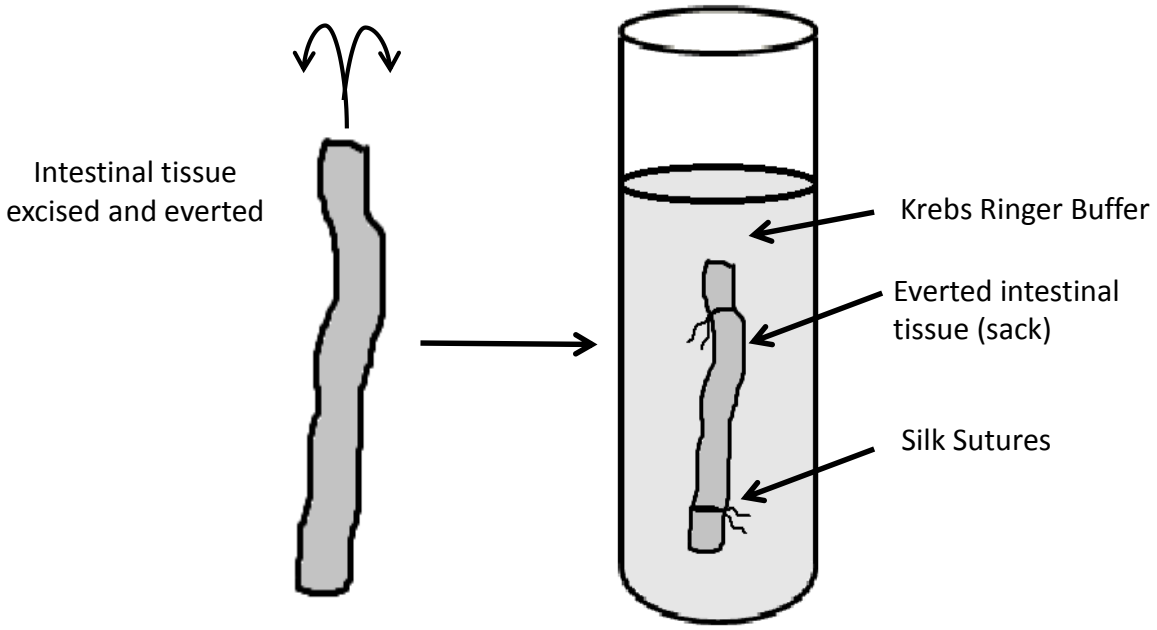

Figure 3. Schematic diagram of the everted gut sack technique

In the original study by Wilson and Wiseman, the intestinal tissue was placed in a simple salt media during incubation (Wilson & Wiseman, 1954). However, this simple salt media resulted in rapid histological degradation, poor tissue viability and ultimately the loss of proper physiological functioning of the intestinal cells. Levine and colleagues (Levine *et al.*, 1970) showed that after 30 min in this medium, 50-75% of the epithelium had become inactive due to degradation and/or separation from the basal layer. After one hour, there was complete loss of the epithelial border. Bridges and colleagues (Bridges *et al.*, 1978) introduced a more complex tissue culture medium, TC 199, to replace the simple salt medium used by Wilson and Wiseman, which resulted in improved tissue viability for up to two hours (Barthe *et al.*, 1998).

The everted gut sack technique has been used to assess the absorption of a range of compounds possessing widely differing physicochemical properties (Blundell *et al.*, 1993; Moshtaghie *et al.*, 2006; Naisbett & Woodley, 1994; Nolon *et al.*, 1977; Rowland & Woodley, 1981). Different aspects of absorption, such as the role of membrane transporters, intestinal enzymes, site as well as area of absorption can

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also be evaluated using this technique (Alam *et al.*, 2012; Barthe, et al, 1998; Bouer *et al.*, 1999; Carreno-Gomez & Duncan, 2000; Cornaire *et al.*, 2004; Da Silva *et al.*, 2009; Li *et al.*, 2011; Moshtaghie et al., 2006; Pento & Johnson, 1983; Uchiyama *et al.*, 1999).

The everted gut sack method can be carried out using GIT tissue from a variety of different animals, such as fish (Kleinow *et al.*, 2006), rabbits (Clauss & Hörnicke, 1984), chickens (Scharrer & Stubenhofer, 1984), pigs (Panichkriangkrai & Ahrens, 1988) guinea pigs (Himukai, 1984) and mice (Iizasa *et al.*, 2003; Mary & Rao, 2002; Yamagata *et al.*, 2007), however rat tissue is still most commonly used for this technique (Carreno-Gomez & Duncan, 2000).

As the tissue used in this technique is not human, interspecies differences could affect the accurate prediction of absorption in humans. However, good correlation between human intestinal absorption has been observed and the everted gut sack method is reported to be a fairly accurate predictive tool (Amidon *et al.*, 1988; Lennernas, 1997).

It is well-known that numerous compounds have different absorption capacities in different regions of the intestines (Lacombe *et al.*, 2004; Quevedo & Briñón, 2009). One advantage of the everted gut sack technique is it allows for absorption in each of the different anatomical regions of the intestine to be assessed separately and simultaneously. The everted gut model also retains all membrane transporter activity for as long as the tissue is viable. Other benefits of the method include that it is relatively simple, rapid and reproducible.

1.6.2 Cultured cell monolayers

Immortalized cell lines, such as Caco-2, HT-29 and T84, are commonly used to assess compound absorption *in-vitro* (Barthe *et al.*, 1999), with Caco-2 cells being the most frequently used in studies involving drug transport (Artursson *et al.*, 2001; Barthe *et al.*, 1999). The Caco-2 cell line was established in 1974 by Fogh

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(Fogh *et al.*, 1977). This immortal cell line was obtained from a human colon cancer tumour and is commonly grown as a monolayer on a porous support surface between two isolated solutions of media. Once a complete monolayer of cells has been formed on the porous support one side, termed the donor compartment, is loaded with the test compound (Figure 4) (Artursson *et al.*, 2001).

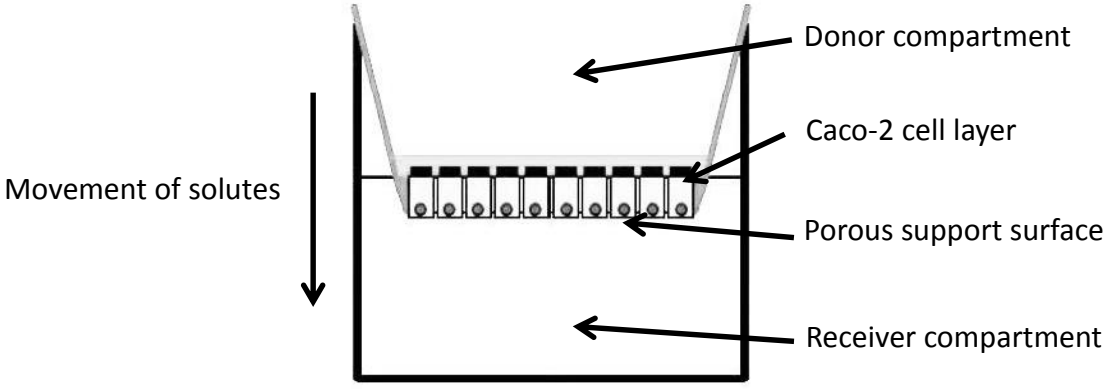

Figure 4. Schematic diagram of a Caco-2 cell monolayer (adapted from Rasgado-Flores et al., 2013)

As Caco-2 cells are derived from human colonic cancer cells, no interspecies differences with regards to the morphological or physiological characteristics should exist between the cells in the model and cells present in the human intestine. Previously, studies (Artusson, 1993; Lennernas *et al.*, 1996; Rubas *et al.*, 1996; Yee, 1997) have shown that Caco-2 cells show good correlation to human intestinal tissue, when comparing the absorption of a variety of drugs, and can be used as a predictive model.

Although Caco-2 cells can be used to predict absorption in humans, an area of inconsistency exists within the cell line itself. Caco-2 cells are a heterogeneous cell population (Vachon & Beaulieu, 1992) with properties of cells differing between populations from different laboratories and sub-populations (Hu *et al.*, 1995; Walter & Kissel, 1995). Various factors can cause the variability in properties between Caco-2 populations from different labs, such as, number of

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passages the cells have undergone (Caro *et al.*, 1995; Walter & Kissel, 1995), time spent in culture medium (Wilson *et al.*, 1990) the type of support (Nicklina *et al.*, 1982) and even slight differences in the cell culture medium (Jumarie & Malo, 1991). It is therefore neither robust, nor reproducible assay method and thus it is not feasible to compare Caco-2 permeability data between laboratories due to the lack of standardization in cell culturing.

Another drawback is that the Caco-2 cells do not closely mimic the environment of an *in-situ* intestine. The monolayers of these clonal cells are without mucus producing goblet cells and are thus without a naturally occurring mucus layer which has been shown to have a significant effect on absorption of many compounds (Madara & Trier, 1982).

1.6.3 Ussing chamber

The Ussing chamber was first developed in the 1950's by Hans Ussing in order to better understand NaCl transport (Ussing & Zerahn, 1951). This system is used to study electrophysiology or the diffusion of substances such as ions, nutrients and drugs.

The system is composed a perfusion system and two chambers separated by a membrane derived from tissue from an animal (Figure 5). Each of the chambers is filled with an equal amount of the same media and the test substance can be placed in one of the chambers to assess its movement from one chamber to the other across the membrane. During electrophysiology studies, the transepithelial response of ion movement across the membrane is measured using an electrical circuit system, whereas the diffusion-based studies measure the net movement of solutes from one chamber to the other and the respective concentrations resulting in each chamber can be compared using methods such as spectroscopy and high performance liquid chromatography (HPLC).

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The test membrane can be derived from a variety of different tissues, which is an advantage of this assay. Common tissue used can be obtained from the stomach, intestinal, bladder, skin and trachea (Dunning-Davies *et al.*, 2013; Lampen *et al.*, 1996; Lester & Rice, 2012; Ussing & Zerahn, 1951).

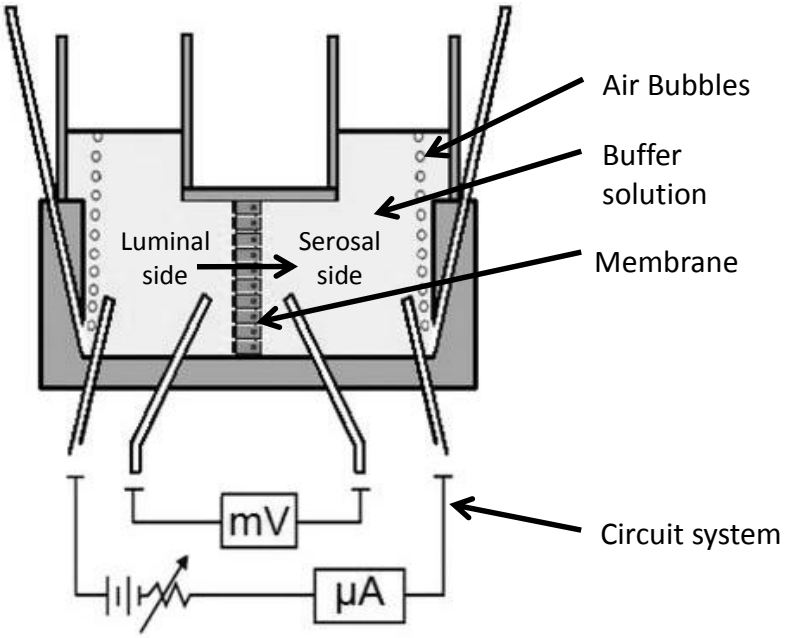

Figure 5. Schematic diagram of the Ussing chamber, (adapted from Rasgado-Flores et al., 2013)

This system only allows for one membrane to be assessed at a time and is time consuming when assessing transport in more than one type of membrane or membranes from different anatomical regions.

1.6.4 PAMPA

Parallel artificial permeability assay, first described by Kansy *et al.* in 1998, is a method used for absorption studies whereby the permeability of a compound is assessed using a lipid-infused artificial membrane (Kansy *et al.*, 1998). The artificial membrane consists of a phospholipid layer on a filter plate, treated with an organic solvent to mimic a cell membrane. Various membranes consisting of different lipids and making use of different solvents can be assessed. The assay is carried out using a modified 96-well plate containing a donor well and an acceptor

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well separated by a lipid-infused membrane (Figure 6). Each compartment of the 96-well plate is customizable for a specific region in the body. This assay allows for high throughput of samples and is used primarily to assess passive, transcellular diffusion (Bermejo *et al.*, 2004). There is no mechanism for active transport, therefore compounds can only be assessed based on permeability properties alone. PAMPA allows for permeability to be assessed at different pH, therefore can mimic the environment of different regions of the GIT (Avdeef, 2005).

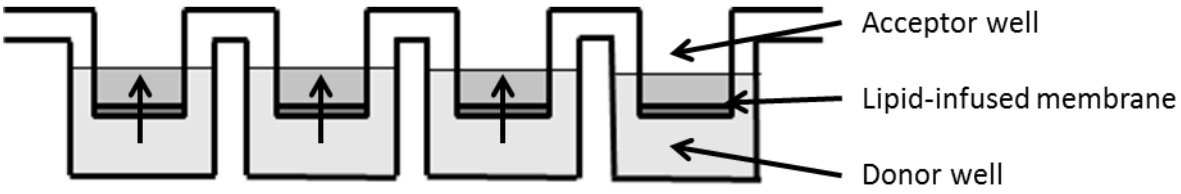

Figure 6. Schematic diagram of the PAMPA method

Although PAMPA has many advantages, such as being a high throughput technique, relatively inexpensive and easily customizable, it has several disadvantages. PAMPA can be used to predict oral absorption in the human GIT, however, it can only be used to predict passive, transcellular absorption (Corti *et al.*, 2006). Furthermore, the artificial membrane is relatively standard and does not allow for an accurate representation of the different regions of the intestine.

1.7 Humic and Fulvic acids

Humic substances (HS) are a group of complex organic macromolecules widely distributed throughout the environment as components of coal (Bergh *et al.*, 1997), surface water (Chin *et al.*, 1994), soil (Sutton & Sposito, 2005), peat (Hartenstein, 1981), compost, sewage and brown coal (Pena-Mendez, 2005). HS are formed through the process of humification, whereby plant and animal matter is continuously broken down by microorganisms (Mayhew, 2004). HS are divided into three main subgroups according to their solubility characteristics: fulvic acid (FA) humic acid (HA) and humin (Stevenson, 1994). FA are soluble in water over all pH values, HA are soluble in neutral and basic solutions and humin is insoluble

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in aqueous solutions (Stevenson, 1994). These subgroups range from yellow to dark brown in colour and share similar elemental composition but are seen to differ in structure and molecular weight (Figure 7) (Stevenson, 1982).

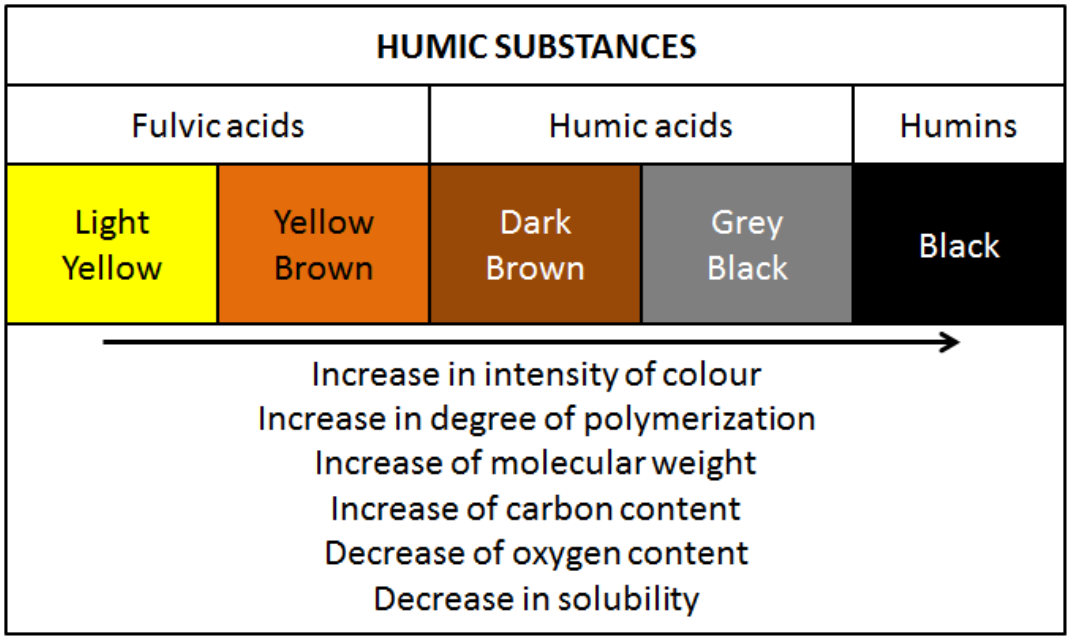

Figure 7. Properties of humic substances, (adapted from Stevenson, 1982)

1.7.1 Humic and fulvic acids as binding agents

The presence of numerous functional groups, especially the more prevalent oxygen-containing groups such as phenolic hydroxyl and carboxyl, allow humic and fulvic acids to easily bind to and form complexes with a variety of compounds. This high binding potential has been seen in the environment as humic and fulvic acids easily bind toxic and other harmful substances. Such substances include herbicides (Martin-Neto *et al.*, 2001), pesticides (Jones, 2003), endocrine disrupting pharmaceutical products (Loffredo & Senesi, 2006; Zhou *et al.*, 2007), organic pollutants (De Paolis, 1997), heavy metals (Pandey *et al.*, 2000; Water & Hiraide, 1992) polyaromatic hydrocarbons (PAH's) (Conte *et al.*, 2001; Perminova *et al.*, 2001) and polychlorinated biphenyls (PCB's) (Klle *et al.*, 1987) and mycotoxins (Jansen van Rensburg *et al.*, 2001). Furthermore, both humic and fulvic acids have the potential to bind and form complexes with many different

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classes of drugs (Aamir *et al.*, 2011; S.P. Agarwal & Anwer, 2008; Anwer *et al.*, 2010; Mirza *et al.*, 2011; Pils & Laird, 2007; Zhang *et al.*, 2012)

1.7.2 Humic and fulvic acid structure

Numerous analytical techniques (Nebbioso *et al.*, 2011) have been used in hopes of revealing the true structure of humic substances, however, none of the techniques was entirely successful and the exact structure and molecular weight of humic substances remains unknown. Large chemical heterogeneity, complex composition as well as geographical variability also add to the difficulties when defining the structure of the humic substances (Pena-Mendez *et al.*, 2007; Piccolo, 2001). It is however clear that all humic substances share very similar basic elemental composition and contain C (40-60%) > O (30-50%) > H (4-5%) > N (1-4%) > S (1-2%) by weight (Rice & MacCarthy, 1991). Literature suggests that molecular weights of humic substances can range from 500 Da to 10^6 Da (Stevenson, 1994) due to large differences in the chemical heterogeneity seen in substances from different origins.

In the past, humic substances were commonly believed to be high molecular weight polymers (Piccolo, 2001), however, more recent studies suggest that the humic substances are supramolecular molecules composed of many small heterogeneous molecules, of less than 1000 Da, held together by relatively weak interaction forces such as van der Waals, π-π interactions and hydrogen bonding (Nebbioso *et al.*, 2011; Piccolo, 2001). Moreover this model accommodates the earlier idea that humic substances possess a micellar structure when in an aqueous solution. This micelle formation explains the occurrence of hydrophilic functional groups and regions on the exterior while the more hydrophobic regions are found in the interior, protected from the surrounding water molecules (Guetzloff & Rice, 1994). The general structure for these compounds consists of condensed aromatic rings having a large variety of functional groups, such as, phenolic hydroxyl, ketones, carboxylic and quinone groups (Hayes & Clapp, 2001; Schepetkin *et al.*, 2002; Stevenson, 1994). Phenolic hydroxyl and carboxyl groups

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are found to be the most commonly occurring functional groups of humic substances from soil and lake sediments.

Although humic and fulvic acids share similar structure and elemental composition, clear differences exists between the two subgroups. Fulvic acids are generally seen to have a lower pKa value, molecular weight, less aromatic structures, lower carbon content, higher oxygen content and less hydrophobic than humic acids (Lubal *et al.*, 1998; Rice & MacCarthy, 1991; Stevenson, 1982)

1.7.3 Applications of humic substances

A review by Pena-Mendez and colleagues (Pena-Mendez *et al.*, 2005) highlights the benefits of humic substances in a variety of agricultural as well as biomedical applications.

1.7.3.1 Applications in agriculture

Humic substances are a common addition to fertilizers used in the agricultural industry as they are observed to have positive effects on plant growth, soil fertility and crop yield. Atiyeh and colleagues (Atiyeh *et al.*, 2002) found that the growth of cucumbers and tomatoes showed significant growth in terms of plant heights, leaf areas, shoot and root dry weights when humic substances were mixed into their growth medium. Another study by Eyheraguibel in 2008 showed similar effects on maize (Eyheraguibel *et al.,* 2008). Increased crop yields were noted by other researchers when plants were grown in fertilizer rich in humic substances. (Pârvan *et al.,* 2012; Shahryari & Mollasadeghi, 2011; Tringovska, 2012). The positive effects seen in these studies have been attributed to increased nutrient uptake and soil fertility brought on by the humic substances (Khaled & Fawy, 2011). Growth promoting effects of humic substances added to agricultural animal feed has also been observed in chickens and pigs (Ji *et al.,* 2006; Kocabağli *et al.,* 2002; Kucukersan *et al.,* 2005).

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1.7.3.2 Applications in medicine

Substances containing humic and fulvic acids have been used in folk medicine throughout history for the treatment of a variety of ailments (Agarwal *et al.* 2007; Schepetkin *et al.,* 2002). Studies carried out on fulvic and humic acids over the past few decades have confirmed that these substances possess a wide range of beneficial properties when used internally or topically. These properties are summarised in the Table 1.

Table 1. Therapeutic properties of humic substances

Property	Reference
Anti-viral	(Klöcking & Sprössig, 1972; Schiller *et al.* 1979; Thiel *et al.*, 1977; Van Rensburg *et al.*, 2002)
Anti-inflammatory	(Jooné & Van Rensburg, 2004; Sabi *et al.*, 2012; Van Rensburg et al., 2001)
Antimicrobial	(Sherry *et al.*, 2012; Ansorg & Rochus, 1978)
Anticancer	(Yang *et al.*, 2004; Pant *et al.* , 2012)
Immunostimulatory	(Vucskits *et al.*, 2010; Jooné *et al.*, 2003)
Antimutigenic	(Ferrara *et al.*, 2006)
Antioxidant	(Vašková *et al.*, 2011; Rodríguez *et al.*, 2011)
Wound healing	(Sabi *et al.*, 2012)

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1.8 Study motivation

Due to the wide range of apparently remarkable medicinal and therapeutic properties possessed by both humic and fulvic acids, an increasing number of medicinal preparations containing these compounds are available on the market.

A web-based search of commercially available humic and fulvic acid preparations resulted in the following list:

Table 2. Commercially sold humic and fulvic acid preparations

Product	Manufacturer
CaFA600	Sherston
CHD-FA	Fulhold
Premium Grade Humic Acid™	Advanced Health Nutraceuticles
Fulvic and humic acid	Nano Health Solutions, Inc.
Wu Jin San	Faust Bio-Agricultural Services, Inc
Fauna Mana	Faust Bio-Agricultural Services, Inc
Supreme Fulvic & Humic Complex™	Supreme Fulvic.com
BEST FULVIC™ and The Gift™	Mother Earth Labs, Inc
Humigold	Natural Nutrition
VFI Humic Acid®	Laub Biochem Organic Defense™
ULTRA Immune	Ultra Nano Humic Acid

These humic and fulvic acid-containing preparations fall into the category of alternative medication and are marketed as nutraceuticals or dietary supplements. Although recently having been revised, the regulations linked to this group of medication are less stringent than with conventional medications and are not formally scheduled. Consequently they do not require a prescription from a healthcare professional, making them easily accessible. Moreover, they are commonly sought as they are often cheaper than conventional medications and are perceived to be safer as they are “natural substances”.

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A large portion of the words population have difficulty obtaining conventional medication due to limited access to a healthcare professional or restricted funds, and thus rely on a variety of alternative medications. Furthermore, a large portion of the South African population use government-provided chronic conventional medication for the treatment of HIV and TB but have cultural beliefs that encourage use of herbal or alternative medication. Subsequently, there is a high potential for patients to simultaneously take alternative medications, like humic and fulvic acid containing preparations, together with conventional medication. Loss in therapeutic effect of these drugs or enhancement of adverse side-effects brought about by altered bioavailability could be detrimental to these patients and the overall health status of the diseased population.

Humic and fulvic acids display strong complexing abilities for a variety of different classes of compounds. It is thus highly possible the humic and fulvic acids contained in medicinal preparations may bind to co-ingested compounds resulting in a direct interaction and altered absorption or pharmacokinetic parameters.

Intestinal absorption of co-ingested nutrient substances, like minerals and vitamins, in the diet or in supplement form, may also be affected by the presence of humic and fulvic acid. These substances are necessary in certain quantities to maintain normal physiological functioning of the body. Similar to drugs, alterations in the amount of nutrients absorbed could potentially have negative effects on health.

It is therefore not only important to assess the potential binding effects that humic and fulvic acids can have on the absorption of drugs, but also the absorption of important nutrients such as minerals and vitamins.

1.8.1 Rationale for the choice in absorption model

This study was performed to assess the potential effects that concomitantly ingested humic or fulvic acids would have on the absorption of several commonly

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administered drugs and nutritionally important vitamins and minerals. The everted gut sack technique was selected as the absorption model for this study.

Even though the method makes use of rodent intestinal tissue, good correlation with human intestinal absorption has been demonstrated, eliminating the concerns of interspecies differences. Unlike in the PAMPA assay, this technique allows the accumulated effect of different transport mechanisms to be assessed for different compounds. Furthermore, absorption in each of the different anatomical regions of the intestine (duodenum, jejunum, ileum and colon) can be assessed. This cannot be achieved using Caco-2 monolayers as they are derived from human colon cancer cells only and thus different GIT regions cannot be assessed. Caco-2 cell monolayers show low reproducibility of the absorption model which also differ between laboratories, highlighting the potential for poor comparison to other researchers work. The everted gut sack technique allows for multiple segments to be assessed simultaneously resulting in a quick overall process, unlike the Ussing chamber, which can only assess transport over a single membrane at a time, resulting in a time-consuming method with a higher requirement for experimental animals. Finally, the everted gut model is relatively simple, reproducible and comparatively inexpensive. For these reasons, the everted gut sack method was chosen to carry out the aims of this study.

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1.9 Aims

1. To assess the effect that concomitant administration of **fulvic acid** would have on the absorption of several different classes of commonly ingested drugs, vitamins and minerals using the everted mouse gut model.

2. To assess the effect that concomitant administration of **humic acid** would have on the absorption of several different classes of commonly ingested drugs, vitamins and minerals using the everted mouse gut model.

1.10 Objectives

1. Develop and validate individual LC-MS/MS methods for quantitation of each test drug selected to represent different drug classes.

2. Develop and validate individual LC-MS/MS methods for quantitation of vitamins of differing polarities.

3. Develop a suitable sample preparation method for the detection of selected physiologically important minerals using ICP-MS.

4. Use the everted mouse gut model to assess the intestinal absorption of the drugs, vitamins and minerals without and in the presence of fixed fulvic or humic acid concentrations

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Chapter 2

2. Materials and methods

2.1 Animal work

2.1.1 Animal ethics

All procedures during this study relating to animals were carried out under the guidelines of the SANS 10386:2008. Ethical clearance for the use of mice for this study was obtained through the Animal Use and Care Committee (AUCC) of the University of Pretoria (see Addendum for approval letters H018-11 and H007-12).

2.1.2 Criteria for use of animals

A total of 90 BALB/c mice were used in this study. Only female mice were used in order to eliminate any gender-related differences in absorption. Only mature and healthy mice between 6 - 8 weeks old and weighing 18 - 20 g were used.

2.1.3 Animal housing and care

Housing and care of mice was undertaken at the University of Pretoria Biomedical Research Centre (UPBRC).

Small groups of female BALB/c were purchased from the breeding facility approximately two weeks prior to the experiment and acclimatized to the local laboratory conditions for at least one week prior to the start of experimentation. Mice were housed in conventional plastic mouse cages with grid tops, in groups of three with 12-hour light/dark cycles, in rooms with controlled environmental conditions (21˚C and 60 – 70% relative humidity). During acclimatization, mice had free access to water and a standard mouse chow diet. Additional nesting and enrichment toys were provided.

2.1.4 Euthanasia

Solid food was withheld for 16 hours prior to euthanasia, in order to clear gut of any solid food, however, mice had free access to drinking water enriched to 5%

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glucose. Mice were euthanized by qualified personnel at the UPBRC using an overdose of anaesthesia (Isoflurane). Isoflurane does not have any effect on the mouse small intestine and was therefore an appropriate euthanizing agent for this study. Six mice were euthanized per week (three mice on two separate days) over non-consecutive weeks.

2.1.5 Dissection and removal of intestinal tissue

Immediately following euthanasia, the abdominal region of the mouse was opened by a midline incision. The entire GIT was carefully excised from the oesophagus to the rectum and the omentum removed. Any remaining content was gently flushed out with cold saline solution introduced using a two millilitre syringe and all remaining mesenteric attachments carefully removed. The intestinal tissue was immediately placed in ice cold Krebs Ringer Buffer (KRB) (pH 7.4) until the remainder of the GIT preparation took place.

2.1.6 Disposal

Animal carcasses were packaged for incineration and disposed of by qualified personnel according to the standard cadaver disposal procedure.

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2.2 Experimental Design

This *in vitro* study involved the use of the everted gut absorption model to assess and compare the uptake of several drugs and nutritional components in the presence or absence of both fulvic and humic acids. The absorption study of each test compound was repeated in triplicate (3 separate animals) using four different GIT region sections in each experiment.

2.2.1 Test compounds

Test compounds chosen for this study will be discussed below but included drugs from different classes, vitamins and minerals to test the possible drug/drug interaction in the presence of a constant concentration of either humic or fulvic acids.

2.2.1.1 Drugs

Six common orally administered drugs, from a range of different drug classes and physicochemical properties were selected (Table 3). These drugs exhibited widely differing structures and physicochemical properties. Physicochemical properties were obtained from ChemSpider and DrugBank databases. (ChemSpider, 2014; DrugBank, 2014). The values obtained from these websites are calculated theoretical values and were not obtained under experimental conditions.

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Table 3. Physicochemical properties of selected drugs

Drug	Drug Class	Molecular mass	LogP*	LogD**	pKa***
Diclofenac	Non-steroidal anti-inflammatory (NSAID)	296.15 g/mol	4.06	0.95	4.00
Penicillin V	Antibiotic	350.39 g/mol	1.88	-1.85	2.79
Warfarin	Anti-coagulant	308.33 g/mol	3.42	0.61	5.08
Rifampicin	Anti-tuberculosis agent	822.94 g/mol	1.09	-1.43	7.53
Valsartan	Angiotensin receptor blocker	435.52 g/mol	4.74	0.01	4.37
Zidovudine	Nucleoside analogue reverse-transcriptase inhibitor (NRTI)	267.24 g/mol	-0.53	-0.53	9.96

***LogP** – the partition coefficient. The lower the value, the more hydrophilic a compound.*

****LogD** - the distribution coefficient represents the lipophilicity of a compound at a specific pH (pH 7.4)*

*****pKa** – the acid dissociation constant. The lower the pKa value, the more acidic the compound*

2.2.1.2 Vitamins

Two vitamins, one water-soluble and one fat-soluble were selected. Physicochemical properties were obtained from ChemSpider and DrugBank databases. (ChemSpider, 2014; DrugBank, 2014). These values are calculated theoretical values and were not obtained under experimental conditions.

Table 4. Physicochemical properties of selected vitamins

Vitamin	Solubility	Molecular mass	LogP*	LogD**	pKa***
Vitamin B_3 (Niacin)	Water-soluble	123.11 g/mol	0.15	-2.93	4.75
Vitamin E (α-tocopherol)	Fat-soluble	430.71 g/mol	11.90	11.90	10.8

***LogP** – the partition coefficient. The lower the value, the more hydrophilic a compound.*

****LogD** - the distribution coefficient represents the lipophilicity of a compound at a specific pH (pH 7.4)*

*****pKa** – the acid dissociation constant. The lower the pKa value, the more acidic the compound*

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2.2.1.3 Minerals

Five dietary minerals, from both major and trace groups were selected.

Major minerals are needed in large amount by the body on a daily basis in order to maintain proper physiological functioning. Major minerals include calcium, phosphorous, sodium, magnesium and potassium. Trace minerals are needed in small amounts on a daily basis and include iron, iodine, manganese, copper, cobalt, zinc, selenium and fluoride.

Table 5. Properties of selected minerals

Mineral	Type	Main physiological functions
Calcium (Ca^{2+})	Major	Formation of bones and teeth Muscle contraction and relaxation Nerve functioning Blood clotting Immune system health
Magnesium (Mg^{2+})	Major	Formation of bones Protein synthesis Muscle contraction Nerve transmission Immune system health
Iron(II) (Fe^{2+})	Trace	Forms part of haemoglobin found in red blood cells responsible for oxygen transport in the body Energy metabolism
Iron(III) (Fe^{3+})	Trace	No physiological function
Zinc (Zn^{2+})	Trace	Forms part of enzymes Cell division and growth Needed for the senses smell and taste Immune system health

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2.3 Materials

All chemical compounds, mineral compounds used were of analytical grade or better and obtained from reputable chemical suppliers. Drugs and vitamins were standards purchased from Sigma Aldrich except for valsartan which was a kind gift from Novartis, Switzerland.

A synthetic carbohydrate derived fulvic acid (CHD-FA) was donated by Fulhold (Stellenbosch, RSA). A batch of a 5% solution was obtained and stored at 4˚C until required.

Humic acid manufactured by Sherston was obtained as a dry formulation from CalPharm, (Centurion, RSA).

2.3.1 Preparation of solutions

Krebs Ringer Phosphate Buffer (pH 7.4)

The following amounts were dissolved and made up to a final volume of 1 L using deionised water:

D-Glucose	1.80 g
Magnesium chloride	0.99 g
Sodium chloride	7.00 g
Potassium chloride	0.34 g
Sodium phosphate dibasic	0.25 g
Sodium phosphate monobasic	0.20 g
Sodium bicarbonate	1.26 g

Control, HA and CHD-FA solutions

A control solution as well as two different solutions containing either HA or CHD-FA were prepared as follows:

Control solution

The control solution consisted of KRB only.

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HA solution

A 5% humic acid solution was prepared by dissolving 5 g humic acid preparation (CalPharm, S.A.) in 100 ml deionised water. The solution was placed in an ultrasonic bath to ensure complete dissolution of the humic acid.

The HA test solution was prepared by mixing one part 5% humic acid solution with 3 parts KRB (1:3).

CHD-FA solution

CHD-FA is a specially prepared form of fulvic acid, derived from a carbohydrate source. CHD-FA is thus clear of heavy metals, pesticides and other contaminants unlike other forms of fulvic acids. A 5% CHD-FA solution was kindly donated for the study (Fulhold, S.A.). The CHD-FA solution was prepared in a similar fashion to the HA test solution by diluting the 5% CHD-FA solution with KRB (1:3).

Preparation of drug, vitamin and mineral solutions

Each drug, vitamin or mineral under study was dissolved in either control solution (KRB), 1.25% CHD-FA in KRB or 1.25% Humic acid in KRB to obtain a final concentration of 1.3 mM (estimated initial concentration in the GIT following oral administration).

2.4 Methods

2.4.1 Everted mouse gut model

The general methodology followed for assessment of absorption of all the drugs, vitamins and minerals, using the everted mouse gut model, were exactly the same. Although a common GIT absorption method was used throughout the study, each of the test compounds from the different experiments were quantified using separately optimised analytical liquid chromatography mass spectrometry (LC-MS/MS) methods. The absorption of each test compound was carried out using GIT segments from four different regions of the GIT and performed in triplicate using separate animals for each repeat.

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The general method for the use of the everted gut model was as follows: Immediately following euthanasia the entire GIT was dissected from the mouse and separated from the omentum. The straightened GIT was then flushed of any remaining solid material using 1 ml of ice cold saline solution followed by KRB. The gut was then divided into four main regions (duodenum, jejunum, ileum and colon) and then further cut into 6 cm segments per region. Individual segments were everted by firstly gently pushing a wetted thin glass rod (diameter 0.2 cm) through the length of the segment. One end of the segment was tied at one end to the rod using a thin silk suture and the free end was carefully pushed back over the rod in the opposite direction. Once everted, segments were again rinsed in KRB solution to remove any remaining solid debris. The thin mucosal layer produced by the goblet cells of the GIT was left to coat the surface of the segments.

Each 6 cm length of everted intestinal segment (duodenum, jejunum, ileum and colon) was tied off at one end using #5 heavy braided suture silk approximately 5 mm from one end. Using a micropipette, 200 µl of KRB was used to fill the segment and the remaining open end tied off (5 mm from the end) creating a sack of ±5 cm in length. The segments were approximately three quarters full, allowing space for an air bubble, after filling with the 200 µl of KRB. Care was taken when tying off the segments as tying too tightly could easily severe the soft intestinal tissue, whereas not tying tight enough resulted in the sutures coming loose during the experiment. Thus both ends were carefully tied off twice to ensure the sack remained closed throughout the duration of the experiment. Continuous moistening of segments was carried out by frequent submersion of the segments in the KRB solution in order to avoid drying out of the intestinal tissue during the process.

Individual GIT segment sacks underwent incubation in one of the three test solutions containing the test drug, vitamin or mineral at a fixed concentration of 1.3 mM in KRB, 1.25% CHD-FA in KRB or 1.25% HA in KRB. The presence of the air bubble in the sack, allowed for the segments to hang vertically while submerged in the respective solutions.

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Incubation tubes were placed in a shaking water bath, kept at a constant temperature (37°C), for 90 min while gently shaken (10 – 12 rpm) in an attempt to mimic the normal gut movement in mice.

Following incubation, sacks were collected, briefly rinsed in fresh KRB to wash off the excess test solution and blotted dry. A volume of 100 µl of the sack contents was collected using a micropipette by piercing the upper end of the sack while being suspended vertically.

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Chapter 3

3. Sample analysis

3.1 Sample analysis of test compounds

The test drug, vitamin or mineral which had crossed the intestinal tissue would have followed the normal luminal to serosal direction for both diffusing and actively transported compounds and would therefore be found inside the GIT segment sacks. Quantitation of the accumulated compound in the sacks that took place during the incubation period was performed. The absorption of the test compounds in the presence of HA or CHD-FA was compared to the control where the carrier solution was KRB with the same concentration of test compound. The absorption in each major region of the GIT (duodenum, jejunum, ileum and colon) was also assessed and compared to each other.

Quantitation of the drugs and vitamins were carried out using LC-MS/MS and quantitation of the minerals was carried out using inductively coupled plasma mass spectrometry (ICP-MS).

3.1.1 Sample preparation

Due to the low volume of samples collected from the intestinal sacks (approximately 100-200 µl), sample clean-up techniques such as solid phase extraction (SPE) or filtering were not used.

3.1.1.1 Sample preparation for drugs and vitamins

A solvent based protein precipitation procedure was carried out in order to remove protein in the collected samples. Protein precipitation is a commonly used sample preparation technique to remove most proteins from biological samples prior to further analysis. The addition of double the volume of organic solvent to the sample results in the precipitation of the proteins, which is removed by centrifugation at 10 000 *g* or higher. Two parts organic solvent (methanol or acetonitrile, depending on the solubility of the test compound) was added to one part sample and allowed to stand at room temperature for 10 min. The mixture

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was centrifuged at 10 000 *g* for 10 min, after which the supernatant was collected and stored at -80°C until analysed.

3.1.1.2 Sample preparation for minerals

Acid digestion was carried out on the mineral samples. This involves the addition of nitric acid to the samples to digest all the organic material during preparation and to ensure that inorganic analytes, such as minerals, are in ionic form for analysis.

A volume of 200 µl of 15% nitric acid (HNO_3) was added to 100.00 µl of sample and vortex mixed thoroughly. Samples were stored overnight at 40°C in sealed Eppendorf tubes. Each sample was diluted by adding exactly 1.70 ml of water.

3.1.2 Sample Analysis of drugs, vitamins and minerals

Quantitation of the drugs and vitamins was carried out using LC-MS/MS and quantitation of minerals was carried out using ICP-MS.

3.1.2.1 Sample Analysis of drugs and vitamins using LC-MS/MS

Quantitation of drugs and vitamins was achieved using LC-MS/MS. LC-MS/MS analysis of the samples was carried out using an Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with an well-plate autosampler, mobile phase degasser, binary pump and column thermostat unit. Void volume of the system was approximately 1.2 min. The detector used was an AB Sciex 4000 QTrap triple-quadrupole mass spectrometer (Applied Biosystems MSD/SCIEX, Concord, Canada) equipped with a TurboV electrospray ionization (ESI) source operated in either positive or negative mode. Analyst 1.5.2. software was used for system control and data analysis.

Separate LC-MS/MS methods were developed for each drug and vitamin under study. All methods made use of an internal standard (IS) and underwent validation procedures.

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Optimized compound-specific mass spectrometry parameters were obtained for all test compounds and internal standards using manual tuning with constant flow infusion of analyte standards at approximately 50 µg/ml concentrations.

3.1.2.1 LC-MS/MS validation

The following validation parameters were assessed for each method:
Selectivity, matrix effect, linearity, accuracy, precision, limit of quantitation (LOQ), limit of detection (LOD) and percentage recovery (% RE) as summarized by Peters in 2007 (Peters *et.al*, 2007).

Selectivity

Selectivity refers to the extent to which a method can determine a particular analyte in mixtures or matrices without interferences from other components (Vessman *et al.*, 2001). The use of retention time, precursor mass and multiple mass transitions during fragmentation for each analyte and internal standard (IS) during mass spectrometry analysis when using a triple quadrupole mass spectrometer allows for a high degree of specificity during quantitation, which increases the overall confidence in results.

Matrix effects

LC-MS/MS analysis is known to be prone to ion suppression or enhancement by co-eluting compounds, especially when using ESI and complex biological samples. A qualitative determination of matrix effect was carried out for each analyte and IS. Extracted blank control sample matrix was injected through the column while a dilute solution of analyte was simultaneously and continuously infused into the eluent flow through a "t" fitting coupled post-column but before the mass spectrometer ionisation source.
The presence of a matrix effect, measured as a change in signal of the infused analyte as either a decreased or enhanced signal, was confirmed not to coincide with the retention time of the analyte or the IS using the chromatographic conditions for the quantitation of the actual samples.

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Linearity

The relationship between the analyte concentration and the resultant detector response was investigated using a set of different analyte concentrations and establishing calibration curves. Calibration curves were constructed using 6 different concentrations of standards of the analyte. Each concentration level was spiked with a constant concentration of the internal standard and injected a total of 6 times. A linear regression model was used for all methods and r^2 values were all >0.99.

Accuracy and Precision

Intra-day accuracy and precision was assessed using six concentration levels of the analyte. This assessment was carried out in triplicate on single days (*n=3*).
Inter-day accuracy and precision was assessed over three different days where different batches of mobile phases were used. This assessment was carried out using six repeats (*n=6*).
The results for precision and accuracy were found to be within the acceptance criteria (precision within 15% CV and accuracy ± 15% variability).

LOD and LOQ

LOD and LOQ were assessed for each method and the signal to noise ratio (S/N) were taken as > 3 and > 10 respectively at the analyte retention time.

Recovery

Percentage recovery was assessed by comparing the peak area obtained from a sample spiked before the extraction procedure and the peak area obtained from a sample spiked with the same amount of analyte after extraction. Determined in triplicate.
"Pre-spiked" samples were prepared by spiking blank sample matrix with the analyte at three different concentration levels before the protein precipitation step of the sample preparation. "Post-spiked" samples were prepared using the same spiking concentrations, however the spiking with the analyte took place after the completion of all the sample preparation steps.

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Percentage recovery was then assessed by comparing the peak area of the pre and post-spiked samples using the following formula:

$$\% \boldsymbol{RE} = \frac{\textbf{Peak area of pre-spiked sample}}{\textbf{Peak area of post-spiked sample}} \textbf{x } 100$$

% Recovery was found to be > 90% for all analytes which falls within the acceptable methodology limits.

3.1.3 Analysis of minerals using ICP-MS

Quantitation of minerals was achieved using inductively coupled plasma mass spectrometry (ICP-MS). ICP-MS analysis of the samples was outsourced and carried out by trained analysts at V & M Analytical Toxicology Laboratory Services (George, Western Cape, South Africa) using an Agilent 7700x ICP-MS run under standard analytical operating parameters using MassHunter software. Analyte concentrations were determined from calibration curves of the analytes made up in nitric acid solutions and were not from matrix matched samples. The quantitative results for the samples where humic and fulvic acid were present during the absorption experiment were compared to the control samples that were collected and treated in the same way. Results were given as ppm of the test minerals for each sample and were then compared to the control samples.

3.2 Statistical analysis of samples

Statistical analysis of the samples was carried out using GraphPad Prism, version 5.00. GraphPad Software, La Jolla California USA.

The results obtained in the presence of CHD-FA or HA were compared to the control. Due to the limited sample size, a non-parametric test was chosen to assess the data generated during sample analysis. A two-tailed Mann-Whitney test was carried using a confidence interval of 0.95.

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Chapter 4

4. Drugs

Medicinal drugs have provided patients with enormous benefits in the treatment and prevention of a variety of illnesses. Furthermore, many people rely on chronic medicine, which if not used on a daily basis, would result in severe symptoms or even early death. Over time, drugs with similar activity, or for treatment of the same illness, have been grouped together into drug classes.

Compounds belonging to different drug classes can have widely differing physical and chemical properties, such as molecular mass, molecular shape, pKa, LogP, LogD and elemental composition. These physicochemical properties largely govern how and the extent to which a drug is absorbed in the intestines after oral administration.

Therapeutic index

Each drug has a specific therapeutic index or therapeutic ratio. The therapeutic index (TI) is calculated by calculating the ratio of TD_{50} (the dose at which half the population experience toxic side effects) to ED_{50} (the minimum dose at which half the population respond therapeutically). Drugs considered safe generally have therapeutic indices of greater than 1000 but there are many effective drugs with a therapeutic index of <5. Drugs showing a small therapeutic index show very little difference between the dose which is therapeutically effective and the dose that is toxic. Small changes in the administered dose or the amount reaching the systemic circulation can lead to significant alteration in the effects of the drug and may result in sub-therapeutic or toxic effects. Drugs with narrow therapeutic indices must therefore be carefully dosed and closely monitored.

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4.1 Diclofenac

Background

Diclofenac is a non-steroidal anti-inflammatory (NSAID) of the phenylacetate class, commonly used in the treatment of pain and inflammation. Diclofenac is indicated for treatment of both acute and chronic disorders. Acute conditions include musculoskeletal and postoperative pain, headaches, migraines and dysmenorrhoea. Chronic illnesses include gout attacks and inflammatory diseases such as osteoarthritis, rheumatoid arthritis and ankylosing spondylitis (Kantor, 1986). Diclofenac can be administered orally, in the form of diclofenac sodium or diclofenac potassium, or topically, intramuscular, intravenous or as a suppository. Diclofenac can be found as an active in a variety of medications and can be easily obtained over the counter without prescription. Diclofenac thus falls into the category of non-prescribed conventional medications.

Mechanism of action

Diclofenac inhibits prostaglandin synthesis via non-specific inhibition of the cyclooxygenase enzymes (both COX-1 and COX-2). Prostaglandins are involved in sensitizing pain receptors thus inhibition of their effects results in analgesia (Cashman, 1996).

Symptoms of toxicity

Gastrointestinal effects, hypersensitivity, blood coagulation problems, oedema, urticaria, and asthma. Higher than recommended doses of diclofenac can result in permanent hepatotoxic and nephrotoxic damage.

Properties

IUPAC name: 2-(2-(2,6-dichlorophenylamino)phenyl)acetic acid

Molecular mass: 296.15 g/mol

Molecular formula: $C_{14}H_{11}Cl_2NO_2$

LogP: 4.06

LogD: 0.95 (pH 7.4)

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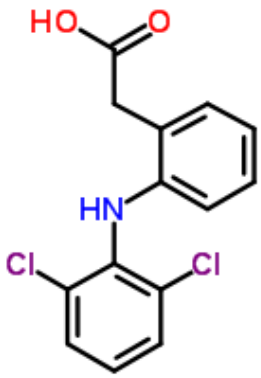

Figure 8. Molecular structure of diclofenac (ChemSpider, 2014)

LC-MS/MS analysis

The conditions and parameters for the LC-MS/MS method for the analysis of diclofenac were as follows:

Table 6. HPLC conditions for diclofenac

Mobile phase	A: 0.1% formic acid, adjusted to pH 3 using dilute ammonium hydroxide (NH_4OH) B: Methanol
Analytical column	Apollo C18 (150 mm x 4. 6mm, 5 µm) (Alltech)
Flow rate	1 ml/min
Injection volume	10 µl
Column temperature	45°C
Gradient	0 min- 0.5 min; 70% A 0.5 min- 1.5 min; 15% A 1.5 min- 3.5 min; 15% A 3.5 min- 4.5 min; 70% A 4.5 min- 7 min; 70% A

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Table 7. Compound specific mass spectrometer conditions for diclofenac

	Diclofenac	Ketoprofen (IS)
Ionisation Mode	Negative	Negative
Precursor ion	294.00	253.00
Product ions	250.00 178.00	209.00 253.00
Declustering Potential	-52 -52	-70 -70
Collision energy	-18 -35	-19 -19

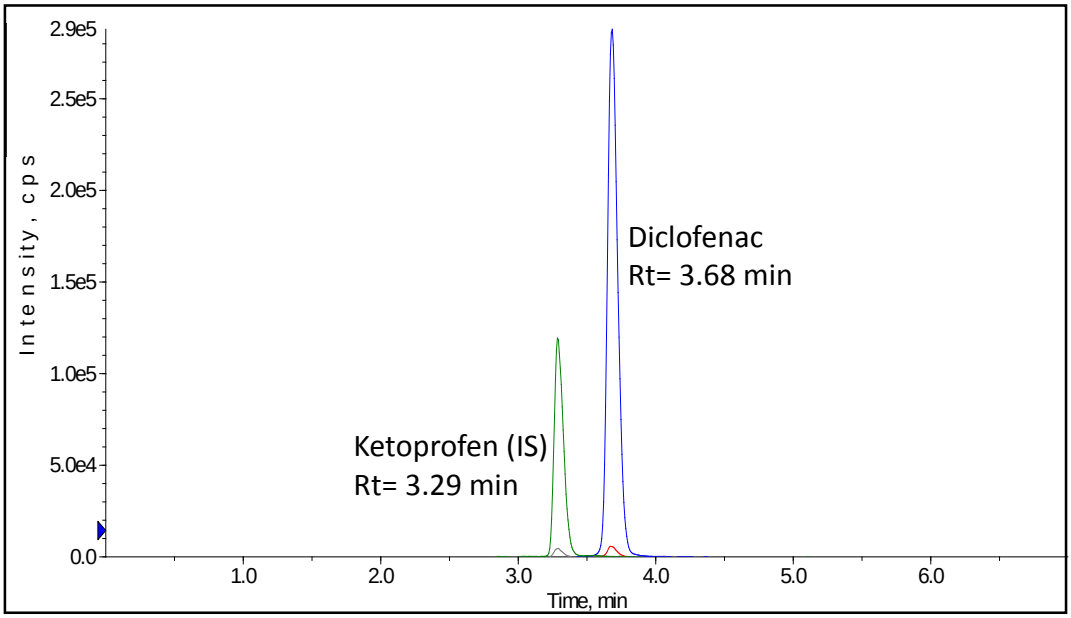

Figure 9. Reversed phased chromatography of diclofenac, retention time (Rt) of 3.68 min, using ketoprofen as the internal standard (IS) (Rt=3.29 min). The total run time was 7 mins, including column re-equilibration. The chromatographic analysis was carried out in order to quantitate the concentration of diclofenac present inside the intestinal sacks after absorption for 90 minutes.

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Method validation

Table 8. Calibration data proving linearity for diclofenac

Slope	Intercept	Correlation coefficient (r^2)
3.57×10^{-3}	1.43×10^{-2}	0.9996

Table 9. Intra- and inter-day precision and accuracy for diclofenac

	Intra-day (*n=3*)			Inter-day (*n=6*)		
Expected concentration (ng/ml)	**Mean concentration (ng/ml) ± SD**	**% CV**	**% Accuracy**	**Mean concentration (ng/ml)**	**% CV**	**% Accuracy**
50	47.5 ± 1.1	2.4	95.1	46.3 ± 0.9	1.8	92.6
100	95.3 ± 3.5	3.7	95.3	96.9 ± 5.0	5.2	96.9
250	250.5 ± 13.3	5.3	100.2	252.9 ± 10.7	4.2	101.2
500	511.1 ± 3.19	0.6	102.2	506.9 ± 7.3	1.4	101.4
750	751.2 ± 13.0	1.7	100.2	750.4 ± 9.3	1.2	100.1
1000	993.3 ± 9.6	0.97	99.3	994.7 ± 8.5	0.9	99.5

Intra- and inter-day precision varied from 0.6% to 5.3% CV and 0.9% to 5.2% CV, respectively.

Intra- and inter-day accuracy ranged from 95.1% to 102.2% and 92.6% to 100.4%, respectively.

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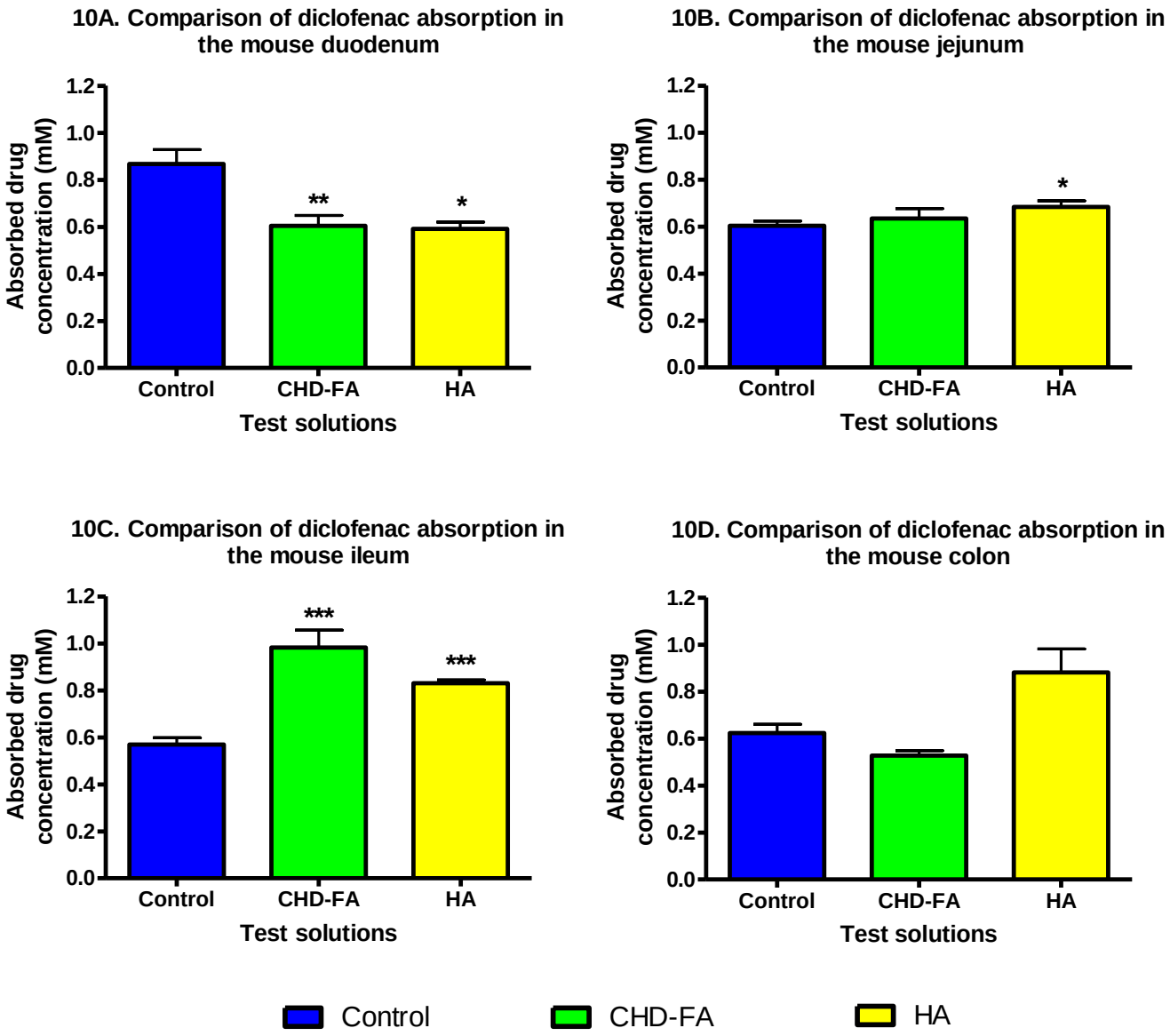

Figure 10 A-D: *Comparison of diclofenac absorption in different regions of the mouse intestine (10A: duodenum, 10B: jejunum, 10C: ileum and 10D: colon). The absorption of the drug alone (control) was compared to the absorption of the drug in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in the duodenum, jejunum and ileum. (n = 3).*

** = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$*

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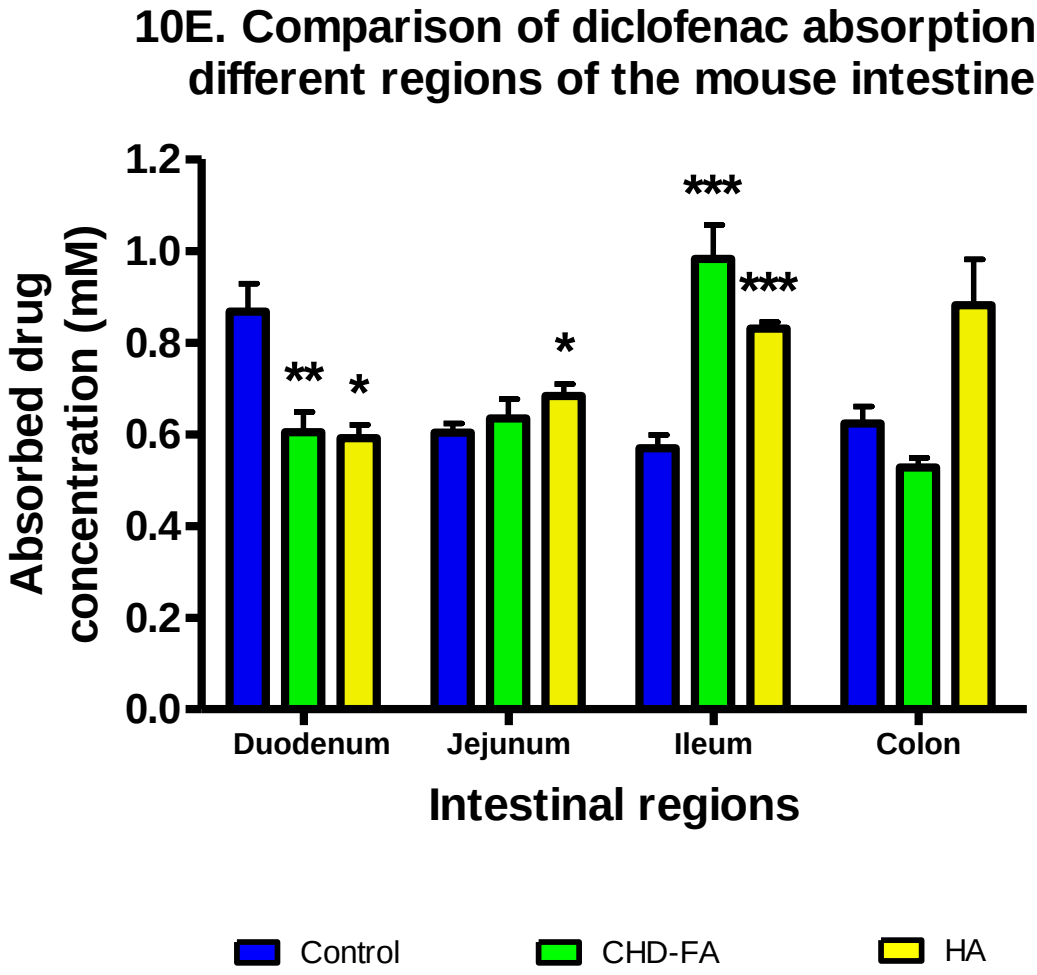

Figure 10E: Combination of graphs 10A-D illustrating difference of diclofenac absorption from different regions of the mouse intestine (duodenum, jejunum, ileum and colon). The absorption of the drug alone (control) was compared to the absorption of the drug in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in the duodenum, jejunum and ileum. (n=3).

** = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$*

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Table 10. Comparison of diclofenac absorption in different regions of the intestine

Intestinal Region	CHD-FA	Significant	HA	Significant
Duodenum	↓ 30%	Yes	↓ 32%	Yes
Jejunum	↑ 5%	No	↑ 13%	Yes
Ileum	↑ 72%	Yes	↑ 46%	Yes
Colon	↓ 15%	No	↑ 41%	Yes

Table 9 shows the comparison of diclofenac absorption in different regions of the mouse intestine in the presence of either CHD-FA or HA. The increase or decrease in absorption is reported as a percentage of the control.
It is evident that the presence of both the HS resulted in changes in the total amount of diclofenac absorbed in all the regions of the intestine when compared to the control. However, neither of the HS elicited a consistent trend in diclofenac absorption in all the regions of the intestine.

Absorption of diclofenac in the presence of CHD-FA was seen to increase in the jejunum and ileum and decrease in the duodenum and colon in comparison to the control. Increases ranged from 5% in the jejunum to 72% in the ileum, while decreases ranged from 15% in the colon to 30% in the duodenum. Consistent significant changes in diclofenac absorption were only seen in the duodenum and ileum.

Absorption of diclofenac in the presence of HA was seen to increase in the jejunum, ileum and colon, and decrease in the duodenum in comparison with the control. Increases ranged from 13% to 46%, with the largest increase being seen in the ileum. Significant changes in diclofenac uptake were seen in all segments except the colon.

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4.2 Penicillin V

Background

Penicillin V, also known as phenoxymethylpenicillin, is a ß-lactam antibiotic used in the treatment of bacterial infections, such as tonsillitis, pharyngitis and rheumatic fever, caused mostly by gram positive bacteria.(Manyemba & Mayosi, 2002). Penicillin V is used for the acute treatment of bacterial infections as there is a risk of developing resistance to the drug when used chronically. Penicillin V can be administered orally as it is more resistant to the acidic conditions of the stomach than other penicillin derivatives. Penicillin V can only be obtained with a prescription and falls into the category of scheduled conventional medication (S4) requiring a prescription.

Mechanism of action

ß-Lactam antibiotics inhibit the formation of peptidoglycan cross-linking in the bacterial cell wall, leading to incomplete formation of the cell wall and cytolysis and death of the bacterial cell due to osmotic pressure (Tenover, 2006).

Symptoms of toxicity

Most common side effect is gastric disturbances and hypersensitivity. Higher than normal doses of penicillin V over a few days can result in confusion, severe skin rash and renal impairment.

Properties

IUPAC name: (2S,5R,6R)-3,3-Dimethyl-7-oxo-6-[(phenoxyacetyl)amino]-4-thia-1-azabicyclo [3.2.0]heptane-2-carboxylic acid

Molecular mass: 350.39 g/mol

Molecular formula: $C_{16}H_{18}N_2O_5S$

LogP: 1.88

LogD: -0.85 (pH 7.4)

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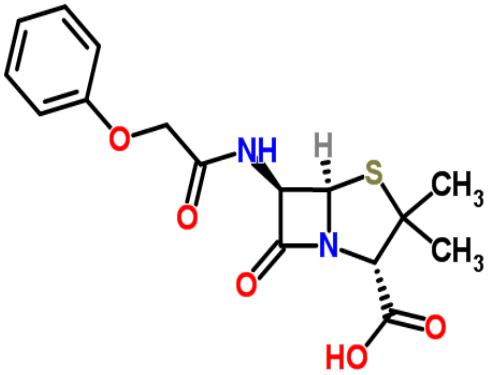

Figure 11, Molecular structure of penicillin V (ChemSpider, 2014)

LC-MS/MS analysis

The conditions and parameters for the LC-MS/MS method for the analysis of penicillin V was as follows:

Table 11. HPLC conditions for penicillin V

Mobile phase	A: 0.1% formic acid (FA), adjusted to pH 3.5 using dilute ammonium hydroxide (NH_4OH) B: Acetonitrile
Analytical column	Apollo C18 (150 mm x 4.6 mm, 5 µm) (Alltech)
Flow rate	1 ml/min
Injection volume	10 µl
Column temperature	35°C
Gradient	0 min- 1 min; 57% A 1 min- 2.2 min; 37% A 2.2 min- 2.6 min; 37% A 2.6 min- 3.2 min; 20% A 3.2 min- 3.5 min; 20% A 3.5 min- 3.8 min; 57% A 3.8 min- 6 min; 57% A

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Table 12. Compound specific mass spectrometer conditions for penicillin V

	Penicillin V	Penicillin G (IS)
Ionisation Mode	Positive	Positive
Precursor ion	351.00	335.00
Product ion	160.00 114.00	289.00 176.00
Declustering Potential	35 35	66 66
Collision energy	15 35	35 35

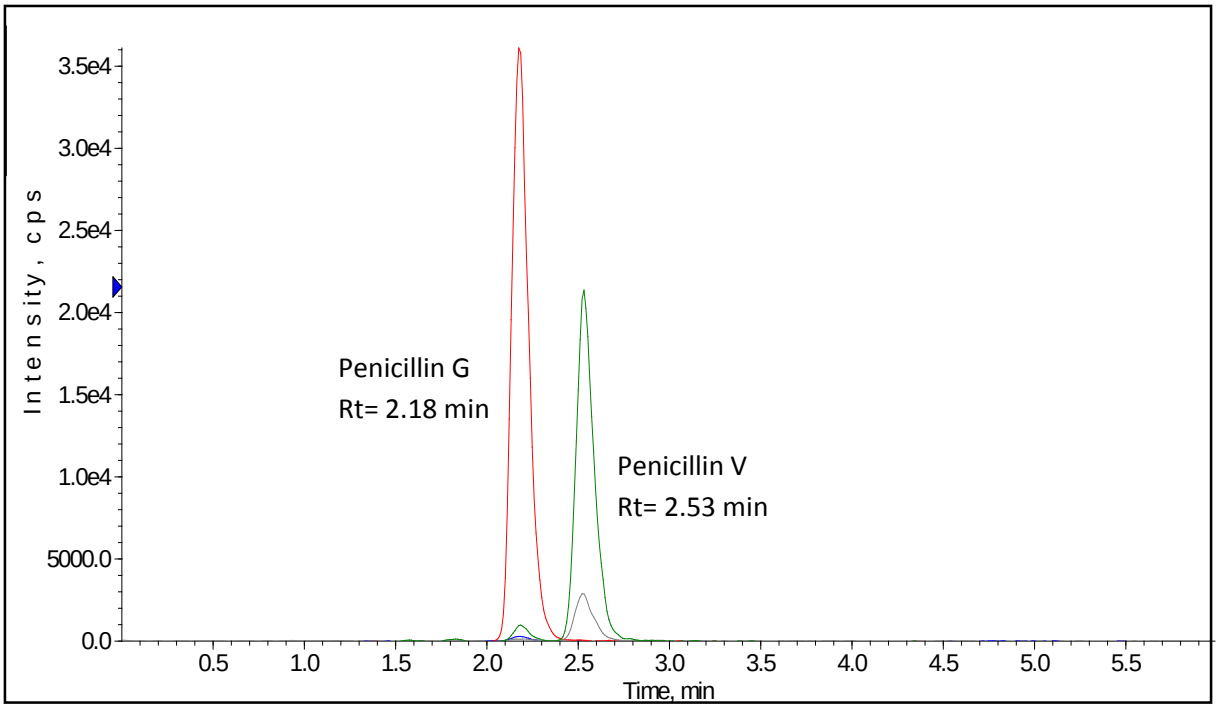

Figure 12. Reversed phased chromatography of penicillin V, retention time (Rt) of 2.53 min, using penicillin G as the internal standard (IS) (Rt= 2.18 min). The total run time was 6 mins, including column re-equilibration. The chromatographic analysis was carried out in order to quantitate the penicillin V present in the intestinal sacks after drug absorption for 90 min.

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Method validation

Table 13. Calibration data proving linearity for penicillin V

Slope	Intercept	Correlation coefficient (r^2)
8.44×10^{-4}	0	0.9998

Table 14. Intra- and inter-day precision and accuracy for penicillin V

	Intra-day (n=3)			Inter-day (n=6)		
Expected concentration (ng/ml)	Mean concentration (ng/ml) ± SD	% CV	% Accuracy	Mean concentration (ng/ml)	% CV	% Accuracy
25	25.8 ± 1.1	4.0	103.2	25.4 ± 0.9	3.4	101.5
50	51.6 ± 0.7	4.1	103.2	51.2 ± 0.6	1.3	102.4
125	123.1 ± 0.8	1.4	98.5	123.9 ± 1.8	1.5	99.1
250	251.5 ± 4.2	0.8	100.6	252.6 ± 5.4	2.1	101.0
500	514.5 ± 7.3	7.3	102.9	500.3 ± 14.7	2.9	100.1
1000	983.5 ± 0.7	0.7	98.3	992.9 ± 14.9	1.5	99.3

Intra- and inter-day precision varied from 0.7 to 7.3% CV and 1.3 to 3.4% CV, respectively.

Intra- and inter-day accuracy ranged from 98.5 to 103.2% and 99.1 to 102.4%, respectively

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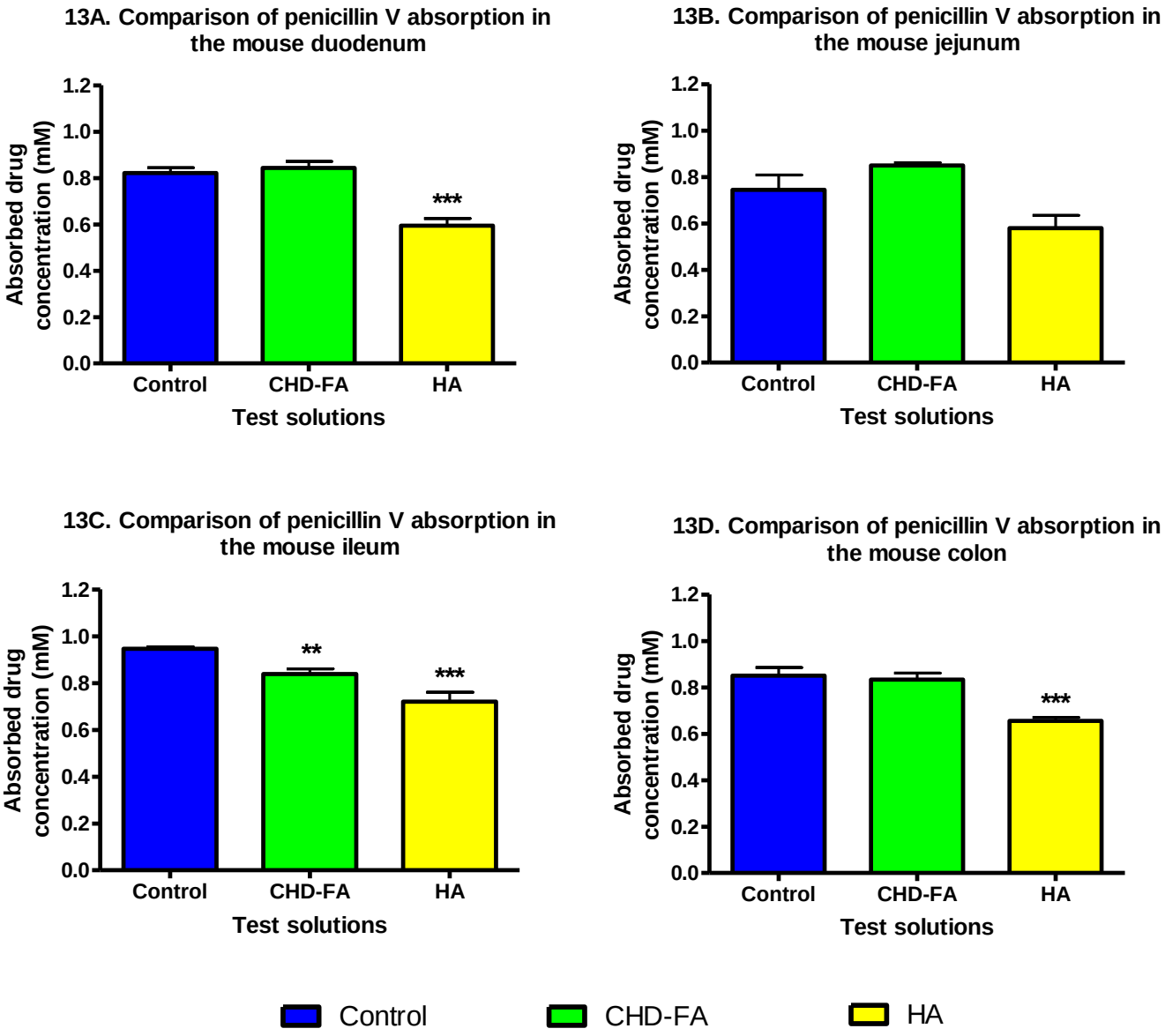

Figure 13 A - D: Comparison of penicillin V absorption in different regions of the mouse intestine (13A: duodenum, 13B: jejunum, 13C: ileum and 13D: colon). The absorption of the drug alone (control) was compared to the absorption of the drug in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in the duodenum, jejunum and ileum, (n = 3).

** = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$*

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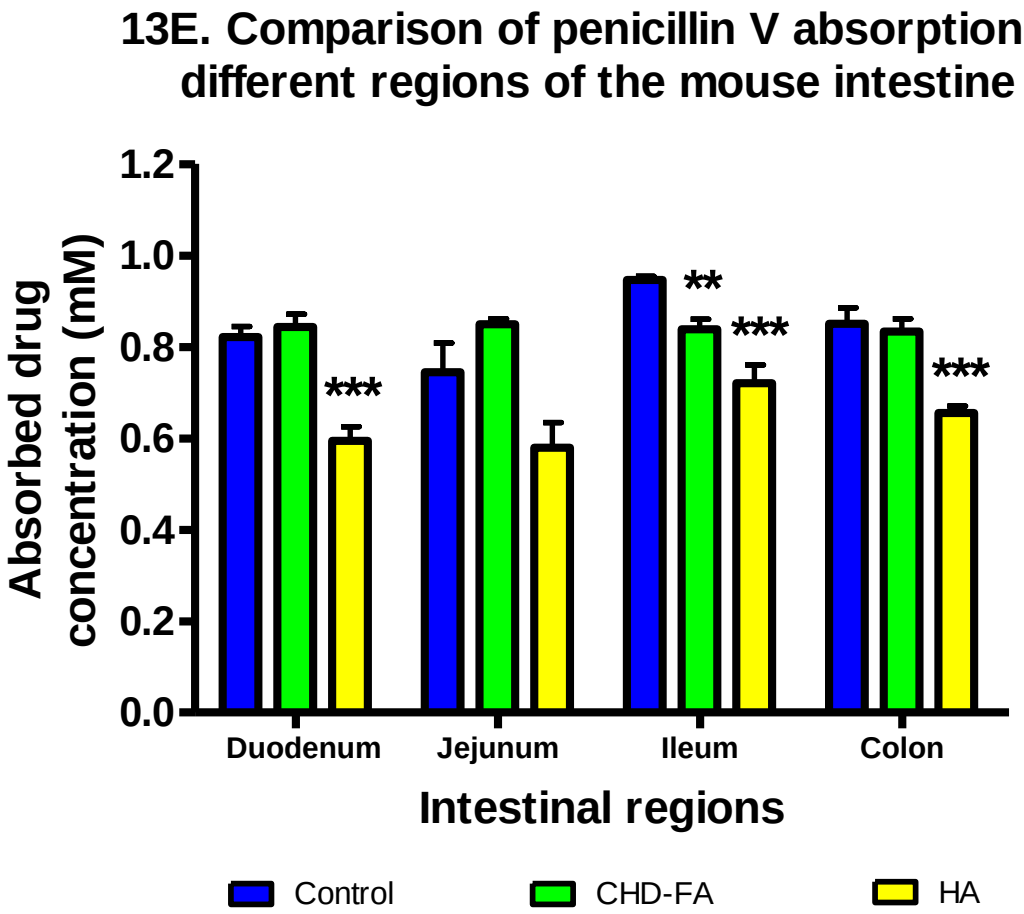

Figure 13E: Combination of graphs 13 A-D showing comparison of penicillin V absorption in different regions of the mouse intestine (duodenum, jejunum, ileum and colon). The absorption of the drug alone (control) was compared to the absorption of the drug in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in the duodenum, ileum and colon, (n = 3).

** = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$*

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Table 15. Comparison of penicillin V absorption in different sites of the intestine

Intestinal region	CHD-FA	Significant	HA	Significant
Duodenum	↑ 3%	No	↓ 28%	Yes
Jejunum	↑ 14%	No	↓ 22%	Yes
Ileum	↓ 11%	Yes	↓ 24%	Yes
Colon	↓ 2%	No	↓ 23%	Yes

Table 14 shows the comparison of penicillin V absorption in different regions of the mouse intestine in the presence of either CHD-FA or HA. The increase or decrease seen in absorption was reported as a percentage of the control. It is evident that the presence of both the HS resulted in changes in the amount of penicillin V absorbed in all regions of the intestine when compared to the control. A small general decreased absorption trend in penicillin V absorption in the presence of HA was observed, while no trend was seen in the presence of CHD-FA.

Absorption of penicillin V in the presence of CHD-FA was seen to have minor effects on absorption in comparison to the control, with the ileum showing the only statistically significant result. The largest increase was seen in the jejunum at 14% and the largest decrease of 11% in the ileum.

Absorption of penicillin V in the presence of HA was seen to decrease in all intestinal regions in comparison to the control. Decreases ranged from 22% to 28% with largest decrease being seen in the duodenum. Significant decreases in all GIT regions were seen for penicillin V uptake in the presence of HA.

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4.3 Warfarin

Background

Warfarin is a commonly employed oral anticoagulant often used in the chronic treatment and prevention of atrial fibrillation, deep vein thrombosis and thromboembolism (Eby *et al.*, 2003). Furthermore, warfarin is routinely administered to patients on a post-operative basis to inhibit clot formation in areas more prone to pooling and slower blood flow.

Warfarin is highly serum protein bound and has a very narrow therapeutic index. It is thus only available through prescription (S4) and patients should be continuously monitored for coagulation times by healthcare professionals. Small fluctuations in warfarin's bioavailability can result in negative effects to the patient as either therapeutic failure or adverse effects.

Mechanism of action

Warfarin inhibits vitamin K recycling thereby reduces carboxylation of clotting factors II, VII, IX and X in the blood, decreasing the potential to completely activate the coagulation cascade that allows blood clotting (Hirsh *et al.*, 2001).

Symptoms of toxicity

Higher than normal doses of warfarin can result in easy bruising, increased bleeding, inability to stop vascular leakage and skin necrosis.

Properties

Molecular mass: 308.33 g/mol

IUPAC name: 4-hydroxy-3-(3-oxo-1-phenylbutyl)chromen-2-one

Molecular formula: $C_{18}H_{16}O_4$ LogP: 3.42

LogD: 0.61 (pH7.4)

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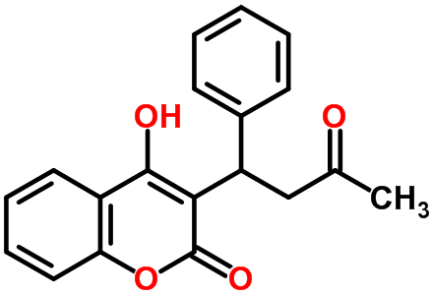

Figure 14. Molecular structure of warfarin (ChemSpider, 2014)

LC-MS/MS analysis

The conditions and parameters for the LC-MS/MS method for the analysis of warfarin were as follows:

Table 16. HPLC conditions for warfarin

Mobile phase	A: 5 mM ammonium formate in water B: Methanol
Analytical column	Eclipse XDB-C18 (150 mm x 4.6 mm, 5 µm) (Agilent)
Flow rate	1 ml/min
Injection volume	10 µl
Column temperature	35°C
Isocratic	0 min - 4 min; 40% A

Table 17. Compound specific mass spectrometer conditions for warfarin

	Warfarin	p-chloro-warfarin (IS)
Ionisation Mode	Negative	Negative
Precursor ion	307	341
Product ions	160 249.7	283 120
Declustering Potential	-60 -60	-60 -60
Collision energy	-28 -34	-34 -30

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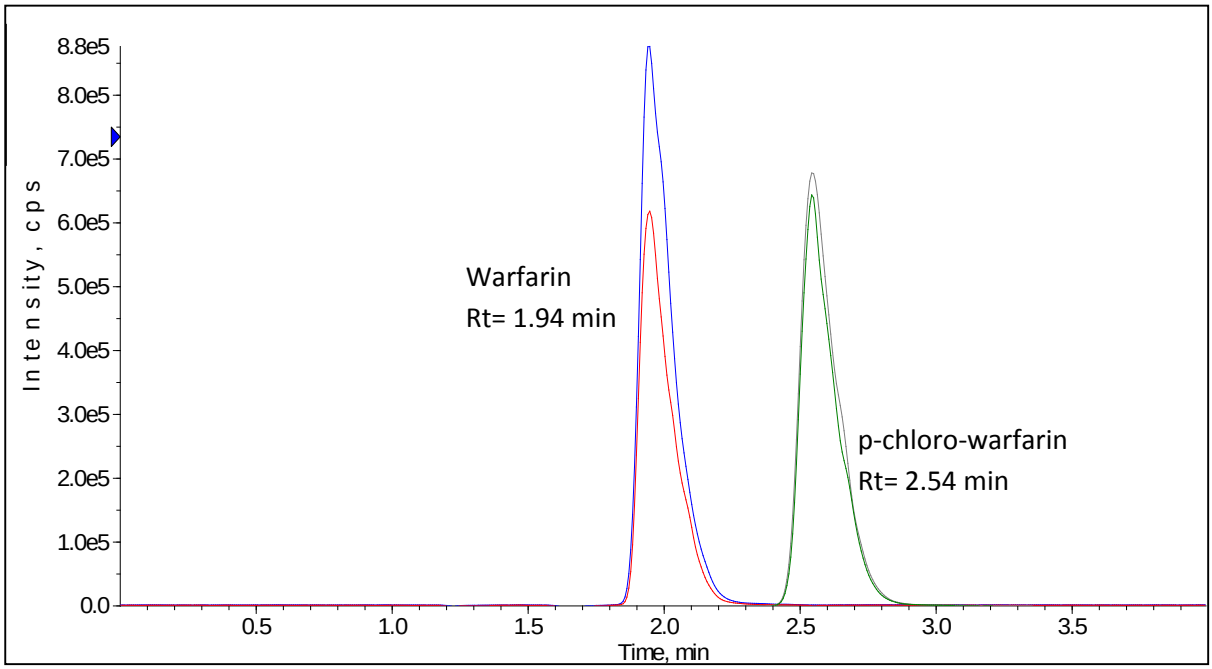

Figure 15. Reversed phased chromatography of Warfarin, retention time (Rt) of 1.94 min, using p-chloro-warfarin as the internal standard (IS) (Rt= 2.54 min). The total run time was 4 mins isocratic. The chromatographic analysis was carried out in order to quantitate the warfarin present in the intestinal sacks after absorption for 90 minutes.

Method validation

Table 18. Calibration data proving linearity

Slope	Intercept	Correlation coefficient (r^2)
5.63 x 10^{-3}	1.22 x10^{-2}	0.9990

Table 19. Intra- and inter-day precision and accuracy

	Intra-day (*n=3*)			Inter-day (*n=6*)		
Expected concentration (ng/ml)	**Mean concentration (ng/ml) ± SD**	**% CV**	**% Accuracy**	**Mean concentration (ng/ml)**	**% CV**	**% Accuracy**
22.5	22.9 ± 0.4	1.6	101.8	21.9 ± 1.2	5.4	97.2
45	45.5 ± 0.2	0.5	101.0	44.9 ± 1.3	2.9	99.8
90	89.7 ± 3.3	3.6	99.7	89.9 ± 3.5	3.9	99.9
115	111.5 ± 2.7	2.4	98.6	114.1 ± 2.5	2.2	101.0
180	179.3 ± 8.4	4.7	99.7	179.9 ± 4.4	2.5	99.9
200	203.6 ± 4.6	2.2	100.3	201.9 ± 4.4	2.2	99.4

Intra- and inter-day precision varied from 0.5% to 4.7% CV and 2.2% to 5.4% CV, respectively.

Intra- and inter-day accuracy ranged from 98.6% to 101.8% and 97.2% to 101.0%, respectively.

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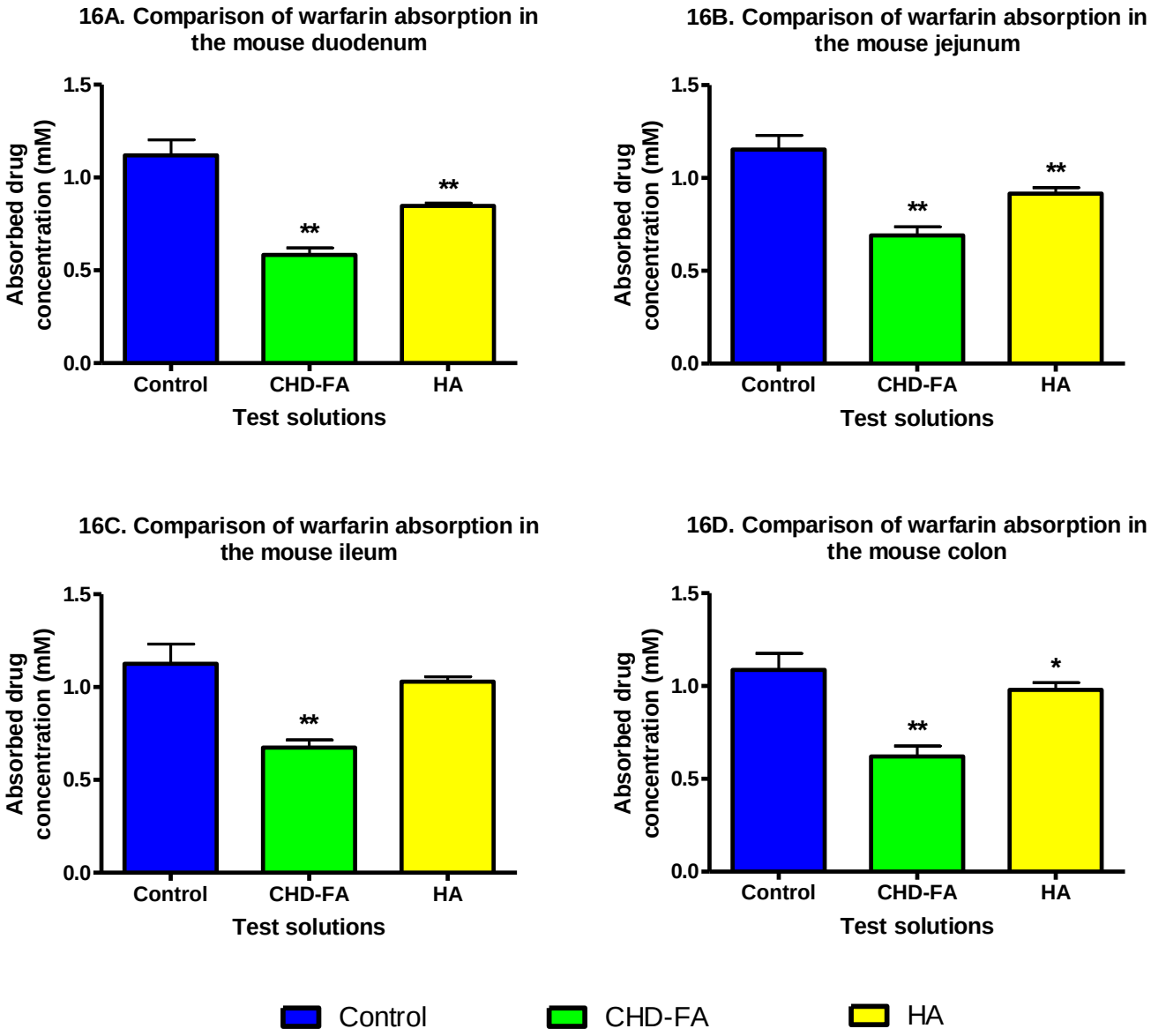

Figure 16 A - D: Comparison of warfarin absorption in different regions of the mouse intestine (16A: duodenum, 16B: jejunum, 16C: ileum and 16D: colon). The absorption of the drug alone (control) was compared to the absorption of the drug in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in the duodenum, jejunum and ileum, (n = 3).

** = p < 0.05 ** = p < 0.01 *** = p < 0. 001*

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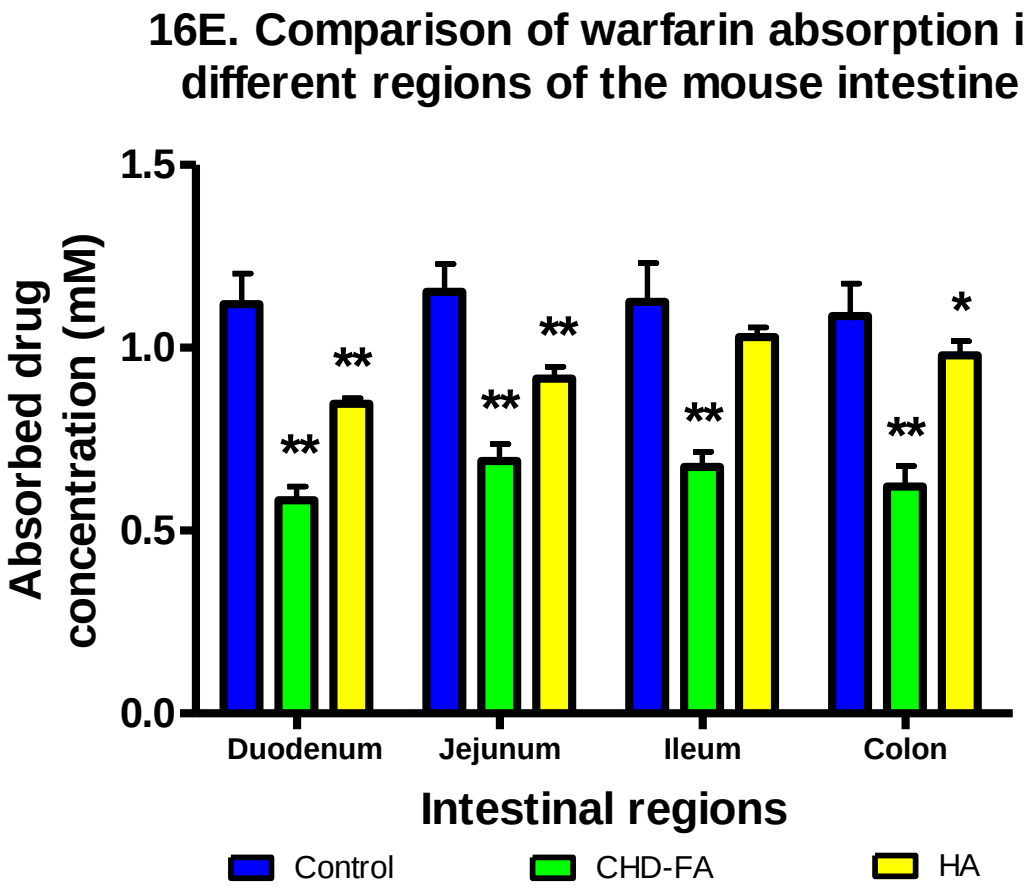

Figure 16E: Combination of graphs 16A-D showing comparison of warfarin absorption in different regions of the mouse intestine (duodenum, jejunum, ileum and colon). The absorption of the drug alone (control) was compared to the absorption of the drug in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in all regions of the intestine, (n = 3).

** = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$*

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Table 20. Comparison of warfarin absorption in different regions of the intestine

Intestinal region	CHD-FA	Significant	HA	Significant
Duodenum	↓ 48%	Yes	↓ 24%	Yes
Jejunum	↓ 40%	Yes	↓ 21%	Yes
Ileum	↓ 40%	Yes	↓ 9%	No
Colon	↓ 43%	Yes	↓ 10%	Yes

Table 19 shows the comparison of warfarin absorption in different regions of the mouse intestine in the presence of either CHD-FA or HA. It is evident that the presence of both the HS resulted in significant decreases in the total amount of warfarin absorbed in all the regions of the intestine when compared to the control. CHD-FA and HA were seen to elicit a general decrease in absorption of warfarin throughout the mouse intestine.

Absorption of warfarin in the presence of CHD-FA was seen to decrease in all the intestinal regions in comparison to the control. Decreases ranged from 40% in the jejunum and ileum to 48% in the duodenum. Significant decreases were seen in all the intestinal regions.

Absorption of warfarin in the presence of HA was also seen to decrease in all the intestinal regions in comparison to the control. Decreases ranged from 9% in the ileum to 24% in the duodenum. Significant changes in warfarin uptake were seen in all regions except the ileum.

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4.4 Rifampicin

Background

Rifampicin is an antibiotic used primarily in the combination treatment of mycobacterial infections such as tuberculosis (TB). Rifampicin, in combination with isoniazid, ethambutol and pyrazinamide make up the first-line treatment for tuberculosis and is used chronically by TB infected patients for a period of approximately six months (World Health Organisation, 2010). Rifampicin is administered orally and is only available with a prescription and thus falls into the category of prescribed conventional medication.

TB and HIV are common co-morbidities in South Africa and a large portion of the population is reliant on chronic medication for the treatment of both disorders. Furthermore, many of these patients also make use of alternative medications such as traditional and over the counter herbal medicines, which may also include humic substances. The risk of potential interactions should thus be further assessed.

Mechanism of action

Rifampicin inhibits RNA synthesis in the mycobacteria by binding to RNA polymerase. Replication of the mycobacteria is thus halted and the infection is restricted (Wehrli, 1983). Due to enzyme mutation rifampicin resistance develops relatively quickly and therefore it is usually given in combination with other antimicrobial drugs.

Symptoms of toxicity

Higher than normal doses of rifampicin can result in flu-like symptoms such as fever, chills, arthralgia and malaise, as well as abdominal disturbances like nausea, vomiting and abdominal cramping.

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Properties

Molecular mass: 822.94 g/mol

IUPAC name: (7S,9E,11S,12R,13S,14R,15R,16R,17S,18S, 19E Pentahydroxy-11-methoxy-3,7,12,14,16,18,22-heptamethyl-26-{(E)-[(4-methyl-1-piperazinyl)imino]methyl}-6,23-dioxo-8,30-dioxa-24-azatetracyclo[23.3.1.14,7.05,28]triaconta-1(28),2,4,9,19,21,25(29),26-octaen-13-yl acetate

Molecular formula: $C_{43}H_{58}N_4O_{12}$

LogP: 1.09

LogD: -1.43 (pH7.4)

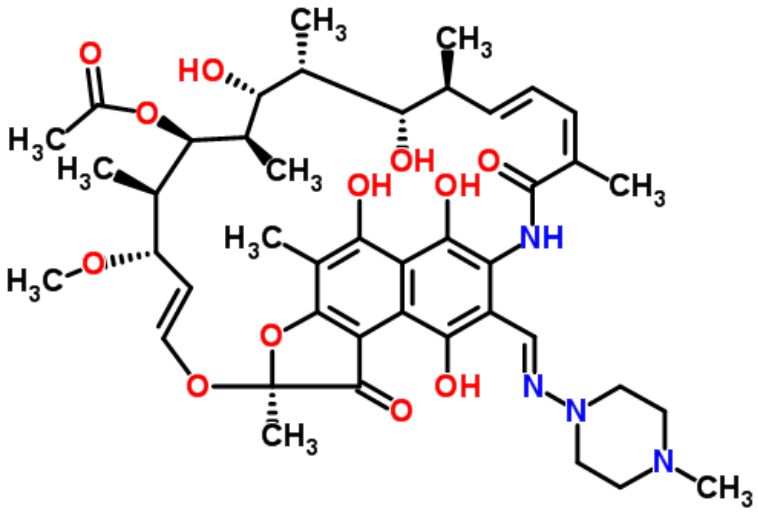

Figure 17. Molecular structure of rifampicin (ChemSpider, 2014)

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LC-MS/MS analysis

The conditions and parameters for the LC-MS/MS method for the analysis of rifampicin were as follows:

Table 21. HPLC conditions for rifampicin

Mobile phase	A: 0.1% formic acid (FA) B: Methanol
Analytical column	Eclipse XDB-C18 (150 mm x 4.6 mm, 5µm) (Agilent)
Flow rate	1 ml/min
Injection volume	10 µl
Column temperature	40°C
Gradient	0 min- 0.75 min; 35% A 0.75 min- 1.5 min; 10% A 1.5 min- 3.0 min; 10% A 3.0 min- 4.0 min; 35% A 4.0 min- 6.5 min; 35% A

Table 22. Compound specific mass spectrometer conditions for rifampicin

	Rifampicin	**Erythromycin (IS)**
Ionisation Mode	Negative	Positive
Precursor ion	821.60	734.00
Product ions	397.00 297.00	576.90 158.00
Declustering Potential	-115 -115	61 61
Collision energy	-60 -69	29 41

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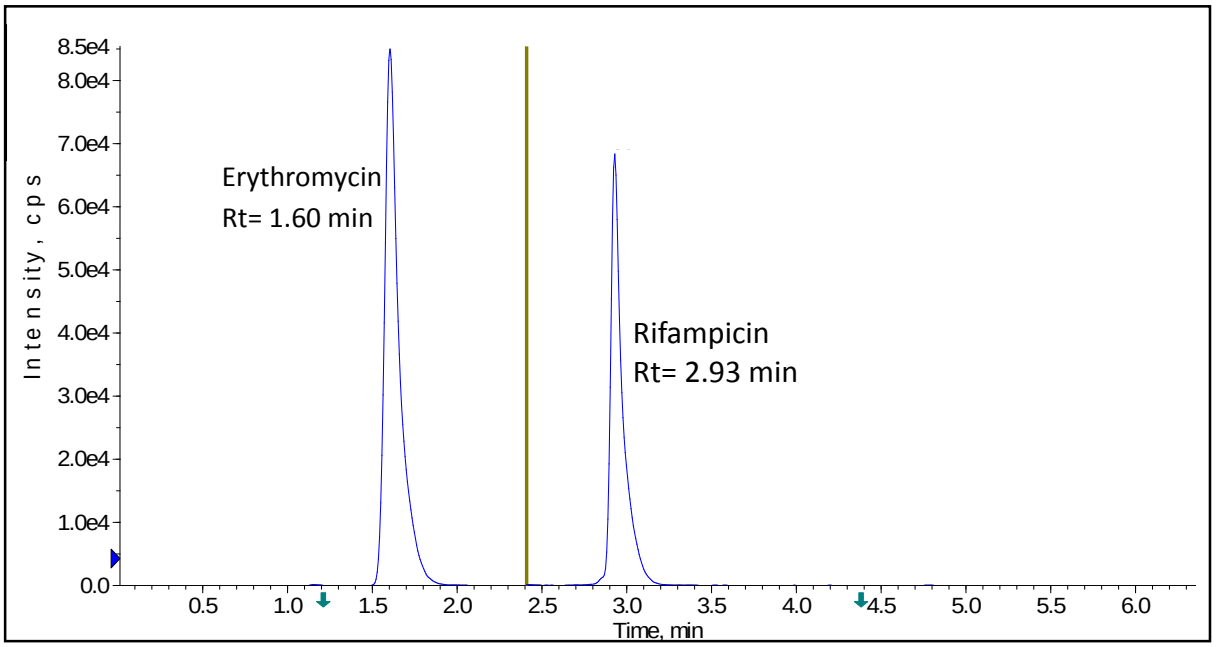

Figure 18. Reversed phased chromatography of rifampicin, retention time (Rt) of 2.93min, using erythromycin as the internal standard (IS) (Rt= 1.60 min). The total run time was 6.5 min, including column re-equilibration. The total run time was split into two periods. Period 1 ran in positive mode from 0 min-2.4 min in order to detect erythromycin and period 2 ran in negative mode from 3.4 min- 6.5 min in order to detect rifampicin. The chromatographic analysis was carried out in order to quantitate rifampicin present in the intestinal sacks after absorption for 90 minutes.

Method validation

Table 23. Calibration data proving linearity for rifampicin

Slope	Intercept	Correlation coefficient (r^2)
3.36×10^{-3}	-4.69×10^{-2}	0.9999

Table 24. Intra- and inter-day precision and accuracy for rifampicin

	Intra-day (n=3)			Inter-day (n=6)		
Expected concentration (ng/ml)	Mean concentration (ng/ml) ± SD	% CV	% Accuracy	Mean concentration (ng/ml) ± SD	% CV	% Accuracy
25	25.9 ± 0.3	1.2	103.7	26.6 ± 4.3	14.1	106.4
55	61.4 ± 2.0	3.2	110.6	59.9 ± 2.0	3.3	107.9
100	105.1 ± 1.9	1.8	105.1	103.4 ± 1.8	1.8	103.4
250	236.0 ± 4.2	1.8	94.4	234.2 ± 4.5	2.0	93.7
1330	1331.0 ± 11.8	0.9	100.1	1330.0 ± 11.2	0.8	100.0
2000	1996.7 ± 7.2	0.4	99.8	2025.4 ± 0.1	0.0	101.3

Intra- and inter-day precision varied from 0.4% to 3.2% CV and 0.0% to 14.1% CV, respectively. Intra- and inter-day accuracy ranged from 94.4% to 105.1% and 93.7% to 106.4%, respectively.

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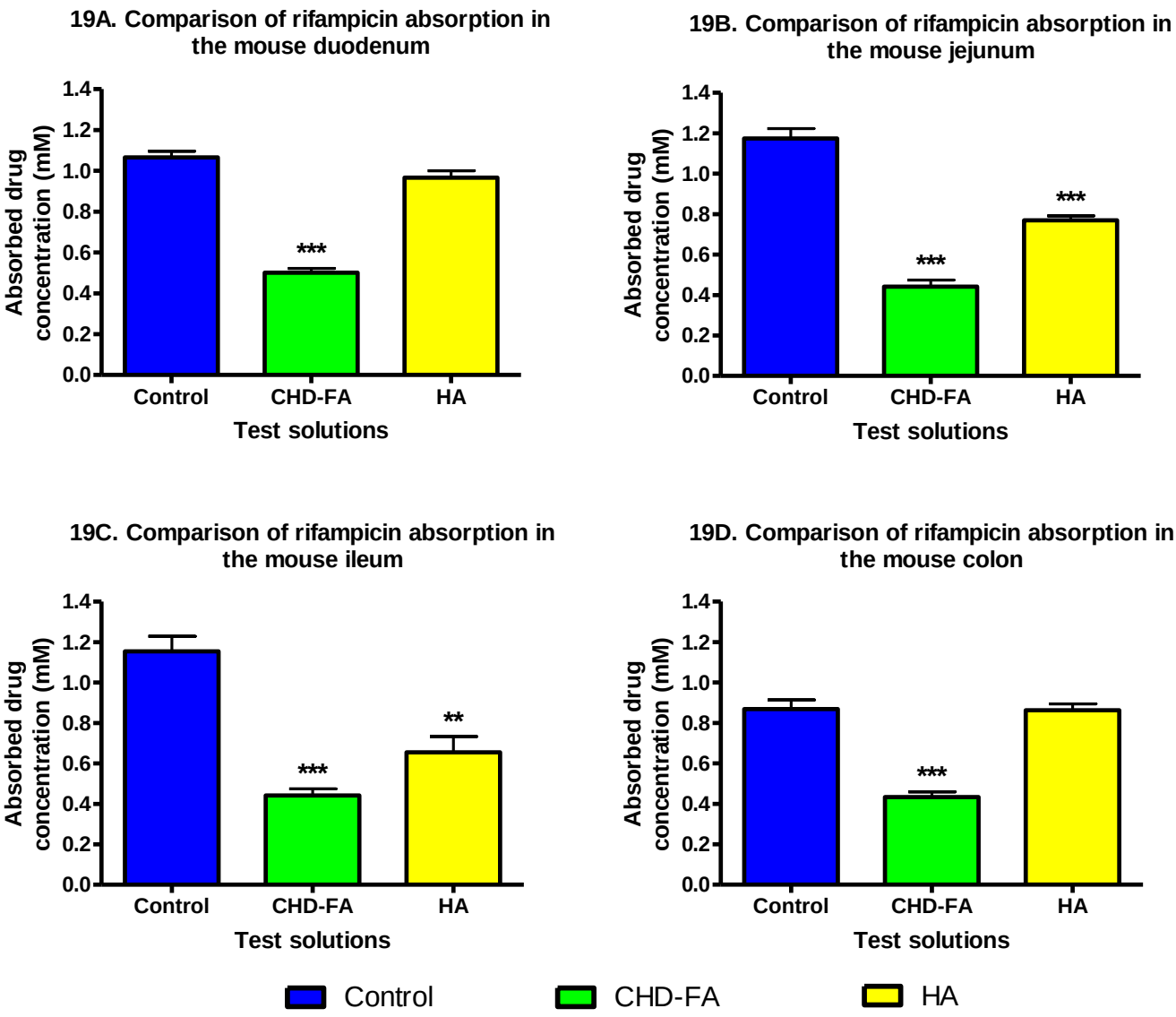

Figure 19A-D: *Comparison of rifampicin absorption in different regions of the mouse intestine (19A: duodenum, 19B: jejunum, 19C: ileum and 19D: colon). The absorption of the drug alone (control) was compared to the absorption of the drug in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in the duodenum, jejunum and ileum, (n = 3).*

** = p < 0.05 ** = p < 0.01 *** = p < 0.001*

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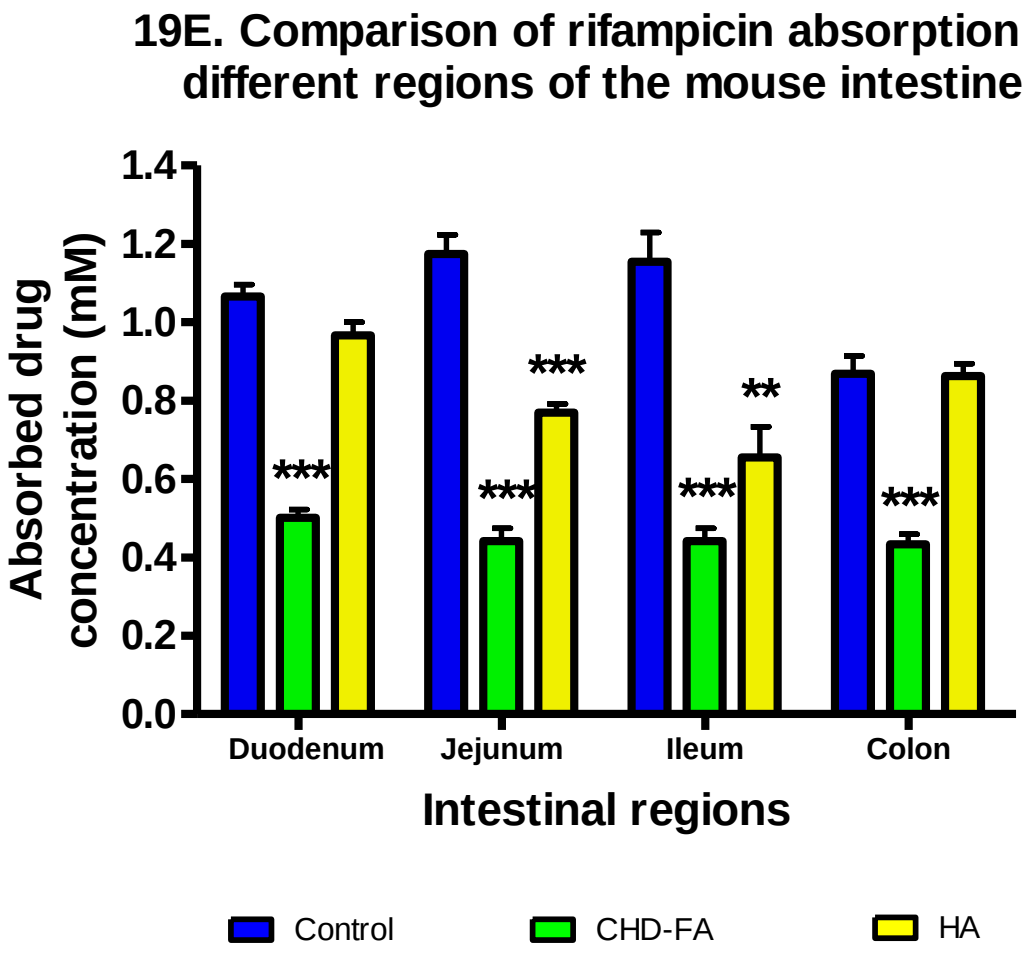

Figure 19E: Combination of graphs 19A-D showing comparison of rifampicin absorption in different regions of the mouse intestine (duodenum, jejunum, ileum and colon). The absorption of the drug alone (control) was compared to the absorption of the drug in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in all regions of the intestine, (n = 3).

** = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$*

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Table 25. Comparison of rifampicin absorption in different regions of the intestine

Intestinal region	CHD-FA	Significant	HA	Significant
Duodenum	↓ 53%	Yes	↓ 10%	No
Jejunum	↓ 62%	Yes	↓ 34%	Yes
Ileum	↓ 62%	Yes	↓ 43%	Yes
Colon	↓ 50%	Yes	↓ 1%	No

Table 30 shows the comparison of rifampicin absorption in different regions of the mouse intestine in the presence of either CHD-FA or HA. The decrease seen in absorption was reported as a percentage of the control. It is evident that the presence of both the HS resulted in changes in the total amount of rifampicin absorbed in all the regions of the intestine when compared to the control. Both the HS elicited a consistent trend in rifampicin absorption throughout the regions of the intestine.

Absorption of rifampicin in the presence of CHD-FA was seen to decrease in all the regions of the intestine in comparison to the control. Large decreases were observed that ranged from 50% in the colon to 62% in the jejunum and ileum. Significant changes in rifampicin absorption were seen in all the regions of the intestine.

Absorption of rifampicin in the presence of HA was seen to decrease in all the regions of the intestine in comparison with the control but not to the same extent as with CHD-FA. Decreases ranged from 1% in the colon to 43% in the ileum. Significant changes in rifampicin uptake were only seen in the jejunum and ileum.

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4.5 Valsartan

Background

Valsartan is an angiotensin II receptor antagonist (ARB) used for the chronic treatment of hypertension, congestive heart failure and myocardial infarction (Cohn & Tognoni, 2001; Holwerda *et al.*, 1996; Pfeffer, 2000). Valsartan falls under the category of prescribed conventional medication.

Mechanism of action

Valsartan acts by binding to and blocking the angiotensin receptor (AT_1-receptor) located in a range of different tissues in the body such as vascular smooth muscle. By binding to the receptor, valsartan inhibits angiotensin II from binding to the receptor. This results in the relaxation of vascular smooth muscle and subsequent dilation and a decrease in blood pressure (Siragy *et al.*, 2000).

Symptoms of toxicity

Higher than recommended doses of valsartan can result in severe hepatotoxicity, hypotension and tachycardia. Occasional other effects include nephrotoxicity, vertigo and hypersensitivity.

Properties

Molecular mass: 435.52 g/mol

IUPAC name:

N-Pentanoyl-N-{[2'-(1H-tetrazol-5-yl)-4-biphenylyl]methyl}-L-valine

Molecular formula: $C_{24}H_{29}N_5O_3$

LogP: 4.74

LogD: 0.01 (pH7.4)

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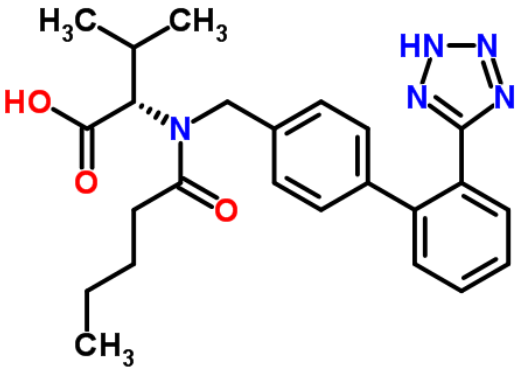

Figure 20. Molecular structure of valsartan (ChemSpider, 2014)

LC-MS/MS analysis

The conditions and parameters for the HPLC-MS/MS method for the analysis of valsartan were as follows:

Table 26. HPLC conditions for valsartan

Mobile phase	A: 0.1% formic acid (FA) B: Methanol
Analytical column	Eclipse XDB-C18 (150 mm x 4.6 mm, 5µm) (Agilent)
Flow rate	1 ml/min
Injection volume	10 µl
Column temperature	40°C
Isocratic	5 min; 43% A

Table 27. Compound specific mass spectrometer conditions for valsartan

	Valsartan	**Gliclazide (IS)**
Ionisation Mode	Negative	Negative
Precursor ion	434.50	322.50
Product ion	178.5 349.7	170.1 105.6
Declustering Potential	-70 -70	-80 -80
Collision energy	-36 -26	-22 -34

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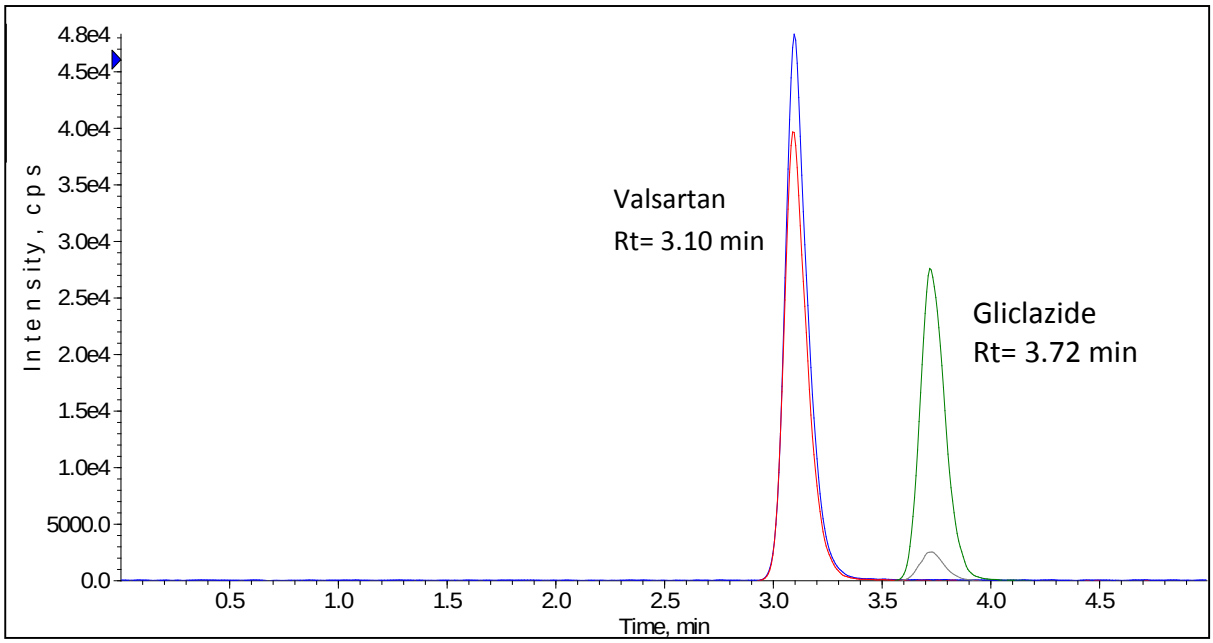

Figure 21. Reversed phased chromatography of valsartan, retention time (Rt) of 3.10 min, using gliclazide as the internal standard (IS) (Rt= 3.72 min). The total run time was 5 mins isocratic. The chromatographic analysis was carried out in order to quantitate valsartan present in the intestinal sacks after absorption for 90 minutes.

Method validation

Table 28. Calibration data to prove linearity for valsartan

Slope	Intercept	Correlation coefficient (r^2)
3.57×10^{-3}	1.43×10^{-2}	0.9996

Table 29. Intra- and inter-day precision and accuracy for valsartan

	Intra-day (*n=3*)			Inter-day (*n=6*)		
Expected concentration (ng/ml)	Mean concentration (ng/ml) ± SD	% CV	% Accuracy	Mean concentration (ng/ml) ± SD	% CV	% Accuracy
50	51.4 ± 1.3	2.6	102.7	47.7 ± 0.9	1.9	95.5
100	102.0 ± 6.7	6.5	102.0	97.4 ± 5.2	5.4	97.4
250	255.8 ± 7.7	3.0	102.3	254.4 ± 9.6	3.8	101.7
500	500.5 ± 12.5	2.5	100.1	503.5 ± 11.3	2.2	100.7
750	747.6 ± 11.1	1.4	99.7	749.1 ± 8.6	1.1	99.9
1000	997.0 ± 11.4	1.1	99.7	997.5 ± 12.4	1.2	99.8

Intra- and inter-day precision varied from 1.1% to 6.5% CV and 1.1% to 5.4% CV, respectively.

Intra- and inter-day accuracy ranged from 99.7% to 102.7% and 95.5% to 101.7%, respectively.

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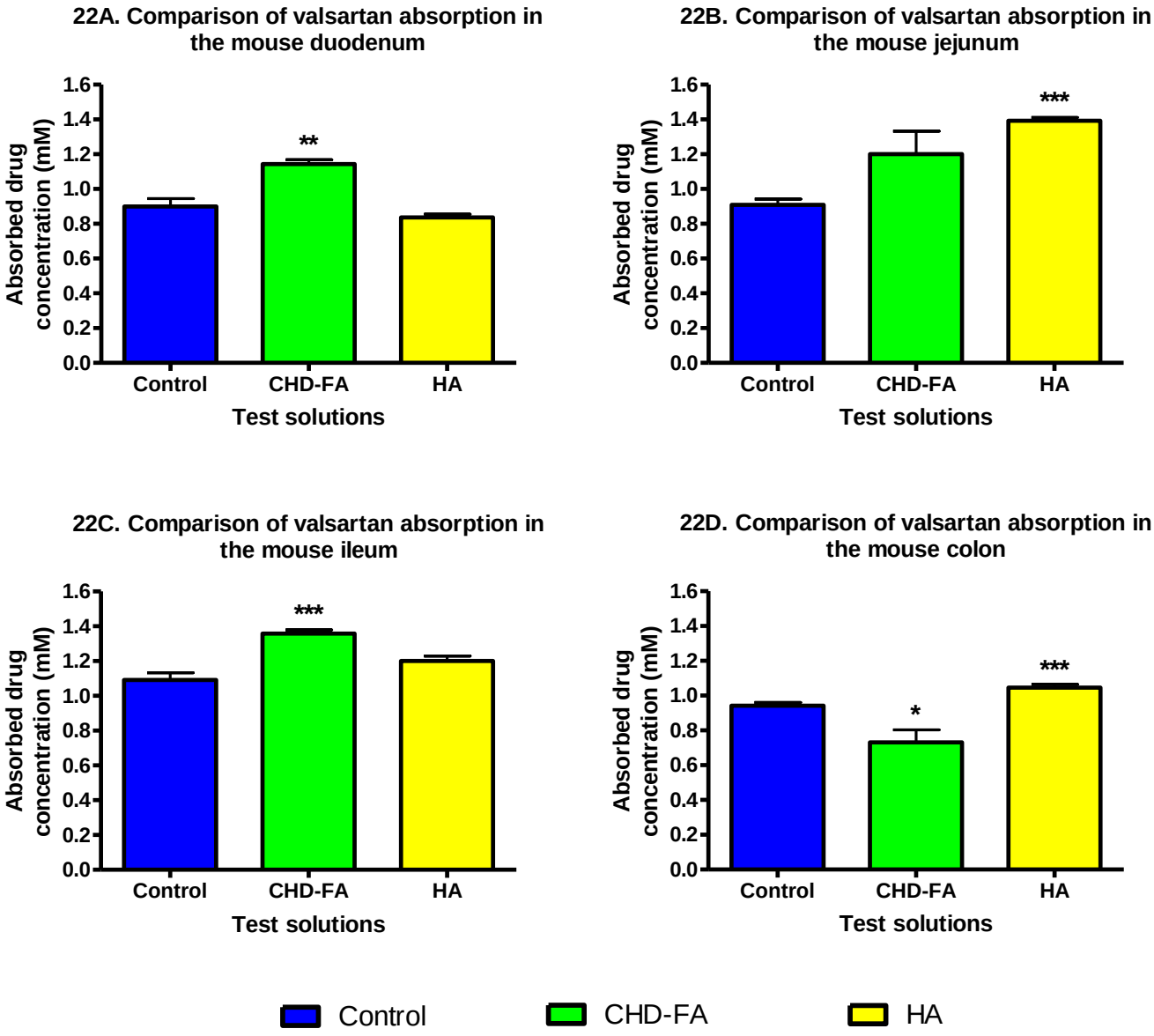

Figure 22 A-D: Comparison of valsartan absorption in different regions of the mouse intestine (22A: duodenum, 22B: jejunum, 22C: ileum and 22D: colon). The absorption of the drug alone (control) was compared to the absorption of the drug in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in the duodenum, jejunum and ileum, (n = 3).

** = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$*

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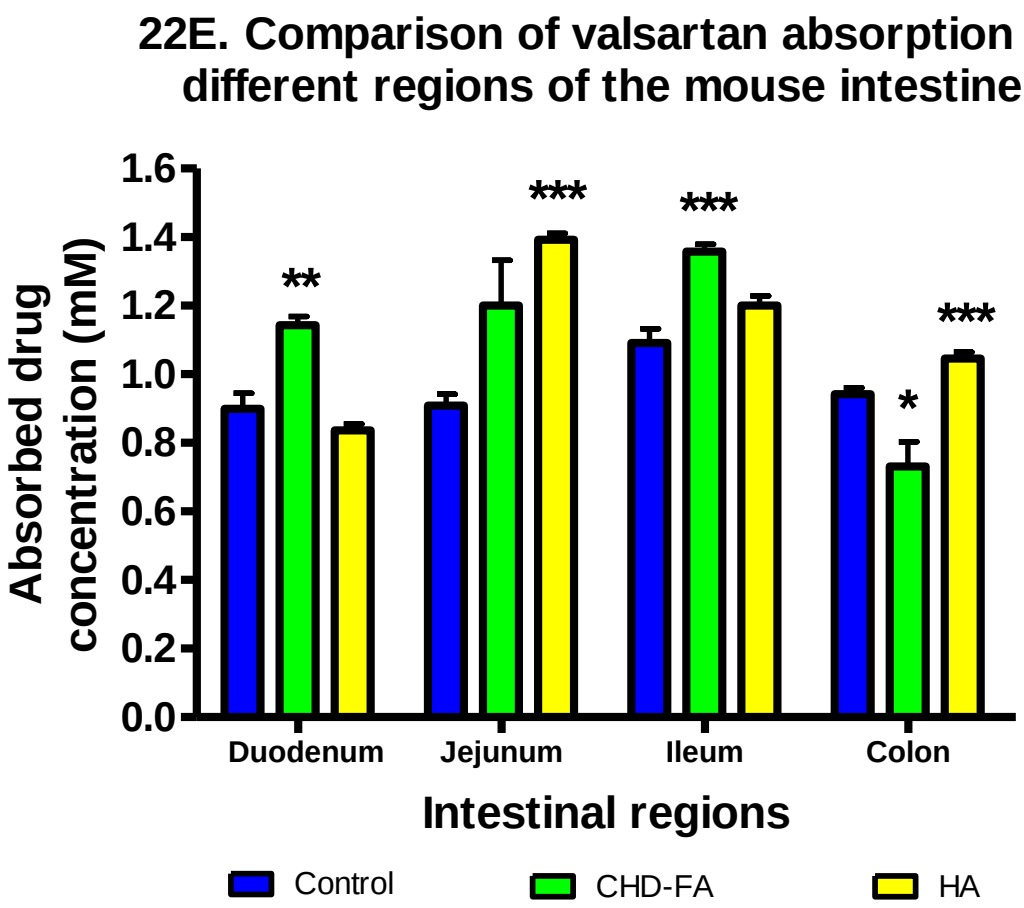

Figure 22E: Combination of graphs 22A-D showing comparison of valsartan absorption in different regions of the mouse intestine (duodenum, jejunum, ileum and colon). The absorption of the drug alone (control) was compared to the absorption of the drug in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in all regions of the intestine, (n = 3).

** = p < 0.05 ** = p < 0.01 *** = p < 0.001*

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Table 30. Comparison of valsartan absorption in different regions of the intestine

Intestinal region	CHD-FA	Significant	HA	Significant
Duodenum	↑ 27%	Yes	↓ 7%	No
Jejunum	↑ 32%	No	↑ 53%	Yes
Ileum	↑ 24%	Yes	↑ 10%	No
Colon	↓ 22%	Yes	↑ 11%	Yes

Table 35 shows the comparison of valsartan absorption in different regions of the mouse intestine in the presence of either CHD-FA or HA. The increase or decrease seen in absorption was reported as a percentage of the control.
It is evident that the presence of both the HS resulted in changes in the total amount of valsartan absorbed in all the regions of the intestine when compared to the control. Neither of the HS elicited a consistent trend in valsartan absorption for all the regions of the intestine.

Absorption of valsartan in the presence of CHD-FA was seen to increase in the duodenum, jejunum and ileum and decrease in the colon in comparison to the control. Increases ranged from 24% in the ileum to 32% in the jejunum, while a 22% decrease was seen in the colon. Significant changes in valsartan absorption were seen in the duodenum, jejunum and ileum only.

Absorption of valsartan in the presence of HA was seen to increase in the jejunum, ileum and colon, and decrease in the duodenum in comparison with the control. Increases ranged from 10% in the ileum to 53% in the jejunum, while a 7% decrease was seen in the duodenum. Significant changes in valsartan uptake were seen in the jejunum and colon only.

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4.6 Zidovudine

Background

Zidovudine is a nucleoside analogue reverse-transcriptase inhibitor (NRTI), an antiretroviral drug used in combination for the first-line treatment of HIV/AIDS infections (World Health Organization, 2014). The combination therapy consists of at least three antiretroviral drugs administered together. These drugs include NRTI's such as tenovir, lamivudine and zidovudine and non-nucleoside reverse transcriptase inhibitors (NNRTI's) such as efavirenz and nivirapine. (World Health Organization, 2014)

The use of these drugs in combination have greatly assisted in reducing the viral load of patients living with HIV, however they do not provide a cure and are often accompanied by a range of side effects. Due to this, many patients concurrently take other medications, like alternative medications, attempting to obtain a combination resulting in a cure or to counteract the negative side effects brought on by the convention medication.

As previously mentioned, TB and HIV are common co-morbidities in South Africa and a large portion of the population is reliant on chronic medication for the treatment of both disorders. Thus the chance of drug interactions with compounds in alternative medications as well as conventional medications is highly likely.

Zidovudine is administered orally and is only available with a prescription.

Mechanism of action

Zidovudine inhibits viral DNA replication by selectively inhibiting the virus's reverse transcriptase, thus halting viral replication. (Furman & Barry, 1988). There have however also been signs of mitochondrial DNA inhibition in several tissues. (Dalakas *et.al*, 1990)

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Symptoms of toxicity

Higher than recommended doses of zidovudine can result in severe hepatotoxicity. Cardiac and skeletal muscle cell myopathy anaemia, neutropenia.

Properties

Molecular mass: 267.24 g/mol

IUPAC name: 3'-Azido-2',3'-dideoxy-3,4-dihydrothymidine

Molecular formula: $C_{10}H_{13}N_5O_4$

LogP: -0.53

LogD: -0.53 (pH7.4)

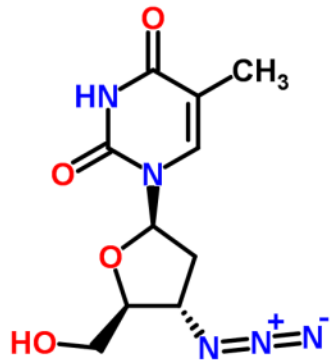

Figure 23. Molecular structure of zidovudine (ChemSpider, 2014)

LC-MS/MS analysis

The conditions and parameters for the LC-MS/MS method for the analysis of zidovudine were as follows:

Table 31. HPLC conditions for zidovudine

Mobile phase	A: 0.1% formic acid (FA) B: Methanol
Analytical column	Eclipse XDB-C18 (150 mm x 4.6 mm, 5 µm) (Agilent)
Flow rate	1 ml/min
Injection volume	10 µl
Column temperature	35°C
Isocratic	0 - 4.5 min; 57% A

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Table 32. Compound specific mass spectrometer conditions for zidovudine

	Zidovudine	Theophylline (IS)
Ionisation Mode	Positive	Positive
Precursor ion	268.3	181.0
Product ions	127.2 110.0	124 96
Declustering Potential	35 35	85 85
Collision energy	10 45	26 26

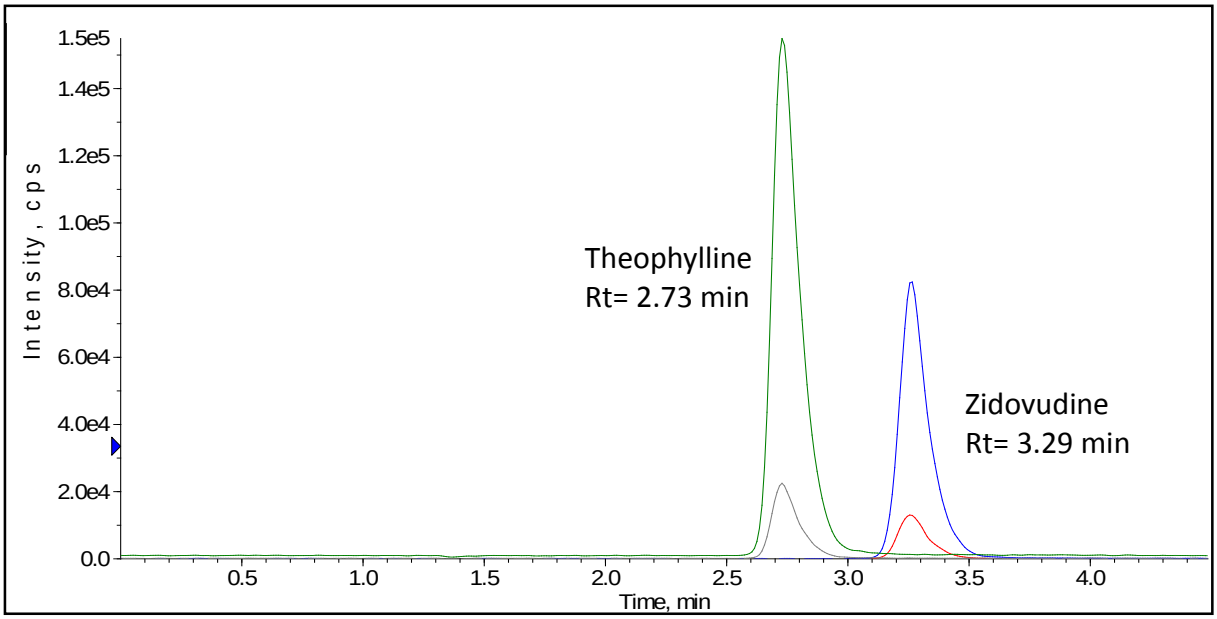

Figure 24. Reversed phased chromatography of zidovudine, retention time (Rt) of 2.73 min, using theophylline as the internal standard (IS) (Rt=3.29 min). The total run time was 4.5 min. The chromatographic analysis was carried out in order to quantitate zidovudine present in the intestinal sacks after absorption for 90 minutes.

Method validation

Table 33. Calibration data proving linearity for zidovudine

Slope	Intercept	Correlation coefficient (r^2)
8.54×10^{-4}	1.08×10^{-4}	0.9999

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Table 34. Intra- and inter-day precision and accuracy for zidovudine

	Intra-day (n=3)			Inter-day (n=6)		
Expected concentration (ng/ml)	Mean concentration (ng/ml) ± SD	% CV	% Accuracy	Mean concentration (ng/ml)	% CV	% Accuracy
25	25.5 ± 0.6	2.2	102.2	24.9 ± 0.8	3.4	99.8
50	51.4 ± 1.0	1.8	102.8	50.5 ± 0.6	1.3	100.9
125	121.6 ± 0.6	0.5	97.3	122.3 ± 1.8	1.5	97.9
250	251.0 ± 7.1	2.8	100.4	249.5 ± 5.4	2.1	99.8
500	495.2 ± 3.5	0.7	99.0	496.0 ± 2.8	0.6	99.2
1000	1006.6 ± 11.9	1.9	100.6	1006.0 ± 0.9	0.9	100.6

Intra- and inter-day precision varied from 0.5% to 2.8% CV and 0.6% to 3.4% CV, respectively.

Intra- and inter-day accuracy ranged from 97.3% to 102.8% and 97.9% to 100.9%, respectively.

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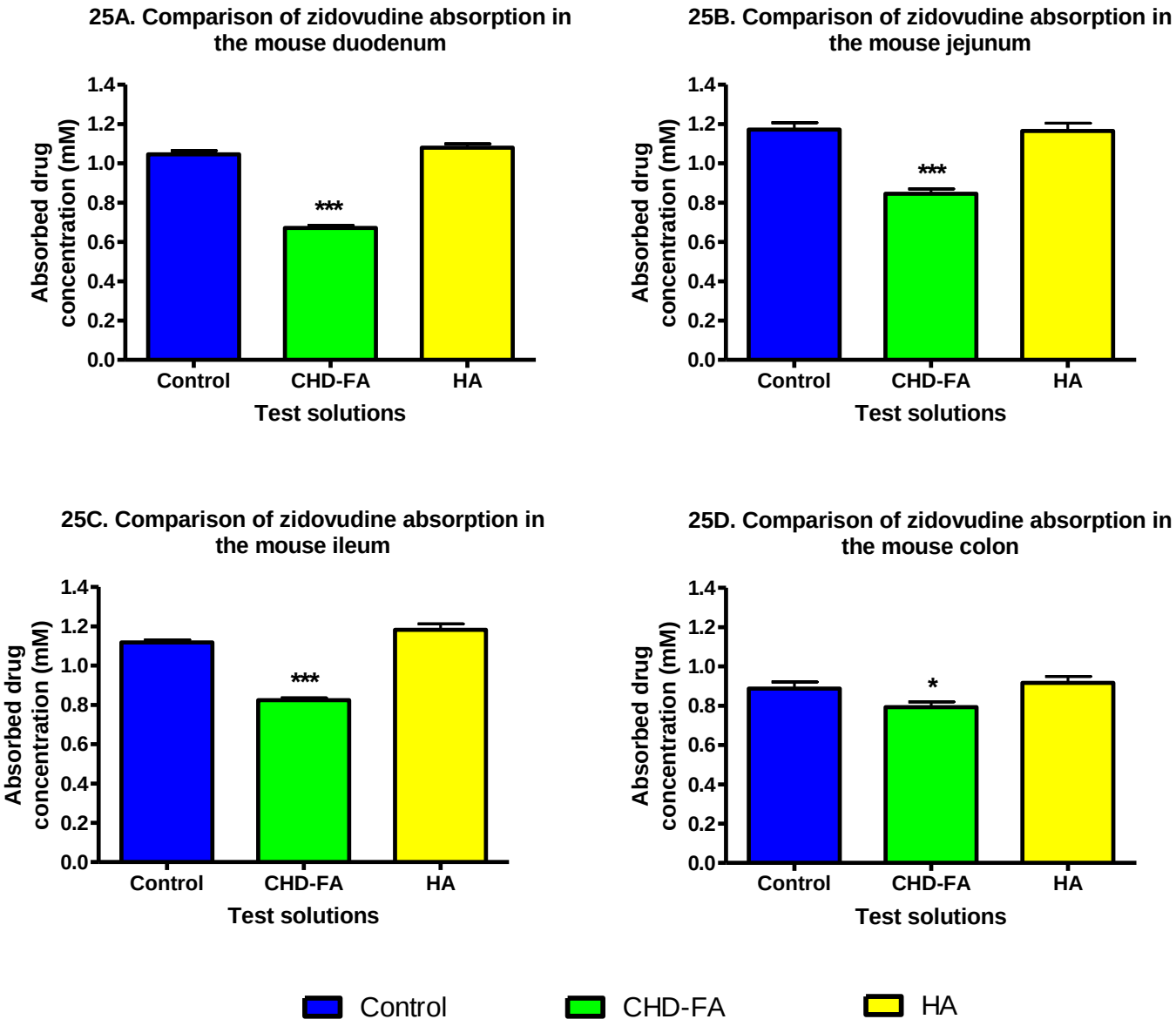

Figure 25 A-D: *Comparison of zidovudine absorption in different regions of the mouse intestine (25A: duodenum, 25B: jejunum, 25C: ileum and 25D: colon). The absorption of the drug alone (control) was compared to the absorption of the drug in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in all regions (n = 3).*

** = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$*

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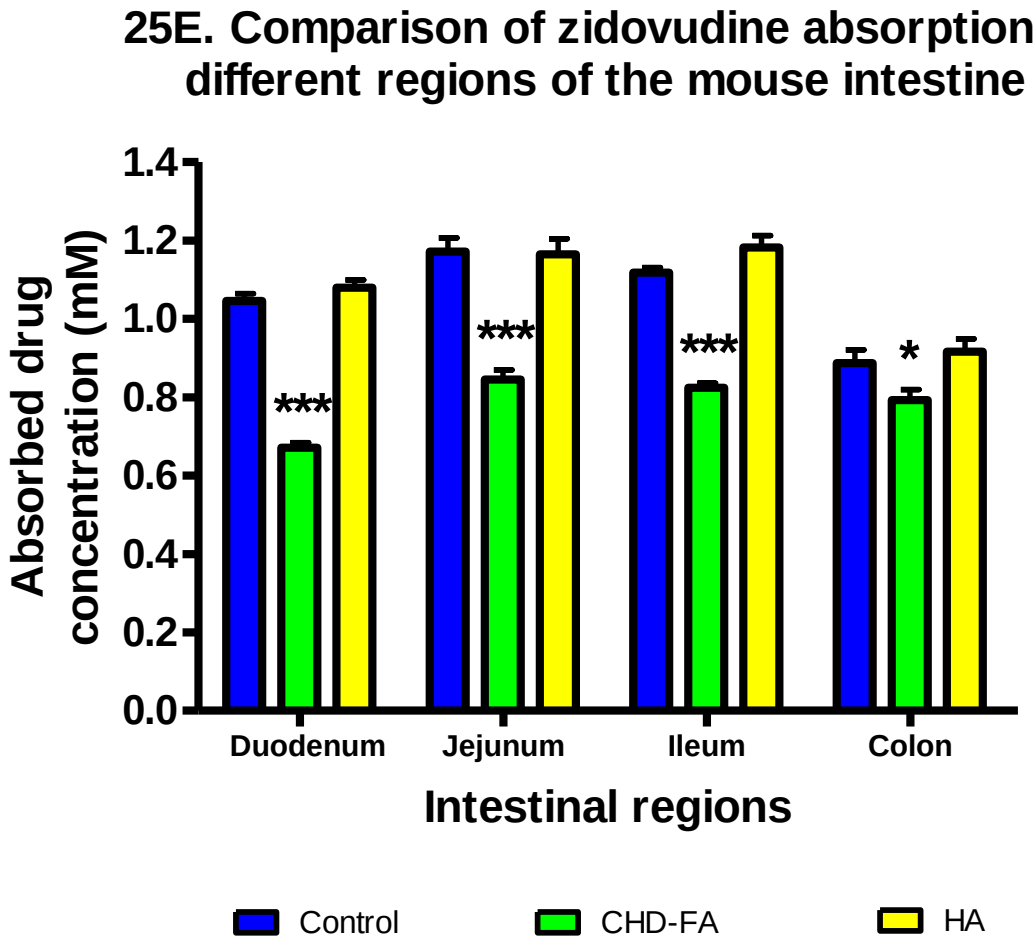

Figure 25E: Combination of graphs 25A-D showing comparison of zidovudine absorption in different regions of the mouse intestine (duodenum, jejunum, ileum and colon). The absorption of the drug alone (control) was compared to the absorption of the drug in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in all regions, (n = 3).

** = p < 0.05 ** = p < 0.01 *** = p < 0.001*

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Table 35. Comparison of zidovudine absorption in different regions of the intestine

Intestinal Region	CHD-FA	Significant	HA	Significant
Duodenum	↓ 36%	Yes	↑ 3%	No
Jejunum	↓ 28%	Yes	↓ 1%	No
Ileum	↓ 26%	Yes	↑ 6%	No
Colon	↓ 11%	Yes	↑ 3%	No

Table 40 shows the comparison of zidovudine absorption in different regions of the mouse intestine in the presence of either CHD-FA or HA. The increase or decrease seen in absorption was reported as a percentage of the control.
It is evident that only the presence of CHD-FA resulted in changes in the total amount of zidovudine absorbed in all regions of the intestine when compared to the control. Both HS showed a consistent yet opposite trends in the absorption of zidovudine throughout all the regions of the GIT.

Absorption of zidovudine in the presence of CHD-FA was decreased in all GIT regions in comparison to the control. Decreases ranged from 11% in the colon to 36% in the duodenum. The changes in zidovudine absorption were significant in all regions.

Absorption of zidovudine in the presence of HA was seen to have no significant effects in any of the regions in comparison with the control. Negligible increases were seen in the duodenum, ileum and colon, with the largest increase being seen in the ileum at 6%. A 1% decrease was seen in the jejunum.

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4.7 Drugs: Combined results summary

Table 36. Summary of drug absorption in different regions of the intestine

Diclofenac				
Intestinal Region	CHD-FA	Significant	HA	Significant
Duodenum	↓ 30%	Yes	↓ 32%	Yes
Jejunum	↑ 5%	No	↑ 13%	Yes
Ileum	↑ 72%	Yes	↑ 46%	Yes
Colon	↓ 15%	No	↑ 41%	No
Penicillin V				
Intestinal Region	CHD-FA	Significant	HA	Significant
Duodenum	↑ 3%	No	↓ 28%	Yes
Jejunum	↑ 14%	No	↓ 22%	Yes
Ileum	↓ 11%	Yes	↓ 24%	Yes
Colon	↓ 2%	No	↓ 23%	Yes
Warfarin				
Intestinal Region	CHD-FA	Significant	HA	Significant
Duodenum	↓ 48%	Yes	↓ 24%	Yes
Jejunum	↓ 40%	Yes	↓ 21%	Yes
Ileum	↓ 40%	Yes	↓ 9%	No
Colon	↓ 43%	Yes	↓ 10%	Yes
Rifampicin				
Intestinal Region	CHD-FA	Significant	HA	Significant
Duodenum	↓ 53%	Yes	↓ 10%	No
Jejunum	↓ 62%	Yes	↓ 34%	Yes
Ileum	↓ 62%	Yes	↓ 43%	Yes
Colon	↓ 50%	Yes	↓ 1%	No
Valsartan				
Intestinal Region	CHD-FA	Significant	HA	Significant
Duodenum	↑ 27%	Yes	↓ 7%	No
Jejunum	↑ 32%	No	↑ 53%	Yes
Ileum	↑ 24%	Yes	↑ 10%	No
Colon	↓ 22%	Yes	↑ 11%	Yes
Zidovudine				
Intestinal Region	CHD-FA	Significant	HA	Significant
Duodenum	↓ 36%	Yes	↑ 3%	No
Jejunum	↓ 28%	Yes	↓ 1%	No
Ileum	↓ 26%	Yes	↑ 6%	No
Colon	↓ 11%	Yes	↑ 3%	No

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4.8 Discussion

Medicinal drugs are required for the treatment and prevention of a range of acute and chronic illnesses. The correct dosing of these drugs is vital in order for a drug to reach the therapeutic threshold to achieve its full efficacy in the body while eliciting the least possible side effects. The therapeutic index of a drug provides a means to assess the relationship between the dose of a drug which is therapeutic and the dose which causes toxic side effects. Changes in the amount of drug reaching systemic circulation, especially in the case of drugs with a small therapeutic index, can lead to significant changes in drug effect which may result in sub-therapeutic or toxic effect.

In this study, the change in the uptake of the test drugs in the presence of CHD-FA or HA in the different regions of the GIT were assessed. The choice of drugs was made based on the commonly administered drugs in the South African adult population. In most cases, both HS were seen to elicit changes in the absorption of the drugs, and this was observed in all regions of the mouse intestine. Some drugs showed consistent changes in absorption in all four regions of the intestine compared to a HS free control, while others did not show a consistent trend in the change in absorption along the length of the intestine.

The changes seen in the absorption of the drugs in the presence of the CHD-FA and HA could be due to various causes. The possibility exists that the binding of the drug to the HS to form a drug-HS complex has an effect on the uptake of the drug. This effect could be due to the potential binding affinity of the different drugs to the HS whereby influencing uptake; or by altering the drugs physicochemical properties. An alteration in LogP can be responsible for changes the drugs solubility whereby a different transport mechanism in the intestinal membrane is utilized. This change may lead to a more or less suitable transport mechanism being used, leading to increases or decreases in absorption.

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Furthermore, complex formation could result in the drug-HS complex being too large to cross the intestinal membrane via the traditional routes used by the free drug. The presence of unbound CHD-FA and HA molecules in the solution may also directly interact with the transport mechanisms, causing saturation and inhibition of drug uptake.

The effects on absorption of each drug in the presence of CHD-FA and HA are discussed below in more detail.

Diclofenac

Diclofenac is a commonly used NSAID for the treatment of a variety of acute inflammatory or pain disorders, as well as chronic disorders such as osteoarthritis and rheumatoid arthritis. Due to the wide range of action against pain and inflammation, drugs containing diclofenac are frequently used throughout the population. It can be found as an active in, among others, the following medications in South Africa: Cataflam, Dicloflam, Veltex and Voltaren. HA formulations are claimed to have anti-inflammatory properties and to be safe and without side effects which makes these substances ideal to supplement the administration of conventional anti-inflammatory drugs that have several well-known adverse side effects including some severe GIT, skin and CNS effects.

Diclofenac is a weakly acidic (pKa=4), lipophilic drug (LogP=4.06). At pH 7.4 (LogD=0.95), the drug becomes more hydrophilic but still maintains its lipophilic tendencies. Due to its lipophilic nature, it is likely that the free drug makes use of transcellular transport to cross the intestinal membrane.

The significant changes in diclofenac absorption in the presence of both HS were seen in the duodenum as a decrease and in the ileum as an increase. As the changes were not consistent throughout the segments, it is unlikely that they were caused by the binding affinity of the drug and HS or due to physicochemical changes brought on by complexation. These significant changes in absorption could however be due to either hindered or assisted transport of the specific

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transport mechanism in the different anatomical sections by the free HS themselves.

Therefore the concurrent ingestion of conventional medication containing diclofenac with alternative medications containing either of these HS may result in changes in diclofenac uptake leading to a lack of therapeutic effect or adverse side effects in patients suffering from pain or inflammatory disorders. As diclofenac-containing drugs are freely available over the counter, product labels should display warnings regarding the drug interaction potential with HS.

Penicillin V

Penicillin V is a narrow spectrum antibiotic used to treat mild to moderate infections and is one of the drugs of choice to combat common gram positive bacteria. It is the active pharmaceutical ingredient in, among others, the following medications available in South Africa: Oracillin VK, Novo-VK, Len V.K. and V-Cil-K. As both FA and HA formulations claim to improve immune responses, it is likely to be taken together with conventional antimicrobial medication in the belief that it could assist in eradicating persistent infections.

Penicillin V is an acidic (pKa=2.79), but relatively lipophilic drug (LogP=1.88). At pH 7.4, penicillin V becomes slightly more hydrophilic (LogD=-1.85). Thus, paracellular transport through the tight junctions would be the most likely mechanisms of transport for the drug in this experiment.

A constant trend in significant decreases in absorption was seen throughout the intestine in the presence of HA only. This consistent decrease in absorption could be due to hindered transport of the transport mechanism by either the size of the complex formed or due to saturation by free HA molecules.

The lack of trend seen in the CHD-FA results compared to the HA results could be due to the difference in structure between CHD-FA and HA. Penicillin V may display a different binding affinity for the two HS, forming complexes more/less

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readily with each. Alternatively, free CHD-FA may not saturate and inhibit the mechanisms of transport involved in the uptake of penicillin V to the same extent as free HA.

The concurrent ingestion of conventional medication containing penicillin V with alternative medications containing these humic substances, especially HA, may result in decreases in penicillin V uptake leading to a lack of therapeutic effect of the antibiotic. This could increase the spread of the infection as well as the possible development of antibiotic resistance by the bacteria. Upon prescribing this antibiotic, healthcare professionals should advise against the concurrent use of the antibiotic with medicinal preparations containing HA.

Warfarin

Warfarin belongs to the coumarin class of compounds and commonly administered as an anticoagulant to inhibit blot clot formation through inhibition of several clotting factors in the blood. It can be found as an active pharmaceutical ingredient in, among others, the following medications in South Africa: Cipla-Warfarin and Lennon-Warfarin. Warfarin has a very narrow therapeutic index and a high protein binding coupled to a long elimination half-life therefore concentrations in the blood need to be closely monitored to maintain the levels within a limited concentration range. Slight increases in plasma concentration of warfarin could lead to internal bleeding, whereas decreases can lead to a lack of anticoagulant activity, increasing the risk of thromboembolism. Many older patients and patients recovering from surgery are dosed chronically with warfarin to inhibit potential thromboembolism. These same patients often self-administer FA or HA formulations due to the claimed wound healing properties and immune modulating effects that enhance recovery after surgery and promote an overall feeling of well-being.

Warfarin is a weakly acidic (pKa=5.08), lipophilic drug (LogP=3.42). At pH 7.4, warfarin decreases in lipophilicity (LogD= 0.61). Transcellular transport through the intestinal membrane is the most likely route of transport in this experiment.

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A constant trend in significant decreases in absorption was seen throughout the intestine in the presence of both CHD-FA and HA. Similarly to penicillin V, these decreases in absorption could be due to hindered activity of the responsible transport mechanisms by either the size of the complex formed or due to saturation by free CHD-FA and HA molecules. CHD-FA was seen to have a more significant effect on warfarin absorption than HA, possibly due to the difference in binding affinity for the two HS, forming complexes more/less readily.

Due to the narrow therapeutic index of warfarin, decreases in absorption of this magnitude have a very high possibility of resulting in therapeutic failure. Therapeutic failure of warfarin would have serious effects on patients undergoing anticoagulation treatment. Thus, healthcare professionals as well as patients should be aware that concurrent ingestion of warfarin and formulations containing these HS should be strictly avoided.

Rifampicin

Rifampicin is an antibiotic used primarily to treat TB infections. Due to the emergence of resistance to TB drugs when administered alone, it is administered in combination with three other drugs: isoniazid, ethambutol and pyrazinamide, to make up the first-line treatment for TB. The combination treatment is used chronically by TB infected patients for a period of approximately 6 months (World Health Organisation, 2010).

In South Africa, a large portion of the population relies on government-provided chronic conventional medication for the treatment of TB. However, many of these patients also follow strong cultural beliefs that encourage use of herbal or alternative medication to treat these and other conditions. Furthermore, due to the numerous drugs taken in each combination, a range of adverse side effects are commonly experienced. The need to counteract these negative side effects brought on by the conventional treatment further promotes the concurrent use of

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alternative medication aimed at relieving the adverse effects or to improve the treatment outcome.

This compound was selected as a model compound to assess how larger lipophilic molecules would be affected by possible complexation with the two HS. Larger molecules possess more functional groups and an increased surface area for interaction and binding with other compounds. This could have a direct effect on the binding affinity of the compounds in complex.

Rifampicin is a weakly basic (pKa= 7.53), relatively nonpolar drug (LogP= 1.09). At pH 7.4, the LogD of rifampicin is estimated at -1.43 according to the drug specific values viewed on ChemSpider (ChemSpider, 2014). This value suggests that at pH 7.4, the drug decreases in lipophilicity and becomes hydrophilic in nature. However, rifampicin is known to readily distribute in cerebrospinal fluid, cross the blood brain barrier and placenta, and be largely excreted in the faeces, confirming the drugs lipophilic nature. The LogD values obtained through ChemSpider are calculated values and are not obtained experimentally. It has been seen in a previous study that the predicted LogD data obtained for rifampicin using a software package differed considerably to values obtained experimentally (Mariappan *et al.,* 2007). Software-predicted values over a wide pH range showed as negative values (indicating hydrophilic tendencies), whereas experimental values were positive (indicating lipophilic tendencies). Thus it is more likely that rifampicin maintains its lipophilicity in the pH 7.4 environment of the experiment and makes use of transcellular transport mechanisms to cross the intestinal membrane.

Decreases in rifampicin absorption were seen in the presence of both HS in all regions of the intestine when compared to the control solution. The decreases in absorption could be due to hindered activity of the responsible transport mechanisms by either the size of the complex formed or due to saturation by free CHD-FA and HA molecules. A potentially stronger binding affinity of the larger drug molecule to the HS could result in a higher amount of the rifampicin drug

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molecule to remain in complex following transport across the intestinal membrane. A diminished concentration of free rifampicin would thus be available in the plasma for therapeutic use in the body. Similarly to the warfarin results, CHD-FA was seen to have a more significant effect on absorption than HA, possibly due to the difference in binding affinity for the two humic substances, forming complexes more/less readily.

The large decreases in absorption would have a very high possibility of causing therapeutic failure of rifampicin which could in turn compromise the combined TB treatment regimen. Furthermore, failure of the treatment can result in the development of resistance to the other antimycobacterials used in the treatment regimen, giving rise to more resistant strains of TB. It is thus advised that concurrent ingestion of rifampicin and medications containing these HS should be strictly avoided.

Valsartan

Valsartan is an angiotensin receptor blocker (ARB) used in the treatment of a range of cardiac related disorders including hypertension, myocardial infarction, coronary artery disease and heart failure. It can be found as an active pharmaceutical ingredient in, among others, the following medications in South Africa: Diovan, Exforge, Tareg and Zomevek.

Valsartan is a weakly acidic (pKa=4.37), nonpolar drug (LogP=4.47). At pH 7.4, valsartan is seen to decrease considerably in lipophilicity (LogD=0.01) according to the drug specific values found in ChemSpider (ChemSpider, 2014). Ingested valsartan molecules would thus most likely be transported via the transcellular route through the intestinal membrane in this experiment.

Changes in the absorption of valsartan were seen in the presence of both HS in all regions of the intestine when compared to the control. Although consistent changes in absorption were not observed in all of the four regions, three of the four regions in the case of both HS showed consistent increases in absorption. As the

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changes were not consistent throughout the segments, it is unlikely that they were caused by the binding affinity of the drug and HS or due to physicochemical changes due to complexation. Valsartan has a poor bioavailability but a very large therapeutic index so fairly large increases in the bioavailability would probably not show a marked clinical effect. The changes in absorption could be due to effects on the transport mechanism in the different anatomical sections by the free HS themselves.

The concurrent ingestion of conventional medication containing valsartan with alternative medications containing these HS is seen to have an effect on the amounts absorbed, whereby three of the four segments showed increase absorption. This increased uptake may improve the clinical outcome due to increased bioavailability giving improved effects in patients. However, healthcare professionals should be aware when prescribing valsartan to patients taking unconventional medication with the possibility of it including HS.

Zidovudine

Similarly to TB, HIV/AIDS infections are common in South Africa and require treatment using a combination of drugs. The treatment regimens consist of a combination of nucleoside reverse-transcriptase inhibitors (NRTIs) and non-nucleoside reverse-transcriptase inhibitors (NNRTIs). Both drug classes inhibit the DNA replication of the virus through different mechanisms during the replication process. Zidovudine is an NRTI, commonly combined with other NRTI's and NNRTI's in the first-line treatment of HIV/AIDS infections.

Similar to TB infections, a large portion of the South African population relies on government-provided chronic conventional medication for the treatment of HIV. Furthermore, many of these patients also follow cultural beliefs that encourage the use of herbal or alternative medication to assist in treatment or to counteract side effects of the treatment combination.

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Zidovudine is a basic (pKa=9.96), polar drug (LogP= -0.53). At pH 7.4, the hydrophilcity of zidovudine is seen to remain the same (LogD= -0.53). It is thus likely that zidovudine is transported across the intestinal membrane via the paracellular route in this experiment.

A constant trend in significant decreases in absorption was seen throughout the intestine in the presence of CHD-FA only. This consistent decrease in absorption could be due to complexation with the acidic CHD-FA or hindered transport of the transport mechanism by either the size of the complex formed or due to saturation by free HA molecules. The results obtained in the presence of HA very closely mirrored the control results and thus no changes were observed.

The lack of trend seen in the HA results compared to the CHD-FA results could be due to the difference in acidity or structure between the two HS. Similarly to penicillin V, Zidovudine may also show a different binding affinity for the two HS, forming complexes more/less readily with each. Alternatively, free HA may not saturate and inhibit the mechanisms of transport involved in the uptake of zidovudine to the same extent as free CHD-FA.

The concurrent ingestion of conventional medication regimens for HIV containing zidovudine along with alternative CHD-FA medications containing will most likely have a decreased effect on the amount of absorbed drug and may result in treatment failure. Treatment failure of this nature could contribute to development of resistance towards the drug combination and healthcare professionals should be aware of whether patients are making use of CHD-FA containing medications prior to prescribing chronic HIV treatment. In contrast, the presence of HA does not seem to have any significant effects on zidovudine uptake, thus concomitant ingestion would not have to be strictly avoided. It should be noted that several HS containing products are marketed specifically into the HIV infected patients.

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Trends in absorption changes

The separate regions of the mouse intestine are known to differ slightly in anatomy as well as in the types of transporters present. This has an effect on the uptake of ingested compounds and results in certain drugs showing better absorption in a specific intestinal region. It is thus possible that the presence of the HS may interact favourably/unfavourably with the specific histology and physiology of each intestinal region whereby resulting a consistent enhancement/ inhibition of absorption in that region.

A consistent enhancement/inhibition could be brought on by the presence of free HS molecules themselves, directly affecting the transport mechanisms in certain GIT regions. Furthermore, due to the changes in the physicochemical properties of the drugs after the formation of the drug-HS complex, it is also possible that certain physicochemical changes will favour/not favour a specific region.

Table 42 displays the intestinal region of the mouse intestine showing the greatest change in absorption in the presence of CHD-FA and HA for each test drug.

In the presence of both HS, there were no trends seen whereby a specific region was consistently favoured/ not favoured. The greatest region of change for each drug was seen to vary between the duodenum, jejunum and ileum. The colon does not appear in the table as a region showing the greatest change for any of the drugs. This result is not unexpected, as the colon is not primarily involved in the uptake of drug molecules, but rather the uptake of water and electrolytes.

In order to assess whether the changes in absorption seen had a direct relationship to any of the main physicochemical properties of the drugs, various tables (Tables 43-46) ranking the drugs according to the different physicochemical parameters were designed. Each table ranks the drugs in increasing values with regards to their molecular weight (Table 43), LogP (Table 44), LogD (Table 45) and pKa (Table 46) values. It would be expected that drugs with similar properties will

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interact with the HS in a similar way and would show similar trends in absorption changes.

Table 37. Intestinal region showing the greatest change in absorption in the presence of CHD-FA and HA

Drug	Intestinal segment showing greatest change (CHD-FA)	Intestinal segment showing greatest change (HA)
Diclofenac	Ileum	Ileum
Penicillin V	Jejunum	Duodenum
Warfarin	Duodenum	Duodenum
Rifampicin	Jejunum/ileum	Ileum
Valsartan	Jejunum	Jejunum
Zidovudine	Duodenum	Ileum

Table 38. Drugs ranked according to increasing molecular mass

Drug	Effect on absorption in the presence of CHD-FA	Effect on absorption in the presence of HA
Zidovudine (267.24 g/mol)	Consistent decrease in all regions	No significant change
Diclofenac (296.15 g/mol)	No consistent changes	No consistent changes
Warfarin (308.33 g/mol)	Consistent decrease in all regions	Consistent decrease in all regions
Penicillin V (350.39 g/mol)	No consistent changes for all regions	Consistent decrease in all regions
Valsartan (435.52 g/mol)	No consistent changes	No consistent changes
Rifampicin (822.94 g/mol)	Consistent decrease in all regions	Consistent decrease in all regions

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Table 39. Drugs ranked according to increasing LogP

Drug	Effect on absorption in the presence of CHD-FA	Effect on absorption in the presence of HA
Zidovudine (LogP -0.53)	Consistent decrease in all regions	No significant change
Rifampicin (LogP 1.09)	Consistent decrease in all regions	Consistent decrease in all regions
Penicillin V (LogP 1.88)	No consistent changes throughout regions	Consistent decrease in all regions
Warfarin (LogP 3.42)	Consistent decrease in all regions	Consistent decrease in all regions
Diclofenac (LogP 4.06)	No consistent changes throughout regions	No consistent changes throughout regions
Valsartan (LogP 4.74)	No consistent changes throughout regions	No consistent changes throughout regions

Table 40. Drugs ranked according to increasing LogD (pH 7.4)

Drug	Effect on absorption in the presence of CHD-FA	Effect on absorption in the presence of HA
Penicillin V (LogD -1.85)	No consistent changes throughout regions	Consistent decrease in all regions
Rifampicin (LogD -1.43)	Consistent decrease in all regions	Consistent decrease in all regions
Zidovudine (LogD -0.53)	Consistent decrease in all regions	No significant change
Valsartan (LogD 0.01)	No consistent changes throughout regions	No consistent changes throughout regions
Warfarin (LogD 0.61)	Consistent decrease in all regions	Consistent decrease in all regions
Diclofenac (LogD 0.95)	No consistent changes throughout regions	No consistent changes throughout regions

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Table 41. Drugs ranked according to increasing pKa

Drug	Effect on absorption in the presence of CHD-FA	Effect on absorption in the presence of HA
Penicillin V (pKa 2.79)	No consistent changes throughout regions	Consistent decrease in all regions
Diclofenac (pKa 4.00)	No consistent changes throughout regions	No consistent changes throughout regions
Valsartan (pKa 4.37)	No consistent changes throughout regions	No consistent changes throughout regions
Warfarin (pKa 5.08)	Consistent decrease in all regions	Consistent decrease in all regions
Rifampicin (pKa 7.53)	Consistent decrease in all regions	Consistent decrease in all regions
Zidovudine (pKa 9.96)	Consistent decrease in all regions	No significant change

Table 43 compares the test drugs, ranked in increasing molecular weight, and the effect on absorption in the presence of CHD-FA and HA. Drug molecular masses used in this study ranged between 200 g/mol and 900 g/mol. Larger mass drugs possess a greater surface area with which to bind to HS, thus it is expected that the binding affinity of the larger drugs to the HS would be greater than that of the smaller drugs and a trend in uptake would be observed. Alternatively, once the larger drug molecules have bound to the HS, the resulting drug-HS complex may be too large to cross the intestinal membrane via the majority of transport mechanisms and a trend in decreasing absorption would be observed with increasing molecular mass.

The results in Table 43 show no clear relationship between the size of the drug and its change in absorption in the presence of either HS, indicating that molecular mass does not have a significant effect on the transport of the drug-HS

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complex across the intestinal membrane and cannot be used as a predictive tool to estimate trends in absorption using other drugs.

Table 44 compares the drugs, ranked in increasing LogP values, and the effect on absorption in the presence of CHD-FA and HA. LogP values of the drugs used in this study ranged between -0.53 and 4.74. These values, however, are calculated values obtained from ChemSpider and may differ slightly from actual values obtained during experimental procedures. For the purpose of this discussion, the calculated values have been used.

A LogP of less than zero indicates that the drug has more hydrophilic tendencies, whereas a LogP of greater than zero indicated lipophilic tendencies. The LogP of a drug governs its solubility as well as the type of transport mechanism used to cross the intestinal membrane.

The results in Table 44 show no clear relationship between the calculated LogP of the drug and its change in absorption in the presence of either HS, indicating that the calculated LogP does not have a significant effect on the transport of the drug-HS complex across the intestinal membrane and cannot be used as a predictive tool to estimate trends in absorption using other drugs.

Table 45 compares the drugs, ranked in increasing LogD values, and the effect on absorption in the presence of CHD-FA and HA. LogD represents the polarity of the drug at a specific pH. In this study, the LogD represents drug polarity at a pH 7.4 which is the pH of the KRB used in the assays.

Similarly to the LogP values, the LogD values of the drugs used in this study are calculated values obtained from ChemSpider and ranged between -1.85 to 0.95. Comparing the deduced LogD values to the LogP values indicates that each of the drugs became less lipophilic in the experimental environment of pH 7.4.

The results in Table 45 show no clear relationship between the calculated LogD of the drug and its change in absorption in the presence of either HS, indicating that the calculated LogD does not have a significant effect on the transport of the drug-

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HS complex across the intestinal membrane and cannot be used as a predictive tool to estimate trends in absorption using other drugs.

Table 46 compares the drugs, ranked in increasing pKa values, and the effect on absorption in the presence of CHD-FA and HA. Drug pKa gives an indication of whether the drug is more basic or acidic. Drugs with a pKa > 7 are basic and drugs with a pKa < 7 are more acidic in nature.

In the presence of CHD-FA it can be seen that the more weakly acidic and basic drugs with a pKa > 5 showed a consistent decrease in absorption. In the presence of HA, no clear relationship was seen. The trend seen only in the presence of CHD-FA could be due to an overall affinity for the HS for more weakly acidic and basic drugs due to their charged state in the pH7.4 environment. However, due to the small number of drugs used in this study, a definite conclusion cannot be made.

Although these results only took into account the main physicochemical properties of 6 drugs, the results suggest that these properties cannot be used to predict the changes in absorption of drugs with similar properties in the presence of the HS. In order to confidently assess if the changes in absorption of a drug in the presence of CHD-FA or HA are linked to one or more physicochemical property, a larger number of drugs will need to be assessed.

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Chapter 5

5. Vitamins

Vitamins are essential to maintain normal physiological functioning in the body and can be obtained from a variety of natural food sources as well as dietary supplements. Thirteen vitamins have been identified as being important in human nutrition and can be classified into two distinct groups based on their solubility characteristics. Vitamins A, D, E and K are classed as fat soluble vitamins, whereas vitamin C, the B complex vitamins (B_1, B_2, B_3, B_6 and B_{12}), pantothenic acid, biotin and folate are classified as water soluble vitamins.

A vitamin deficiency can occur following limited dietary intake over prolonged periods. Deficiencies may be a common occurrence, especially in poorer regions or developing countries and are linked to many negative symptoms. Beriberi, scurvy, pellagra and rickets are examples of diseases resulting from a lack of sufficient vitamin intake. It is, however, also possible to suffer from toxic symptoms from excessive vitamin intake (DiPalma & Ritchie, 1977). These high levels are usually reached due to excessive supplementation rather than through dietary intake.

In order to ensure proper physiological functioning as well as to avoid toxic symptoms, a specific amount of each vitamin should be ingested at regular intervals. Similarly to drugs, humic substances ingested concurrently with food or vitamin supplements may alter the uptake and bioavailability.

In this study, the effect of fulvic and humic acids on the absorption of one water-soluble vitamin (vitamin B_3) and one fat-soluble vitamin (vitamin E) were selected for assessment.

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5.1 Vitamin B_3 (Niacin)

Background

Vitamin B_3 is a water-soluble vitamin, also referred to as niacin or nicotinic acid. It can be synthesized in the body from L-tryptophan, an essential amino acid obtained through a normal diet by eating protein rich foods like lean red meat, poultry, liver and legumes and is a common additive in a variety of dietary supplements labelled as vitamin B complex.

Functions in the body

Vitamin B_3 is an important component of nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$) which are involved in important oxidoreductase reactions where they act as co-enzymes. This includes various dehydrogenase reactions of the glycolysis and Krebs cycle as well as the cytochrome P450 catalyzed reactions in the body. It is necessary for the breakdown reactions of fat, carbohydrates and proteins, and also aids reactions of fatty acid and cholesterol synthesis (Kei *et al.*, 2011). Furthermore, it is also required for the synthesis of various sex and stress-related hormones in the body (Zieve & Eltz, 2011) and is necessary for proper brain function (Bourre, 2006).

Deficiency and high doses

The average amount of vitamin B_3 for male/ female adults above the age of 18 years is approximately 14 - 16 mg per day. Mild deficiency of this vitamin includes symptoms such as fatigue and depression. A moderate to severe deficiency results in pellagra which is characterized by cracked, scaly skin, dementia, and diarrhoea. An excess of vitamin B_3 can show mild side effects with symptoms including flushing, GIT disorders and hyperglycaemia (Kei *et al.*, 2011).

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Properties

Other names: Niacin, Nicotinic acid

Molecular mass: 123.11 g/mole

Molecular formula: $C_6H_5NO_2$

pKa: 2.79

LogP: 0.15

LogD: -2.93 (pH 7.4)

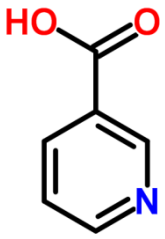

Figure 26. Molecular structure of vitamin B_3 (ChemSpider, 2014)

LC-MS/MS analysis

The conditions and parameters for the LC-MS/MS method for the analysis of vitamin B_3 was as follows:

Table 42. HPLC conditions for vitamin B_3

Mobile phase	A: 0.1% formic acid (FA) B: Methanol
Analytical column	Apollo C18 (150 mm x 4.6 mm, 5 µm) (Alltech)
Flow rate	1 ml/min
Injection volume	10 µl
Column temperature	45°C
Isocratic	0 min - 2.8 min; 84% A

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Table 43. Compound specific mass spectrometer conditions for vitamin B_3

Compound	Vitamin B_3	Theophylline (IS)
Ionisation Mode	Positive	Positive
Precursor ion	124.1	181.0
Product ions	78.0 80.1	124.0 96.0
Declustering Potential	60 60	85 85
Collision energy	40 30	26 26

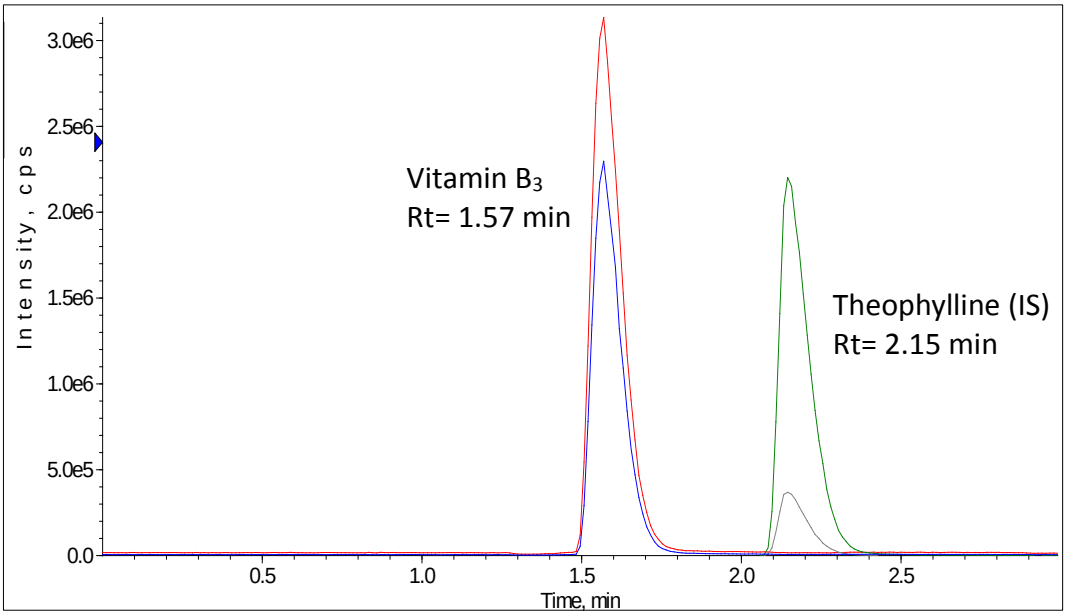

Figure 27. Reversed phased chromatography of vitamin B_3, retention time (Rt) of 1.57 min, using theophylline as the internal standard (IS) (Rt= 2.15 min). The total run time was 2.8 mins isocratic. The chromatographic analysis was carried out in order to quantitate absorption of vitamin B_3 present in the intestinal sacks after absorption for 90 min.

Method validation

Table 44. Calibration data proving linearity for vitamin B_3

Slope	Intercept	Correlation coefficient (r^2)
6.25×10^{-3}	2.44×10^{-2}	0.9996

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Table 45. Intra- and inter- day precision and accuracy for vitamin B_3

	Intra-day (n=3)			Inter-day (n=6)		
Expected concentration (ng/ml)	Mean concentration (ng/ml) ± SD	% CV	% Accuracy	Mean concentration (ng/ml)	% CV	% Accuracy
50	45.4 ± 1.5	3.4	90.9	46.7 ± 1.6	3.4	93.5
100	98.5 ± 0.7	0.8	98.5	98.0 ± 1.1	1.1	98.0
125	122.4 ± 2.0	1.6	98.0	121.9 ± 2.1	1.8	97.5
250	256.0 ± 2.0	0.8	102.4	254.6 ± 2.9	1.2	101.8
500	515.8 ± 12.0	2.3	103.2	514.1 ± 12.7	2.4	102.8
1000	991.6 ± 3.5	0.4	99.2	991.4 ± 17.6	1.8	99.1

Intra- and inter-day precision varied from 0.4% to 3.4% CV and 1.1% to 3.4% CV, respectively.

Intra- and inter-day accuracy ranged from 90.9% to 103.2% and 93.5% to 102.8%, respectively.

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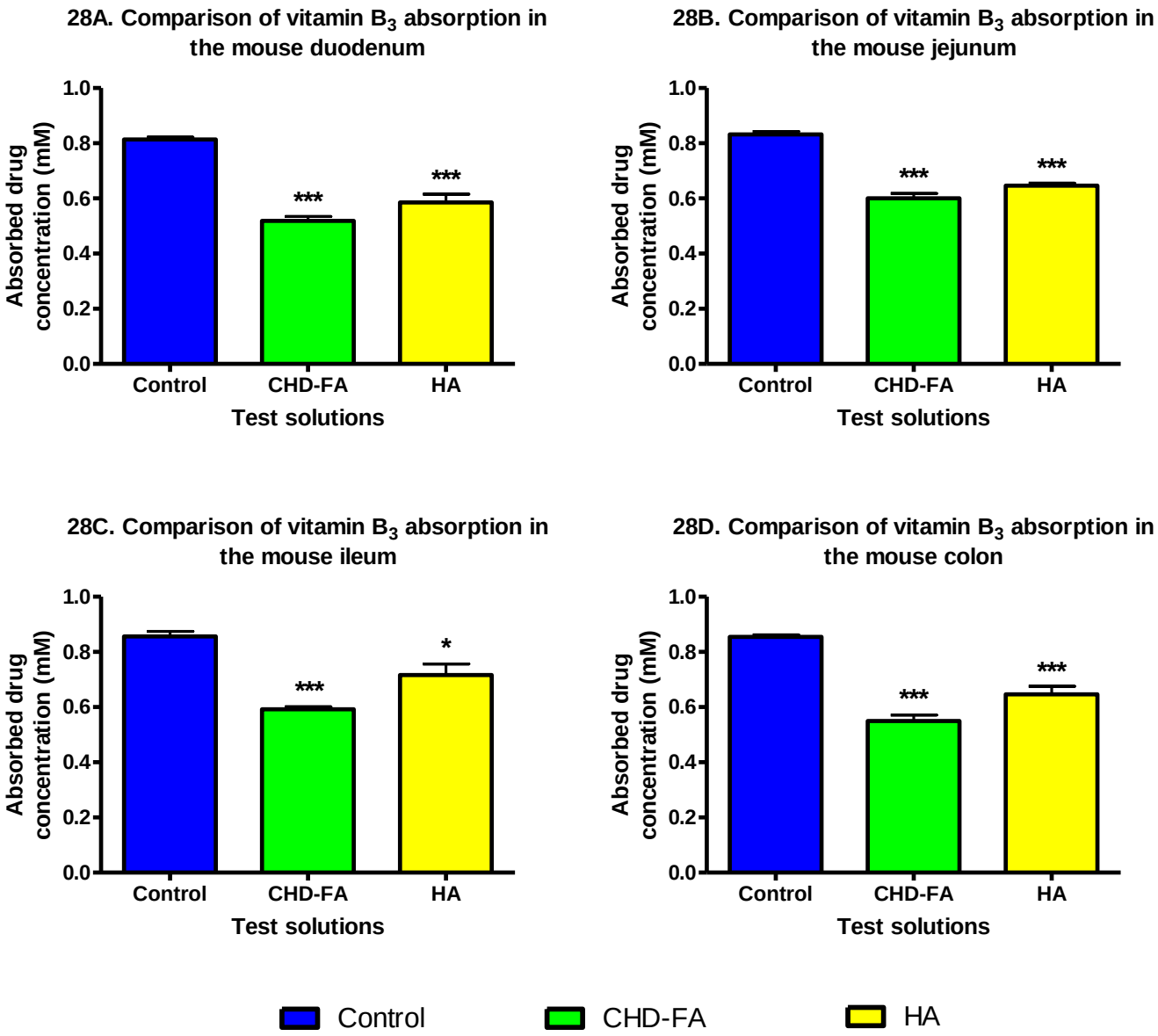

Figure 28 A-D: *Comparison of vitamin B_3 absorption in different regions of the mouse intestine (28A: duodenum, 28B: jejunum, 28C: ileum and 28D: colon). The absorption of the vitamin alone (control) was compared to the absorption of the drug in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in all segments of the intestine, (n=3).*

** = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$*

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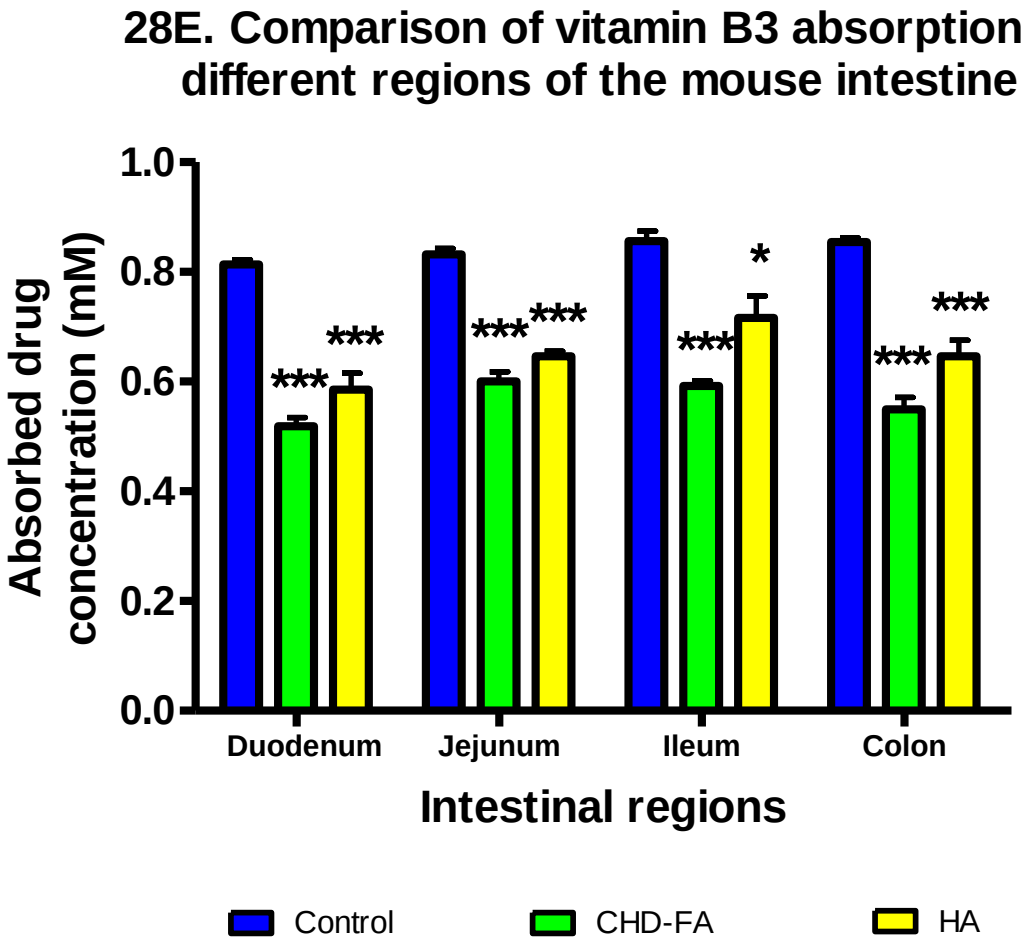

Figure 28E: Combination of graphs 28A-D showing comparison of vitamin B_3 absorption in different segments of the mouse intestine (duodenum, jejunum, ileum and colon). The absorption of the vitamin alone (control) was compared to the absorption of the drug in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in all segments of the intestine, (n=3).

** = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$*

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Table 46. Comparison of vitamin B_3 absorption in different regions of the intestine

Intestinal Region	CHD-FA	Significant	HA	Significant
Duodenum	↓ 36%	Yes	↓ 28%	Yes
Jejunum	↓ 28%	Yes	↓ 22%	Yes
Ileum	↓ 31%	Yes	↓ 24%	Yes
Colon	↓ 36%	Yes	↓ 23%	Yes

Table 51 shows the comparison of vitamin B_3 absorption in different regions of the mouse intestine in the presence of either CHD-FA or HA. The decrease seen in absorption was reported as a percentage of the control.

It is evident that the presence of both the HS resulted in decreases in the total amount of vitamin B_3 absorbed in all the regions of the intestine when compared to the control. Both of the HS elicited a consistent lower absorption trend in vitamin B_3 absorption throughout the regions of the intestine.

Absorption of vitamin B_3 in the presence of CHD-FA was seen to decrease in all regions in comparison to the control. Decreases ranged from 28% in the jejunum to 36% in the duodenum and colon compared to control samples where no HS was present. Significant decreases in vitamin B_3 absorption were seen in all regions.

Absorption of vitamin B_3 in the presence of HA was also seen to decrease in all regions in comparison with the control. Decreases ranged from 22% in the jejunum to 28% in the duodenum. Significant changes in vitamin B_3 uptake were seen in all regions of the mouse intestine.

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5.2 Vitamin E (α-tocopherol)

Background

"Vitamin E" is a group of fat-soluble vitamins also referred to as tocopherol with differences in the methylation number and position on the phenyl ring. Four different forms of tocopherol exist, each having different levels of biological activity. α-Tocopherol is the only form required in the human diet (National Institutes of Health, 2014). This vitamin is present in many different dietary foods such as liver, eggs and nuts as well as various fats and oils. Like vitamin B_3, it is also a common constituent of dietary supplements.

Functions in the body

Vitamin E is a lipophilic antioxidant and can provide cells with protection against the damaging effects of free radicals (Burton & Traber, 1990).

Deficiency and high doses

A mild deficiency of this vitamin may include symptoms like muscle weakness, abnormal eye movements and problems with vision.

Vitamin E demonstrates a range of side effects, such as depressed thyroid functioning, reticulocytosis and depressed calcification in bones, at excessive concentrations when tested on chicks (March *et al.*, 1972).

Properties

Other names: α-tocopherol

Molecular mass: 430.71 g/mol

Molecular formula: $C_{29}H_{50}O_2$

pKa: 11.40

LogP: 11.90

LogD: 11.90 (pH 7.4)

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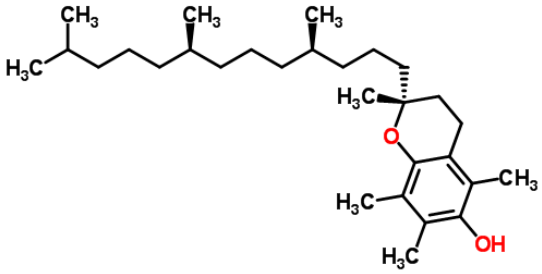

Figure 29. Molecular structure of vitamin E (ChemSpider, 2014)

LC-MS/MS analysis

The conditions and parameters for the LC-MS/MS method for the analysis of vitamin E were as follows:

Table 47. HPLC conditions for vitamin E

Mobile phase	A: Acetonitrile B: Methanol
Analytical column	Eclipse XDB-C18 (150 mm x 4.6 mm, 5µm) (Agilent)
Flow rate	1 ml/min
Injection volume	10 µl
Column temperature	45 °C
Isocratic	0 min- 5 min; 50% A

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Table 48. Compound specific mass spectrometer conditions for vitamin E

Compound	Vitamin E	Theophylline (IS)
Ionisation Mode	Positive	Positive
Precursor ion	431.7	181.0
Product ions	165.0 137.0	124.0 96.0
Declustering Potential	35 35	85 85
Collision energy	40 60	26 26

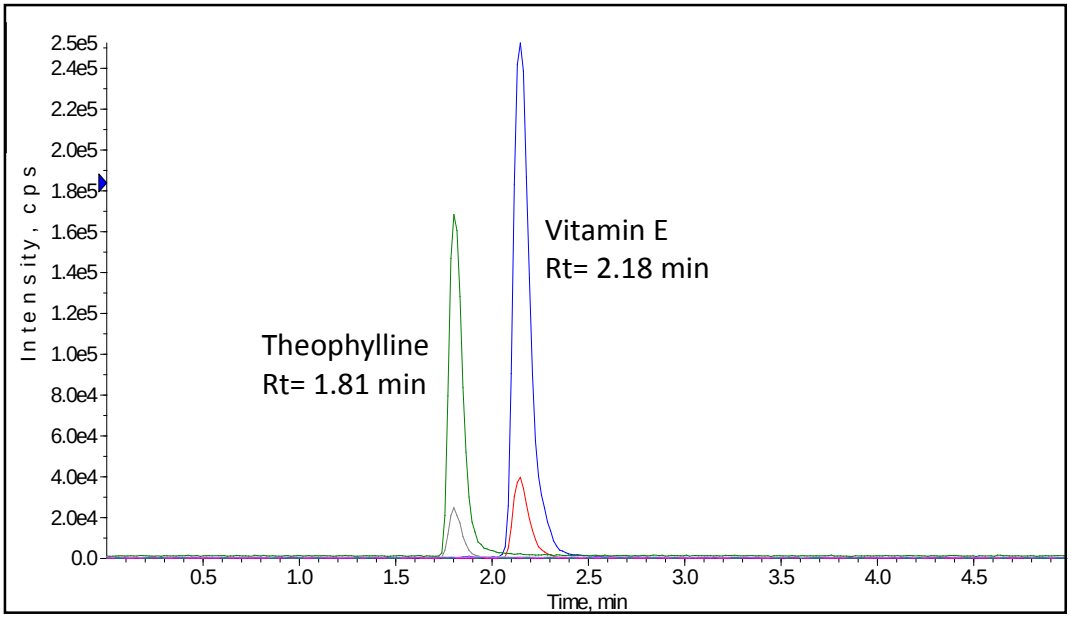

Figure 30. Reversed phased chromatography of vitamin E, retention time (Rt) of 2.18 min, using theophylline as the internal standard (IS) (Rt= 1.81 min). The total run time was 5 mins isocratic. The chromatographic analysis was carried out in order to quantitate vitamin E in the intestinal sacks after absorption for 90 min.

Method validation

Table 49. Calibration data proving linearity for vitamin E

Slope	Intercept	Correlation coefficient (r^2)
2.11×10^{-3}	6.25×10^{-2}	0.9996

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Table 50. Intra- and inter-day precision and accuracy for vitamin E

	Intra-day (n=3)			Inter-day (n=6)		
Expected concentration (ng/ml)	Mean concentration (ng/ml) ± SD	% CV	% Accuracy	Mean concentration (ng/ml)	% CV	% Accuracy
25	22.7 ± 0.5	2.1	90.1	25.00 ± 0.6	2.3	99.9
50	48.1 ± 1.1	2.3	96.2	49.7 ± 1.0	2.1	99.4
125	122.1 ± 6.3	5.2	97.7	119.1 ± 2.7	2.3	95.3
250	257.0 ± 7.6	3.0	102.8	257.0 ± 10.8	4.2	102.8
500	512.7 ± 10.6	2.1	102.5	506.8 ± 10.4	2.0	101.4
1000	994.6 ± 12.5	1.3	99.5	998.9 ± 13.6	1.4	99.9

Intra- and inter- day precision varied from 1.3% to 5.2% CV and 1.4% to 4.2% CV, respectively.

Intra- and inter-day accuracy ranged from 90.1% to 102.8% and 95.3% to 102.8%, respectively.

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Vitamin E was selected as a test compound in this study in order to assess the intestinal uptake of a highly lipophilic fat soluble vitamin in the presence of CHD-FA and HA.

Challenges were faced when attempting to solubilize the fat soluble vitamin in the KRB solution. Following the addition of vitamin E to the KRB solution, the large majority of the vitamin was found to be insoluble and remain floating on the surface. Despite using vigorous magnetic stirring with gentle heating for 20 min then further sonicated for 20 min to aid solubility, the bulk of the vitamin remained insoluble.

Due to the highly lipophilic nature of vitamin E (LogD 11.90), better solubility would be expected in a more nonpolar solution as opposed to the highly aqueous KRB solution. However the everted mouse gut model is used to mimic physiological conditions and KRB at pH 7.4, has been demonstrated to retain proper physiological functioning of the intestinal tissue for extended time. Deviations in the composition of this buffer solution, resulting in changes in the polarity and pH, would result in undesirable environmental conditions that negatively affect the ability and time that intestinal transport would still represent the normal physiological process after excision from the mouse. Thus changes to the KRB solution could not be made in order to accommodate better solubility of very lipophilic compounds such as vitamin E.

Following LC-MS/MS analysis, the quantity of vitamin E in all samples was found to be well below the limit of quantitation due to the poor solubility of the vitamin. Results for the absorption of vitamin E could thus not be obtained.

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5.3 Discussion

Vitamins are necessary to maintain normal physiological functioning in the body. There are thirteen principal vitamins, falling under two different groups depending on their solubility characteristics: water soluble and fat soluble vitamins. Humans must ensure regular intake of both water and fat soluble vitamins from their diet and are therefore commonly acquired through ingestion of vitamin supplements. Chronic low levels of vitamins as well as excessively high levels of vitamins alter normal physiological functioning and both conditions are accompanied by a range of negative symptoms.

In this study, the overall increase or decrease in the total amount of one water-soluble and one fat-soluble vitamin absorbed in the presence of CHD-FA and HA was assessed.

Water-soluble vitamin: Vitamin B_3

Vitamin B_3 is an essential vitamin required for various important anabolic and catabolic reactions, the synthesis of certain hormones as well as ensuring proper brain functioning.

Decreases in vitamin B_3 absorption was observed for all regions of the intestine when in the presence of either HS when compared to the control. Decreases in the presence of CHD-FA and HA ranged from 28% to 36% and 22% to 28% respectively.

Vitamin B_3 is a weakly acidic (pKa=2.79), water soluble vitamin. At pH 7.4, Vitamin B_3 is highly hydrophilic (LogD=-2.93). Thus, paracellular transport through the tight junctions or active transport would be the most likely mechanisms of transport for this vitamin in this experiment.

A constant trend of significant decreases in absorption was seen throughout the intestine in the presence of both CHD-FA and HA. This consistent decrease in absorption could be due to solubility changes of the vitamin once in complex.

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In order to promote general health, a large portion of the population ingests vitamin supplements on a daily basis. As CHD-FA and HA are also thought to possess various health benefits, it would be natural to assume that combining these compounds into a single formulation would result in an improved health supplement. However, the presence of both HS was seen to have a significant decrease on the absorption of vitamin B_3 and it is thus recommended that these compounds should not be administered within two hours of each other and should definitely not be formulated into a single health supplement.

Furthermore, ingestion of preparations containing CHD-FA and HA by patients may result in an overall decrease in absorption of vitamin B_3 from a standard diet. A decrease of this scale may not have an immediate marked effect on a healthy patients' physiological functioning, however this decrease may add to negative physiological symptoms in patients lacking a consistent and well-balanced diet. Small decreases in the amount of the vitamin absorbed in patients already prone to decreased absorption, may exacerbate negative symptoms. These patients include the elderly or patients suffering from various chronic GIT disorders.
Supplements containing CHD-FA and HA should not be ingested within two hours of a meal or vitamin supplements containing vitamin B_3.

Fat-soluble vitamin: Vitamin E

Due to the insolubility of the highly lipophilic vitamin in the aqueous KRB solution, results for vitamin E could not be obtained. Multiple attempts to solubilize the vitamin without solvents or solubilisation enhancers were carried out with no success.
The buffer conditions used for suspending the everted mouse gut cannot be altered without sacrificing the integrity of the tissue which would change the absorptive capabilities of the intestinal tissue, thus this absorption model will show little success when used to assess the absorption of highly lipophilic compounds.

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Chapter 6

6. Minerals

Similar to vitamins, the constant intake of minerals is vital for maintaining proper physiological functioning of the human body. There are two main categories of minerals: major (macro) minerals and trace (micro) minerals. Major minerals are required by the body in relatively large amounts and include calcium, phosphorous, sodium and magnesium. Only small quantities of trace minerals are required in the body, these include iron, copper, cobalt, potassium, iodine, zinc, manganese, molybdenum, fluoride, chromium, selenium and sulphur (Soetan *et al.*, 2010). Minerals cannot be synthesized in the body, thus adequate amounts are needed to be taken in through external sources through a balanced diet or additional supplementation.

As with vitamins, a mineral deficiency can occur when the RDI of a mineral is not provided over a prolonged period of time. Symptoms of mineral deficiencies are a common occurrence, especially in poorer regions or developing countries. Anaemia and osteoporosis are common results of deficiencies resulting from insufficient intake of iron and calcium respectively. These disorders are not only found in poorer regions or developing nations, but are also common in first world countries (Injrmary, 2001; Killip *et al.*, 2007). Often, in order to combat mineral deficiencies, various mineral supplements are ingested on a regular basis.
It is also possible to ingest an excess amount of mineral and is common due to excessive mineral supplementation. These high doses may result in toxicity and give rise to various negative symptoms depending on the mineral.

It has been seen that the presence of humic substances in soil promotes the uptake of minerals by plants and has a positive effect on overall growth (Eyheraguibel *et al.,* 2008). This increased mineral uptake could be due to binding of the mineral with the HS. Reactions of HS with metal ions result in the formation of stable metal-HS complexes through chelation (Pandey *et al.*, 2000). It is likely that

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chelation reactions will occur in the human GIT when these substances are concurrently ingested.

For this study, the effect of fulvic and humic acids on the absorption of two major minerals (calcium and magnesium) and two trace minerals (zinc and iron(II)/ iron(III)) were assessed. The absorption assays were carried out in the same way as for the drugs and the vitamins but the analysis of the samples was done using ICP-MS methodology that required digestion of the organic compounds with analytical grade nitric acid. The prepared samples were sent to a contract laboratory for the quantitative analysis of the minerals.

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6.1 Major minerals

6.1.1 Calcium

Calcium is the most abundant and one of the most important minerals in the human body. It is found in a variety of food sources, such as: dairy products, green leafy vegetables, nuts and fish. The primary function of calcium in the body is to form and maintain healthy bones and teeth; however, it also plays an important role in the blood clotting process facilitating the conversion of prothrombin to thrombin and enzyme activation in a number of metabolic pathways. Furthermore, calcium is required for membrane permeability, to facilitate muscle contractions as well as ensuring normal nerve and brain function.

The calcium RDI for adults over the age of 18 is approximately 1000 mg/day, with an UL of 2500 mg/day.

Calcium absorption following ingestion is relatively poor, with only 30%, on average, being absorbed in the human GIT. Age and gender have a direct effect on calcium uptake, with decreased absorption being seen in females over 50 years, as well as both males and females over the age of 70 years (National Institutes of Health, 2014). In order to counteract this poor absorption, a range of dietary supplements containing calcium are freely available and may be additionally ingested.

Deficiency and high doses

Short term calcium deficiency does not result in any noticeable symptoms. However, a chronic calcium deficiency gives rise to symptoms such as bone softening leading to rickets, osteoporosis, lethargy, poor appetite, as well as muscle weakness and cramps. Postmenopausal as well as amenorrheic women are at risk of calcium deficiencies due the negative calcium balance effect brought on by reduced levels of estrogen (National Institutes of Health, 2014). Other groups prone to a calcium deficiency include lactose intolerant individuals, the elderly and vegetarians.

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Excessively high levels of calcium in the body may result in renal insufficiency, gastrointestinal disturbances, kidney stones, memory loss, depression as well as bone aches and pains. Calcium levels above the UL are seldom a result of excessive calcium obtained from the diet, but rather from the use of calcium containing supplements.

The results of the absorption assays are shown in Figures 31A -31E below.

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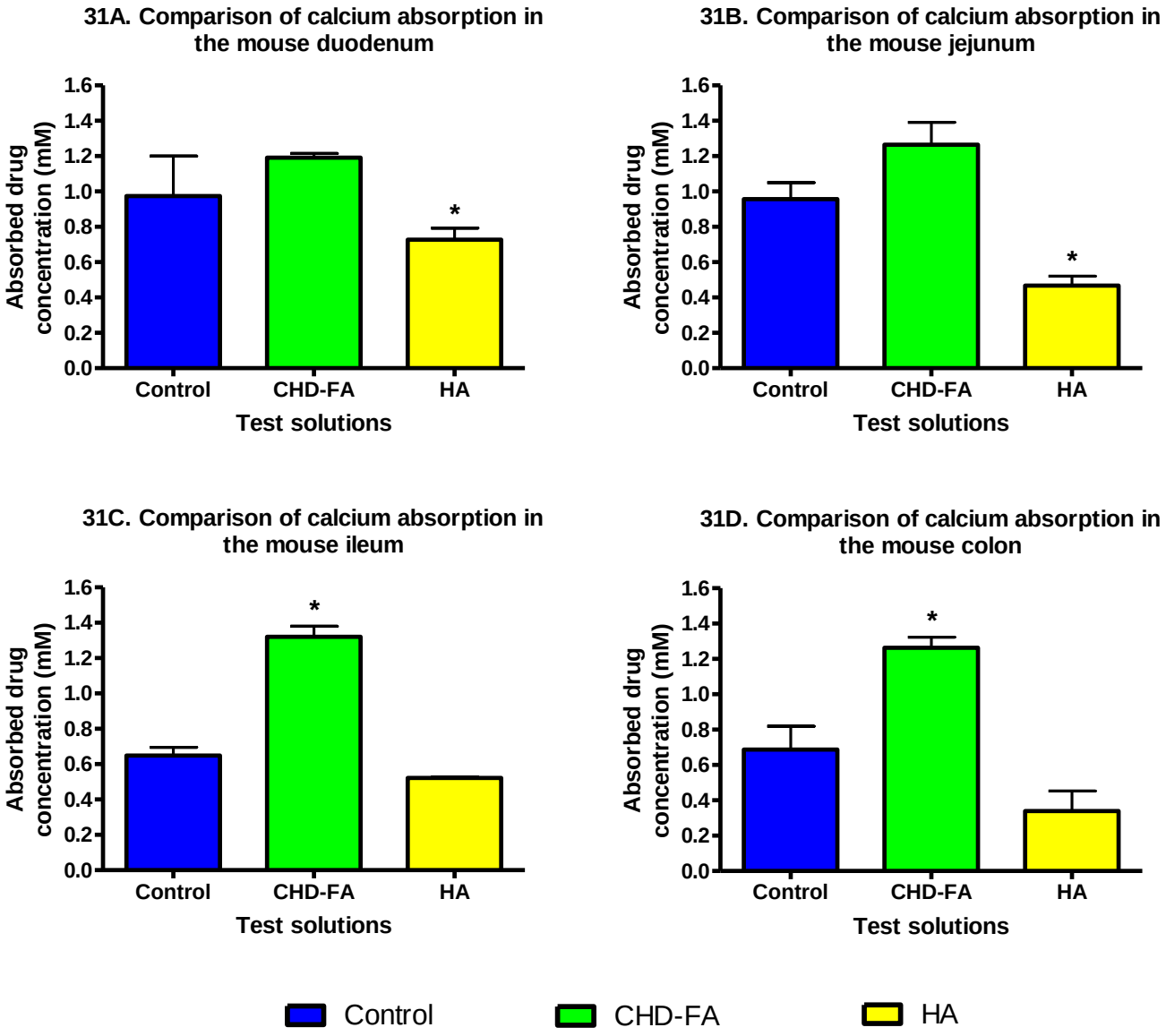

Figure 31 A-D: *Comparison of calcium absorption in different regions of the mouse intestine (31A: duodenum, 31B: jejunum, 31C: ileum and 31D: colon). The absorption of the mineral alone (control) was compared to the absorption of the mineral in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in all segments of the intestine, (n=3).*

*= $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$*

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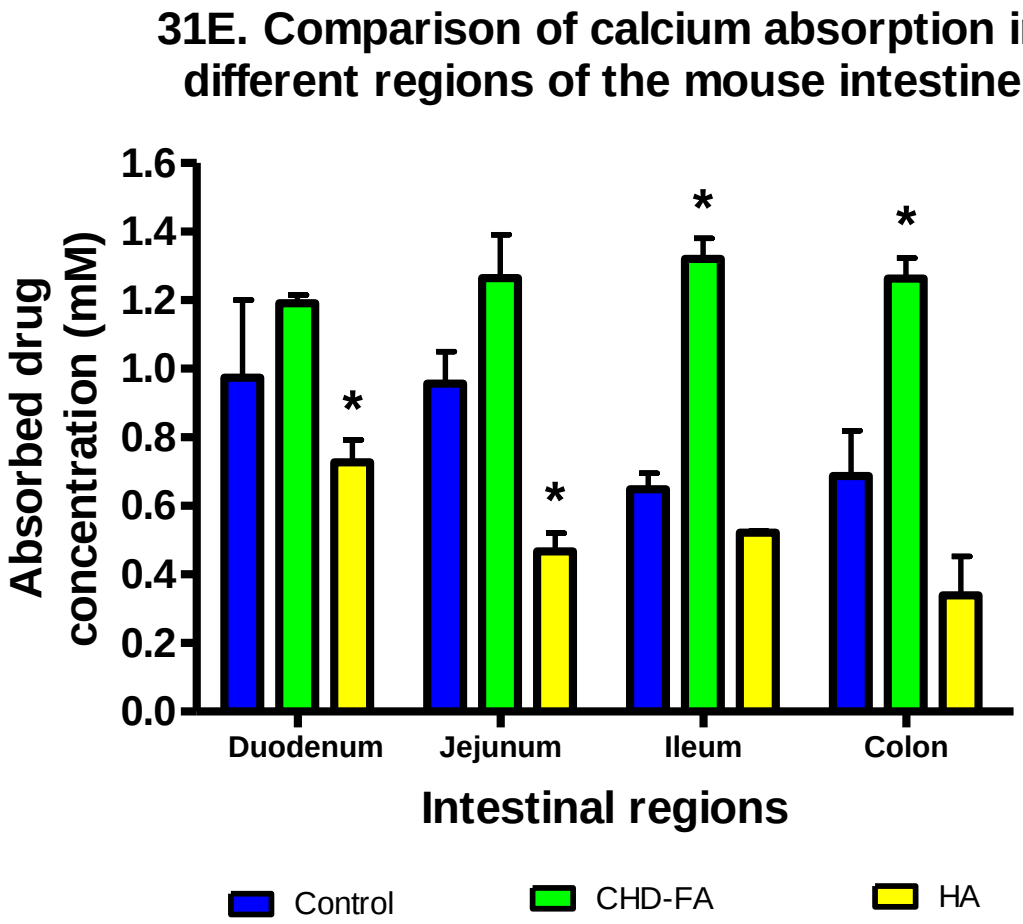

Figure 31E: Combination of graphs 231A-D showing comparison of calcium absorption in different segments of the mouse intestine (duodenum, jejunum, ileum and colon). The absorption of the mineral alone (control) was compared to the absorption of the mineral in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in all segments of the intestine, (n=3).

*= $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$*

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Table 51. Comparison of calcium absorption in different regions of the intestine

Intestinal Region	CHD-FA	Significant	HA	Significant
Duodenum	↑ 22%	No	↓ 25%	Yes
Jejunum	↑ 32%	No	↓ 51%	Yes
Ileum	↑ 104%	Yes	↓ 20%	No
Colon	↑ 84%	Yes	↓ 51%	No

Table 56 shows the comparison of calcium absorption in different regions of the mouse intestine in the presence of either CHD-FA or HA. The increases or decreases seen in absorption were reported as a percentage of the control.

It is evident that the presence of both the HS resulted in changes in the total amount of calcium absorbed in certain regions of the intestine when compared to the control. Opposite trends in absorption of calcium were seen in the presence of fulvic acid verses humic acid.

Absorption of calcium in the presence of CHD-FA was seen to increase in all regions in comparison to the control. Increases ranged from 22% in the duodenum to 104% in the ileum. Only the increases in calcium absorption in the ileum and colon showed significant changes.

Absorption of calcium in the presence of HA was seen to decrease in all regions in comparison with the control. Decreases ranged from 20% in the ileum to 51% in the jejunum and colon. Significant changes in calcium uptake were only seen in the duodenum and jejunum.

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6.1.2 Magnesium

Magnesium is widely distributed in various food sources, particularly in green leafy vegetables, legumes, nuts and whole grains.

This macromineral functions as a coenzyme in numerous biochemical processes in the body including protein synthesis, glucose control and energy production. Magnesium also plays an important role in maintaining normal muscle and nerve functioning. Like calcium, it contributes largely to the formation of healthy bones and teeth.

The magnesium RDI for adults over the age of 18 is approximately 300-400 mg/day, with an UL of 350 mg/day. The UL for magnesium is found to be less than or equal to the RDI in certain gender-age combinations further highlighting the uncertainty of dietary reference intake tables.

A study by Baily *et al.* in 2011 (Bailey *et al.*, 2011) showed that the amount of magnesium obtained from a normal diet alone was below the RDI for both males and females. Only with additional magnesium supplementation were the levels comparable to the RDI of the mineral.

Deficiency and high doses

It is uncommon for a magnesium deficiency to be the result of poor dietary intake alone, as the kidneys have been shown to be efficient in regulating magnesium loss (Fitzgerald & Fourman, 1956). A deficiency in magnesium can however be a result of poor dietary intake in combination with certain disorders and health conditions. Specific groups at risk of magnesium deficiencies include individuals suffering from GIT diseases (such as Crohn's disease, celiac disease and enteritis) due to inhibited absorption, individuals suffering from type 2 diabetes who show higher than average magnesium excretion (Tosiello, 1996), individuals with chronic alcohol dependency (Kalbfleisch *et al.*, 1963) and the elderly.

Symptoms of short term magnesium deficiency may include loss of appetite, fatigue, agitation, anxiety, nausea and vomiting. A prolonged shortage results in more serious effects such as abnormal heart rhythms, seizures, muscle weakness

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and cramps. Certain groups of the population which are more at risk of a magnesium deficiency include individuals suffering from GIT diseases, type 2 diabetes, alcoholics and the elderly ("National Institutes of Health," 2013).

Excessive intake of magnesium in the body can cause various GIT disturbances such as diarrhoea, nausea and vomiting. If extremely high doses are present, more severe symptoms are seen in the form of respiratory paralysis, extreme low blood pressure, cardiac arrhythmias, cardiac arrest leading to death.

The results of the absorption assay for magnesium in the presence of either of the two HS compared to the control are shown in Figures 32A – 32E below.

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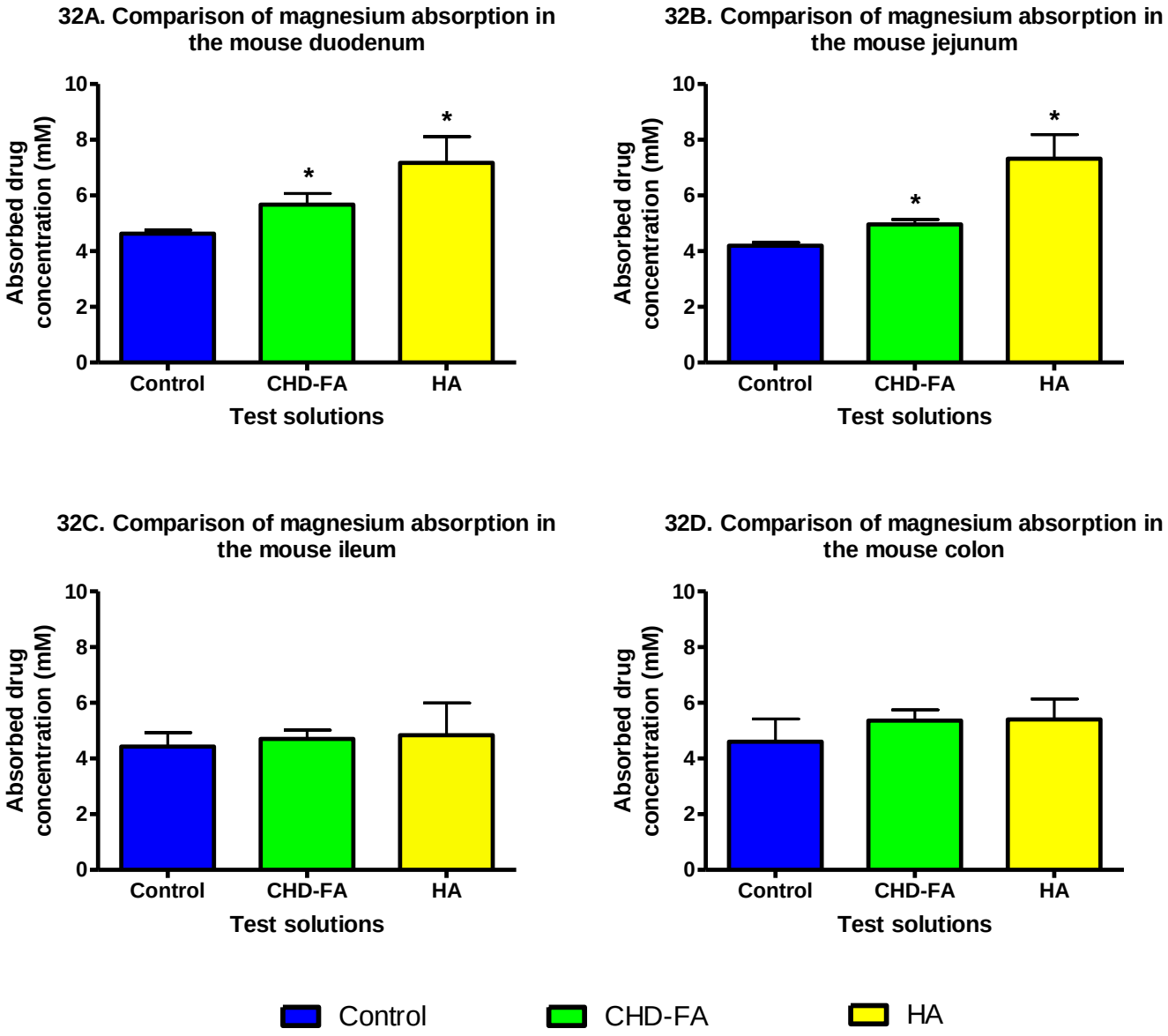

Figure 32 A-D: Comparison of magnesium absorption in different regions of the mouse intestine (32A: duodenum, 32B: jejunum, 32C: ileum and 32D: colon). The absorption of the mineral alone (control) was compared to the absorption of the mineral in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in certain segments of the intestine, (n=3).

*= $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$*

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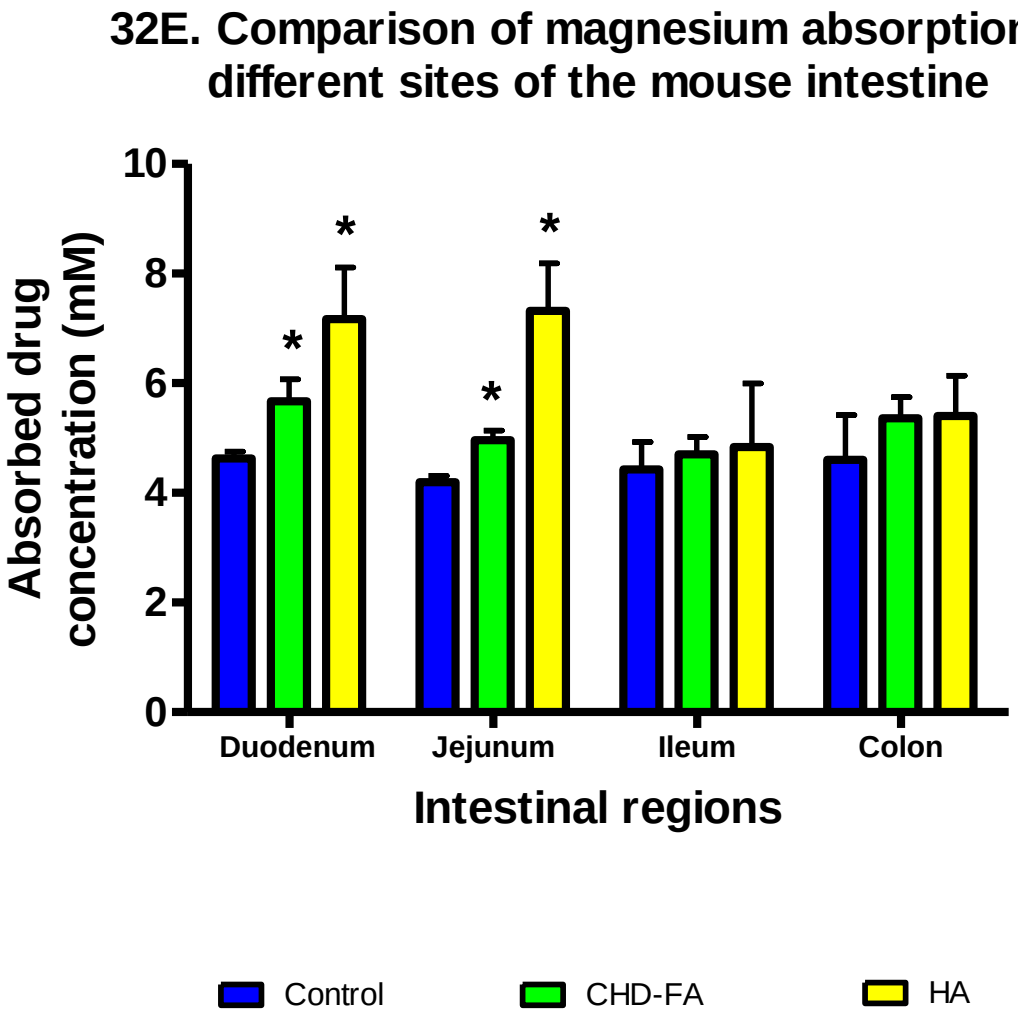

Figure 32E: Combination of graphs 32A-D showing comparison of magnesium absorption in different segments of the mouse intestine (duodenum, jejunum, ileum and colon). The absorption of the mineral alone (control) was compared to the absorption of the mineral in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in certain segments of the intestine, (n=3).

*= $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$*

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Table 52. Comparison of magnesium absorption in different regions of the intestine

Intestinal Region	CHD-FA	Significant	HA	Significant
Duodenum	↑ 22%	Yes	↑ 54%	Yes
Jejunum	↑ 18%	Yes	↑ 74%	Yes
Ileum	↑ 6%	No	↑ 9%	No
Colon	↑ 16%	No	↑ 17%	No

Table 57 shows the comparison of magnesium absorption in different regions of the mouse intestine in the presence of either CHD-FA or HA. The increases seen in absorption were reported as a percentage of the control.

It is evident that the presence of both the HS resulted in consistent increases in the total amount of magnesium absorbed in all regions of the GIT when compared to the control.

Absorption of magnesium in the presence of CHD-FA was seen to increase in all regions in comparison to the control. Increases ranged from 6% in the ileum to 22% in the duodenum. Significant increases in magnesium absorption were seen in the duodenum and jejunum only.

Absorption of magnesium in the presence of HA was seen to increase in all regions in comparison to the control. Increases ranged from 9% in the ileum to 74% in the jejunum. Significant changes in magnesium uptake were seen in the duodenum and jejunum only which was the same as for the fulvic acid treated samples.

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6.2 Trace minerals

6.2.1 Iron

Iron is an important minor mineral in the human body and is essential for the formation of certain proteins as well as metabolic pathways. Iron is a component of the protein haemoglobin, which is responsible for the transport of oxygen from the lungs to the tissues and to the muscles respectively. Iron is also necessary for proper growth and development, normal cellular functioning, formation of neurotransmitters, production of connective tissue and plays a role in maintaining a healthy immune system.

Dietary iron is available in two forms, haeme and nonhaeme. Haeme iron is derived from haemoglobin and myoglobin found in meat, seafood and poultry. Nonhaeme iron can be found in plants. Haeme iron has a higher estimated absorption (15-35%) than nonhaeme iron (3- 20%), most likely due to differences in mechanisms of absorption.
The RDI for adults over the age of 18 years is approximately 8 -18 mg/day, with a UL of 45 mg/day.

Deficiency and high doses

Iron deficiencies are largely associated with poor diet, disorders causing malabsorption (celiac disease, ulcerative colitis and Crohn's disease) and blood loss. A chronic low intake of iron is the primary cause for anaemia which is associated with symptoms such as GIT disturbances, as well as impaired cognitive and immune functioning.

The majority of healthy individuals take in an adequate amount of iron from their diets, however there are certain groups within the population which are more at risk of suffering from an iron deficiency, such as: young children, girls in their teens, pregnant women and premenopausal women (Blanck *et al.*, 2005; Mei *et al.*, 2011). Additional iron supplementation is often required for these groups.

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Healthy adults with normal GIT functioning have little risk of suffering from the iron overdose effects obtained through the diet. However, toxic symptoms of excessive iron, such as, gastric upset, constipation, abdominal pain and vomiting are often seen when too much iron is ingested through supplements or medication. Hemochromatosis is an inherited genetic disease associated with excessive iron accumulation in the body. Due to a much greater daily absorption of iron, an accumulation of the mineral forms in the body and can result in severe symptoms such as heart failure, cirrhosis of the liver, and diabetes. Additional supplementation in this group must be strictly avoided.

Iron supplementation

Iron is present in one of two oxidation states: iron(II) (ferrous) and iron(III) (ferric). Ferrous iron is fairly soluble in the aqueous solutions of any pH, including the acidic environment of the stomach and the alkaline environment of the intestine. However when ferric iron is in an aqueous solution, it may complex to produce ferric hydroxide, an insoluble complex which easily precipitates out of aqueous solutions, greatly lowering the potential for absorption in the body.

Alternatively, ferric iron may undergo a reduction reaction to ferrous iron by one or more reductases present in the intestines in order to be absorbed by the enterocyte (Miret *et al.*, 2003). Nonhaeme iron is predominantly present as ferric iron in food and thus must undergo this reaction in order to be absorbed through the diet (Santiago, 2012). The need for reduction prior to absorption is a contributing factor to the lower absorption levels seen for nonhaeme iron in comparison to haeme iron.

Although iron supplements are available in both ferric and ferrous forms, supplements containing ferrous iron are preferred as the bioavailability is 3-4 times higher than that of supplements containing ferric iron (Nagpal & Choudhury, 2004).

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In this study, the absorption of both iron(II) (ferrous) and iron(III) (ferric) iron was assessed. Iron(III) was included in this study, as a substance with well-known poor solubility and uptake in an aqueous solution, in order to assess the effects that binding with the HS would elicit.

The results of the absorption assay for ferrous iron in the presence of either of the two HS compared to the control are shown in Figures 33A – 33E below and the results of the absorption assay for ferric iron in the presence of either of the two HS compared to the control are shown in Figures 34A – 34E below.

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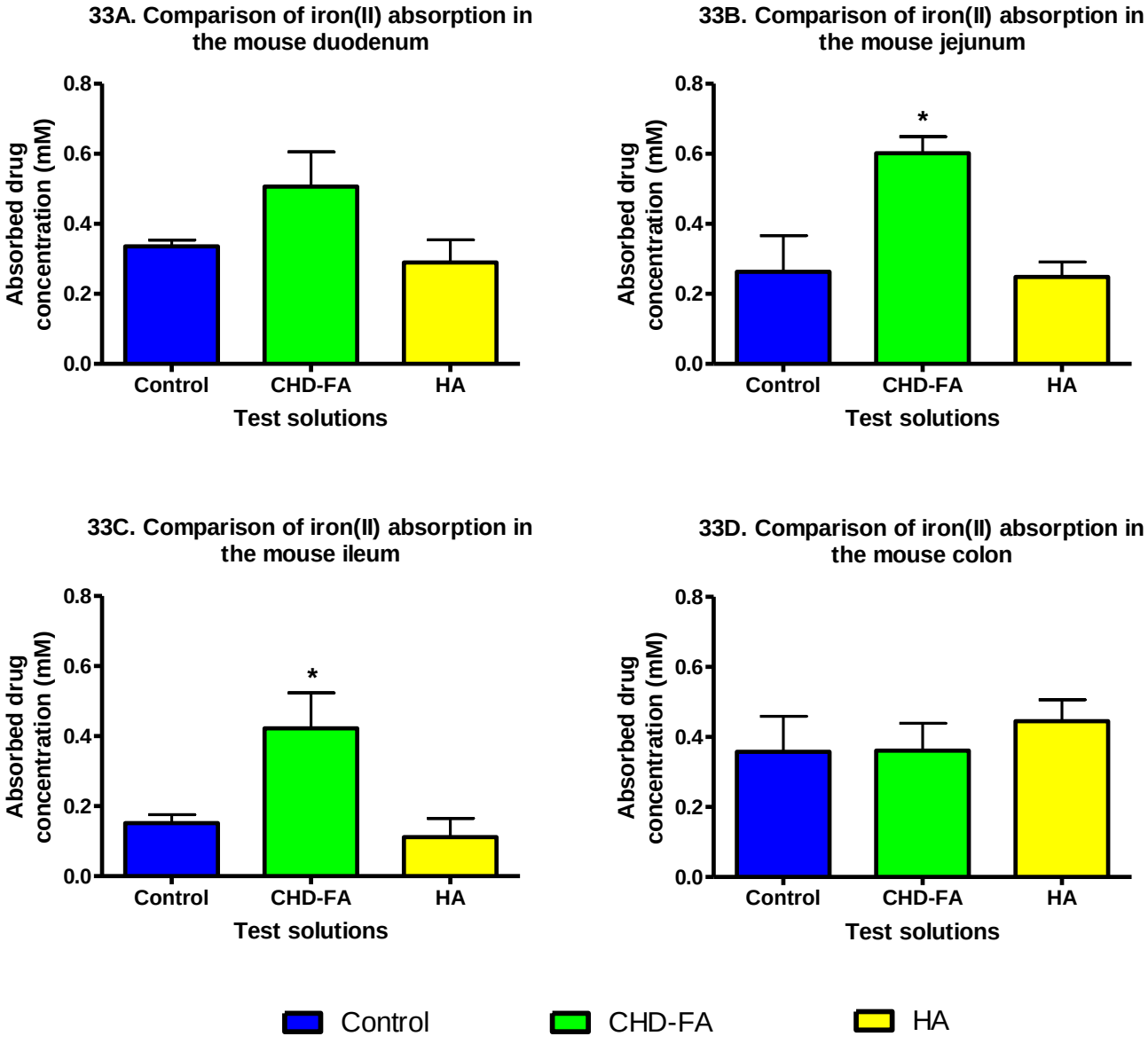

Figure 33 A-D: Comparison of iron(II) absorption in different regions of the mouse intestine (33A: duodenum, 33B: jejunum, 33C: ileum and 33D: colon). The absorption of the mineral alone (control) was compared to the absorption of the mineral in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in certain segments of the intestine, (n=3).

*= $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$*

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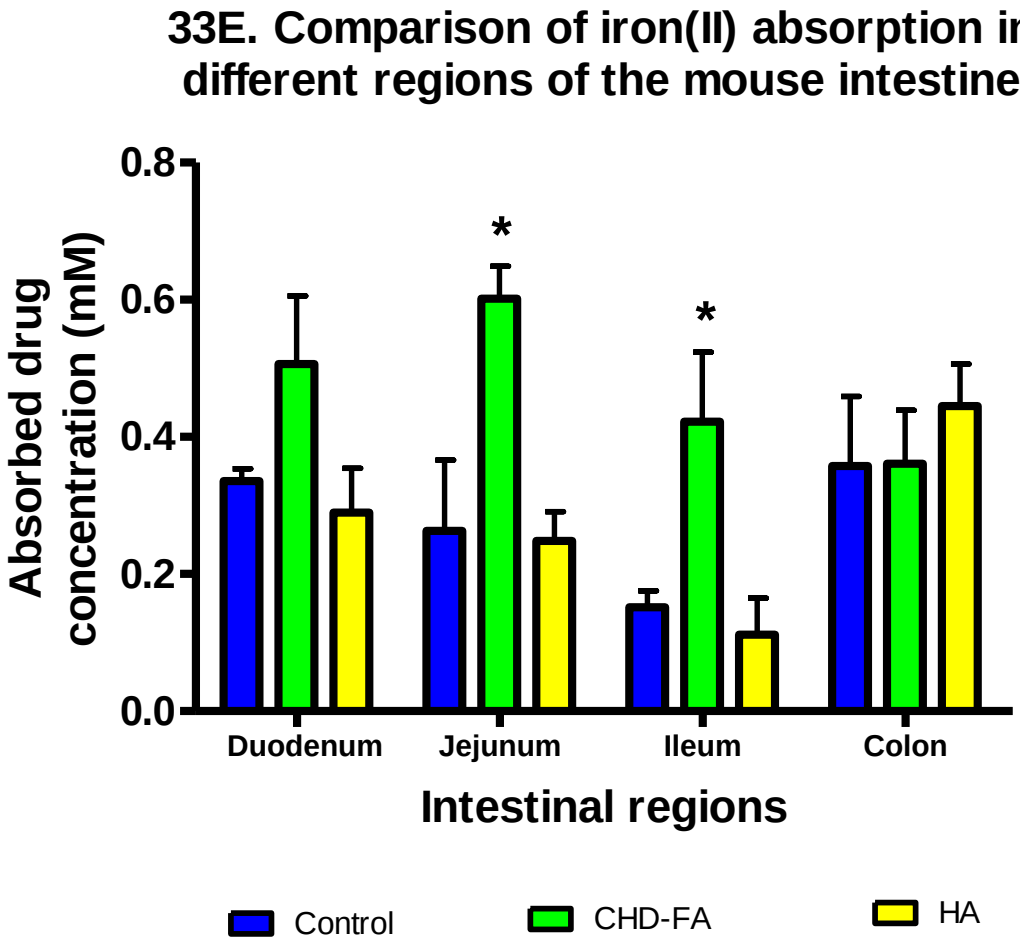

Figure 33E: Combination of graphs 33A-D showing comparison of iron(II) absorption in different segments of the mouse intestine (duodenum, jejunum, ileum and colon). The absorption of the mineral alone (control) was compared to the absorption of the mineral in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in certain segments of the intestine, (n=3).

*= $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$*

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Table 53. Comparison of iron(II) absorption in different regions of the intestine

Intestinal Region	CHD-FA	Significant	HA	Significant
Duodenum	↑ 50%	No	↓ 13%	No
Jejunum	↑ 128%	Yes	↓ 6%	No
Ileum	↑ 178%	Yes	↓ 26%	No
Colon	↑ 1%	No	↑ 25%	No

Table 58 shows the comparison of iron(II) absorption in different regions of the mouse intestine in the presence of either CHD-FA or HA. The increases and decreases seen in absorption were reported as a percentage of the control.

It is evident that the presence of both the HS resulted in changes in the total amount of iron(II) absorbed in all regions of the intestine when compared to the control. These changes were seen in the majority of segments of the intestine, with the fulvic and humic acids showing an opposite trend in iron(II) uptake.

Absorption of iron(II) in the presence of CHD-FA was seen to increase in all regions in comparison to the control, with considerable increases in the first three regions. Absorption in the colon was seen to match that of the control. Increases ranged from 50% in the duodenum to 178% in the ileum. However, significant increases in iron(II) absorption were seen in the jejunum and ileum only.

Absorption of iron(II) in the presence of HA was seen to decrease in the first three regions and increase in the colon only. Decreases ranged from 6% in the jejunum to 26% in the ileum. Iron(II) uptake in the presence of HA demonstrated no statistically significant absorption changes which is probably due to the small sample size and fairly large variability within the method.

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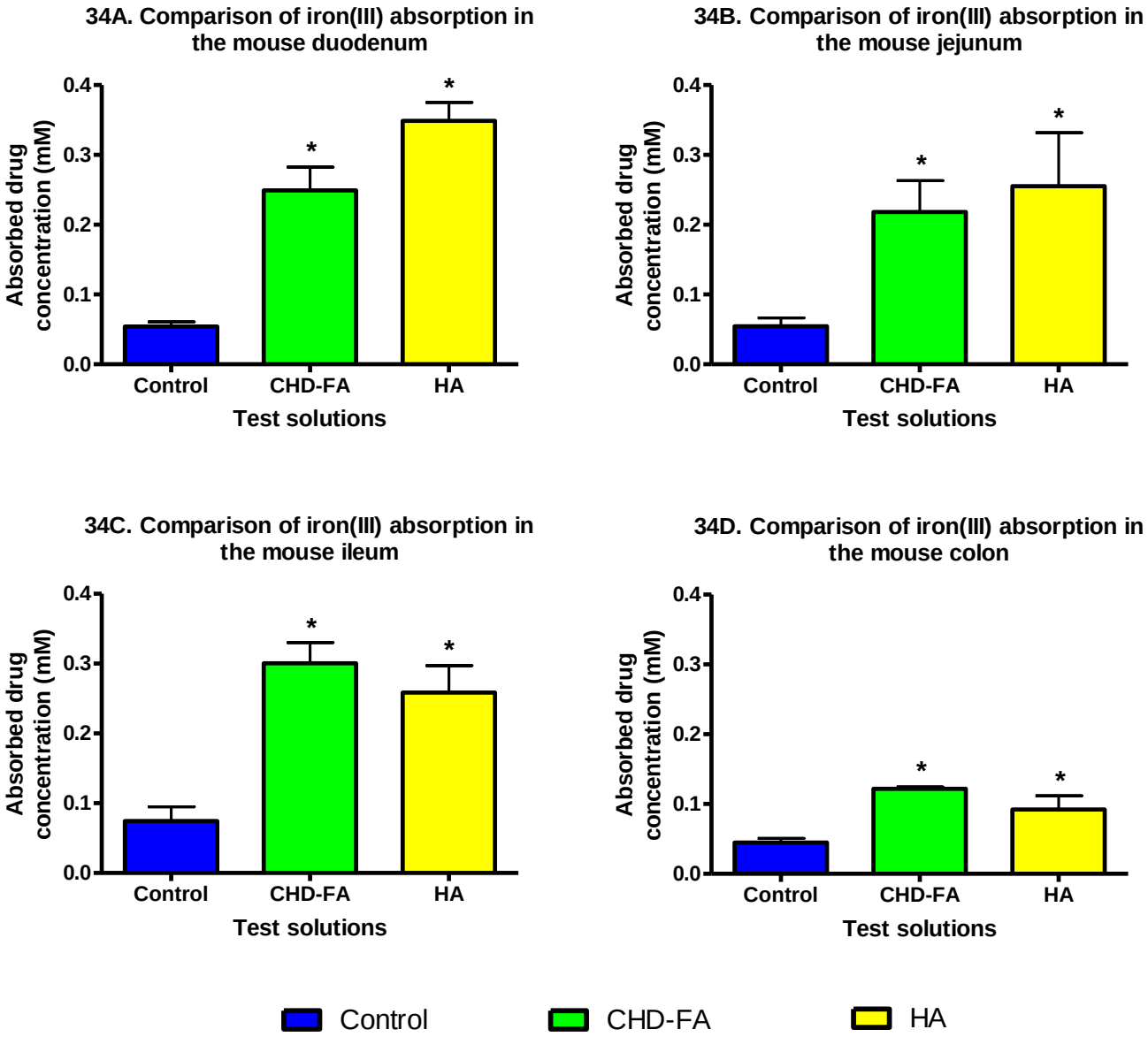

Figure 34 A-D: *Comparison of iron(III) absorption in different regions of the mouse intestine (34A: duodenum, 34B: jejunum, 34C: ileum and 34D: colon). The absorption of the mineral alone (control) was compared to the absorption of the mineral in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in all segments of the intestine, (n=3).*

*= $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$*

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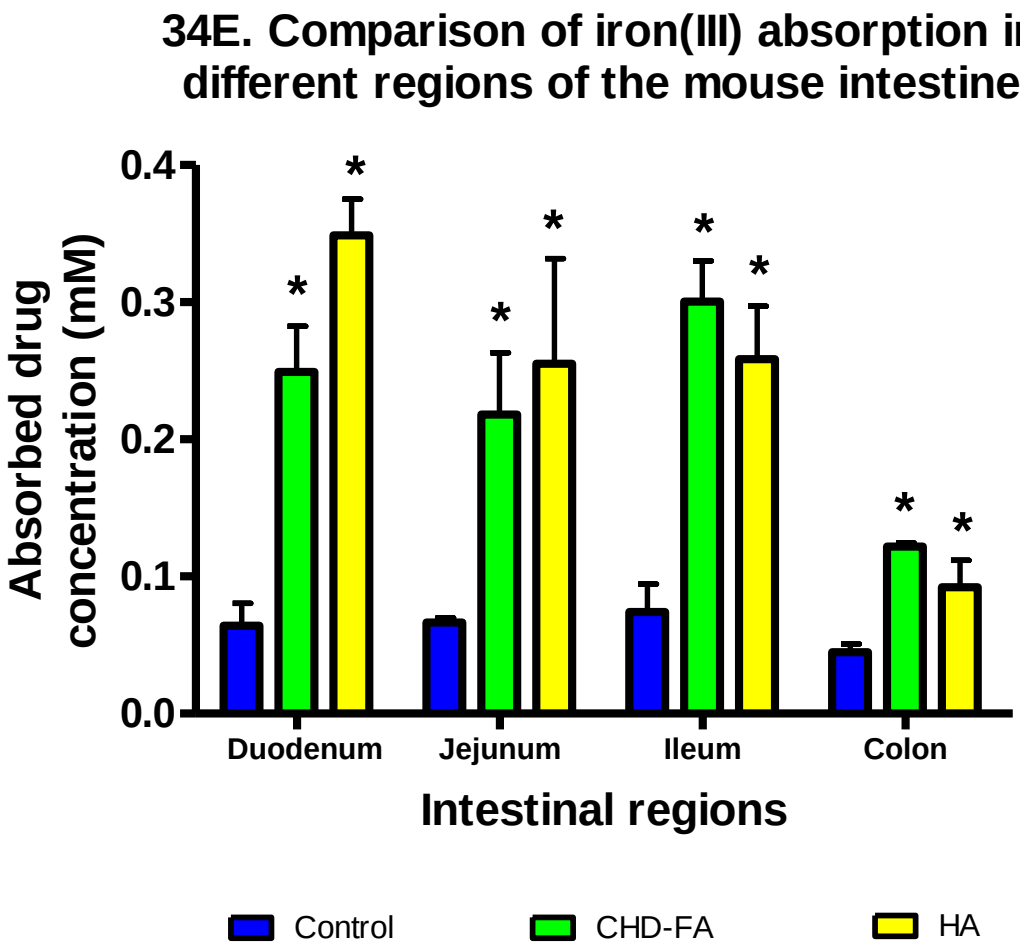

Figure 34E: Combination of graphs 34A-D showing comparison of iron(III) absorption in different regions of the mouse intestine (duodenum, jejunum, ileum and colon). The absorption of the mineral alone (control) was compared to the absorption of the mineral in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in all regions of the intestine, (n=3).

** = $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$*

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Table 54. Comparison of iron(III) absorption in different regions of the intestine

Intestinal Region	CHD-FA	Significant	HA	Significant
Duodenum	↑ 289%	Yes	↑ 444%	Yes
Jejunum	↑ 229%	Yes	↑ 284%	Yes
Ileum	↑ 304%	Yes	↑ 248%	Yes
Colon	↑ 172%	Yes	↑ 105%	Yes

Table 59 shows the comparison of iron(III) absorption in different regions of the mouse intestine in the presence of either CHD-FA or HA. The increases seen in absorption were reported as a percentage of the control.

It is evident that the presence of both the HS resulted in large increases in the total amount of iron(III) absorbed in all regions of the intestine when compared to the control. These changes were seen in all the regions of the intestine, with this trend in iron(III) uptake seen in the presence of both HS.

Absorption of iron(III) in the presence of CHD-FA was seen to increase considerably in all regions in comparison to the control. Increases ranged from 172% in the colon to 304% in the ileum. All increases in iron(III) absorption observed in all GIT regions were significant for both HS.

Absorption of iron(III) in the presence of HA was also seen to increase considerably in all regions in comparison to the control. Increases ranged from 105% in the colon to 444% in the duodenum. Significant increases in iron(III) absorption were seen in all regions.

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6.6.2 Zinc

Zinc is an essential trace mineral vital for a range of biological functions. It is found in cells throughout the body and plays an important role in the formation of various enzymes and other proteins as well as genetic material. Furthermore, zinc is required to maintain a healthy immune system as is partly responsible for wound healing by contributing to cell growth and division. The trace mineral also assists with taste and smell. Key sources of dietary zinc can be obtained through meat, poultry, fish, seafood, cereals and dairy products.

The RDI for zinc in adults over the age of 18 is approximately 8-11 mg/day, with an UL of 40 mg/day.

Deficiency and high doses

Zinc intake among older adults was below the recommended average, even with additional supplementation (National Institutes of Health, 2014). Furthermore certain population groups are more at risk of experiencing a zinc deficiency, these include individuals suffering from GIT disorders, vegetarians as well as pregnant and lactating women. Short term symptoms of decreased zinc intake may include loss of appetite, delayed wound healing, problems with taste and impaired immune function, whereas a chronic shortage may result in growth retardation, delayed sexual maturation and hypogonadism in males.

Acute zinc toxicity may result in GIT disturbances such as vomiting, abdominal cramps and diarrhoea. Chronic excessive levels of zinc are common with extreme supplementation and can result in hospitalization (National Institutes of Health, 2014).

Results of the absorption assay for zinc in the presence of either of the two HS compared to the control are shown in Figures 35A – 35E below

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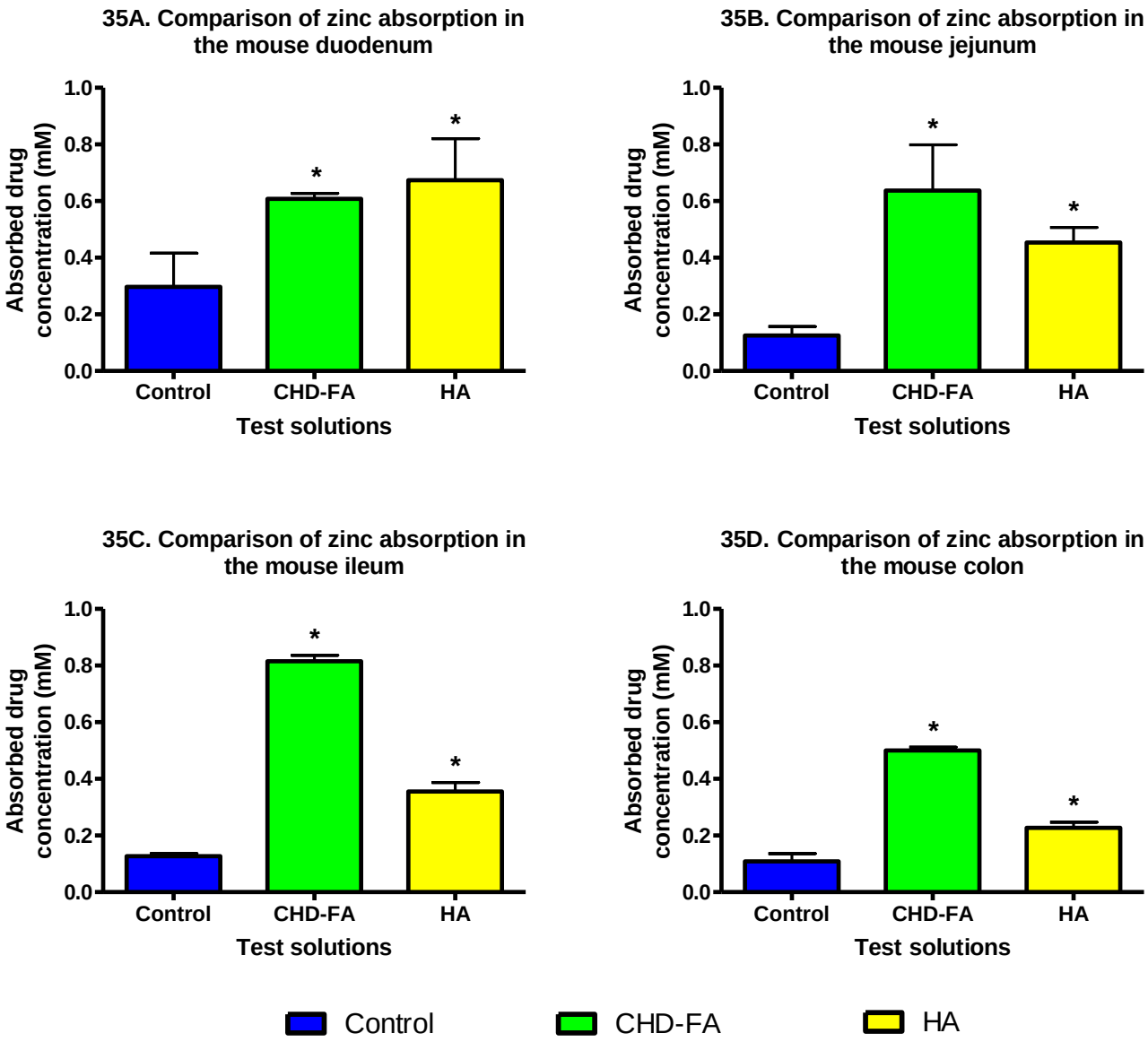

Figure 35 A-D: *Comparison of zinc absorption in different regions of the mouse intestine (35A: duodenum, 35B: jejunum, 35C: ileum and 35D: colon). The absorption of the mineral alone (control) was compared to the absorption of the mineral in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in all segments of the intestine, (n=3).*

*= $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$*

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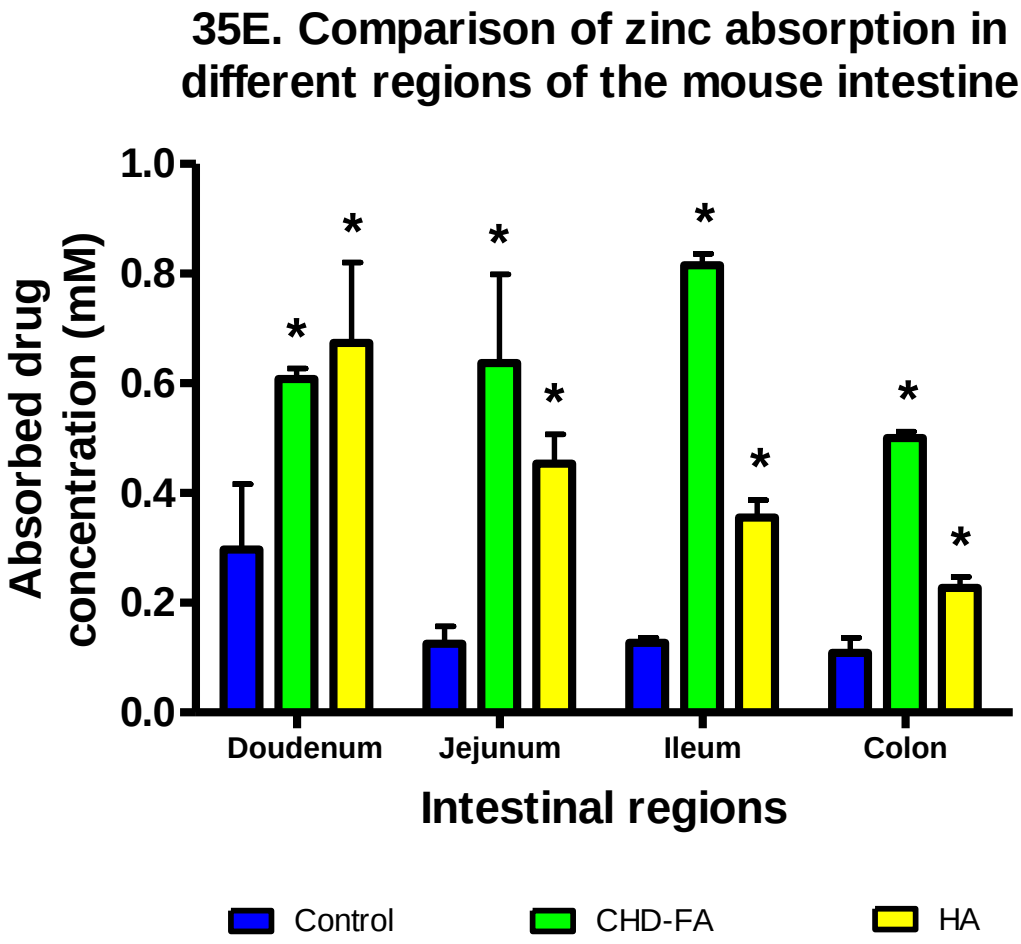

Figure 35E: Combination of graphs 35A-D showing comparison of zinc absorption in different regions of the mouse intestine (duodenum, jejunum, ileum and colon). The absorption of the mineral alone (control) was compared to the absorption of the mineral in the presence of both carbohydrate derived fulvic acid (CHD-FA) and humic acid (HA). A two-tailed Mann-Whitney non-parametric test was used to analyse the results, significant differences were evident in all regions of the intestine, (n=3).

*= $p < 0.05$ ** = $p < 0.01$ *** = $p < 0.001$*

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Table 55. Comparison of zinc absorption in different regions of the intestine

Intestinal Region	CHD-FA	Significant	HA	Significant
Duodenum	↑ 105%	Yes	↑ 127%	Yes
Jejunum	↑ 408%	Yes	↑ 261%	Yes
Ileum	↑ 541%	Yes	↑ 179%	Yes
Colon	↑ 360%	Yes	↑ 109%	Yes

Table 60 shows the comparison of zinc absorption in different regions of the mouse intestine in the presence of either CHD-FA or HA. The increases seen in absorption were reported as a percentage of the control.

It is evident that the presence of both the HS resulted in large increases in the total amount of zinc absorbed for all regions of the intestine when compared to the control.

Absorption of zinc in the presence of CHD-FA was seen to increase considerably in all regions in comparison to the control. Increases ranged from 105% in the duodenum to 541% in the ileum. Increases in zinc absorption were significant in all regions in the presence of CHD-FA.

Absorption of zinc in the presence of HA was also seen to increase considerably in all regions in comparison to the control. Increases ranged from 127% in the duodenum to 261% in the jejunum. Increases in zinc absorption were significant in all GIT regions in the presence of humic acid.

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Minerals: Combined results

Table 56. Summary of drug absorption in different regions of the intestine

Calcium				
Intestinal Region	CHD-FA	Significant	HA	Significant
Duodenum	↑ 22%	No	↓ 25%	Yes
Jejunum	↑ 32%	No	↓ 51%	Yes
Ileum	↑ 104%	Yes	↓ 20%	No
Colon	↑ 84%	Yes	↓ 51%	No
Magnesium				
Intestinal Region	CHD-FA	Significant	HA	Significant
Duodenum	↑ 22%	Yes	↑ 55%	Yes
Jejunum	↑ 18%	Yes	↑ 74%	Yes
Ileum	↑ 6%	No	↑ 9%	No
Colon	↑ 16%	No	↑ 17%	No
Iron(II)				
Intestinal Region	CHD-FA	Significant	HA	Significant
Duodenum	↑ 51%	No	↓ 13%	No
Jejunum	↑ 129%	Yes	↓ 6%	No
Ileum	↑ 179%	Yes	↓ 26%	No
Colon	↑ 1%	No	↑ 25%	No
Iron(III)				
Intestinal Region	CHD-FA	Significant	HA	Significant
Duodenum	↑ 289%	Yes	↑ 444%	Yes
Jejunum	↑ 289%	Yes	↑ 284%	Yes
Ileum	↑ 304%	Yes	↑ 248%	Yes
Colon	↑ 172%	Yes	↑ 105%	Yes
Zinc				
Intestinal Region	CHD-FA	Significant	HA	Significant
Duodenum	↑ 105%	Yes	↑ 127%	Yes
Jejunum	↑ 408%	Yes	↑ 261%	Yes
Ileum	↑ 541%	Yes	↑ 179%	Yes
Colon	↑ 360%	Yes	↑ 109%	Yes

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6.3 Discussion

Minerals are important in maintaining normal physiological functions in the body. Both major and trace minerals ensure proper functioning of a variety of different organs and biological processes. It has been established that deficiencies of any of the minerals lead to a variety of negative effects (Soetan *et al.*, 2010).Excess levels can also result in toxicity and can be equally detrimental to an individual's health. It is thus essential to ensure appropriate amounts of each mineral daily, either through a well-balanced diet or by using dietary supplements.

In this study, the overall increase or decrease in the total amount of absorbed major (calcium and magnesium) and trace minerals (iron(II)/ iron(III) and zinc) in the presence of fulvic and humic acids were assessed.

Major mineral: Calcium

The presence of CHD-FA and HA were seen to have opposite effects on the uptake of calcium. Only increases in calcium absorption were recorded in the presence of CHD-FA (between 22-140%) and only decreases were seen in the presence of HA (between 20-51%).

The results suggest that ingesting medicinal preparations containing CHD-FA together with food or calcium supplements may have an enhanced effect on the mineral's absorption. This improved uptake would be particularly beneficial in certain groups of individuals who are at risk of calcium deficiencies, such as: postmenopausal and amenorrheic woman, the elderly, lactose intolerant individuals and vegetarians. Furthermore, combining both CHD-FA and calcium in a single supplement formulation may be more beneficial on the overall uptake of the mineral than when compared to traditional supplements containing calcium alone.

The increased amount of calcium absorbed in the presence of CHD-FA is not likely to bring about the negative symptoms of excessive absorption.

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One reason for the decrease in absorption of calcium in the presence of humic acids can be the reports that humic acids form insoluble calcium salts (Petrovic & Kastelan-Macan, 1996). Although there was no obvious precipitate in the test samples after addition of the calcium to the humic acid during the absorption assay there may well have been some form of interaction that did form larger complexes that did not aggregate sufficiently to precipitate during the 90 minutes duration of the assay. It could be speculated that this calcium complex is the reason for the reduced absorption observed in the study. It is thus not recommended to ingest medicinal HA-containing preparations together with food or calcium supplements, especially in the case of the above-mentioned groups prone to deficiency.

Major mineral: Magnesium

The presence of CHD-FA and HA were seen to have similar effects on the uptake of magnesium. Increases in magnesium absorption were recorded in the presence of CHD-FA and HA, ranging between 6-22% and 9-74% respectively.

These results suggest that ingesting medicinal preparations containing either of these HS, together with food or magnesium supplements, may have an enhancing effect on the mineral's absorption. This improved uptake would be particularly beneficial in certain groups who are at risk of magnesium deficiencies, such as: individuals suffering from GIT disorders, type 2 diabetes, alcoholism and the elderly. Furthermore, supplements combining either of the HS together with magnesium in a single formulation may be more beneficial on the overall uptake of the mineral than when compared to traditional supplements containing magnesium alone.

As the UL for magnesium uptake falls very close to the RDI values for adults, the increased amount of magnesium absorbed in the presence of both HS could possibly bring about mild negative symptoms of excessive absorption.

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Trace mineral: Iron

The absorption of both iron(II) (ferrous iron) and iron(III) (ferric iron) were assessed in this study. Iron(II) is known to have poor solubility and uptake in the GIT when compared to iron(III).

Iron(II)

The presence of CHD-FA and HA were seen to have similar effects on the uptake of iron(II). Increases in iron(II) absorption were recorded in the presence of CHD-FA and HA, ranging between 1-178% and 6-26% respectively.

These results suggest that ingesting medicinal preparations containing this CHD-FA together with food or iron(II) supplements may have an enhanced effect on the mineral's absorption. This improved uptake would be particularly beneficial in certain groups of individuals who are at risk of iron deficiencies, such as young children, girls in their teens, pregnant women and premenopausal women. Furthermore, supplements combining CHD-FA together with iron(II) in a single formulation may be more beneficial on the overall uptake of the mineral than when compared to traditional supplements containing iron(II) alone.

Similarly to calcium, a general decrease was observed in the presence of HA. This may also be the result of the formation of an insoluble complex with iron(II). Due to the decreased absorption observed, it is not recommended to ingest medicinal HA-containing preparations together with food or iron supplements, especially in the case of groups at risk of suffering from an iron deficiency.

As the UL for iron uptake ranges between 40 and 45 mg/g, far above the 8 – 18 mg/day RDI values for adults, thus the increased amount of iron(II) absorbed in the presence of both HS would most likely not result in the negative symptoms of excessive iron intake.

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Iron(III)

The presence of CHD-FA and HA were seen to have similar effects on the uptake of iron(III). Considerable increases in iron(III) absorption were recorded in the presence of CHD-FA and HA, ranging between 172-304% and 105-444% respectively.

Iron(III) is known to be insoluble in aqueous solutions and must first be reduced to iron(II) in order to be absorbed by the intestinal tissue, however, these results suggest that the presence of CHD-FA and HA greatly assist with iron(III) solubility in the aqueous environment of the GIT.

Haemoglobin can be formed using either iron(II) or iron(III). Methemoglobin refers to haemoglobin formed with iron(III). Unlike haemoglobin, methaemoglobin lacks the ability to transport oxygen in the body. Thus higher than average amounts of iron(III) (ferric iron) in the body and can result in varying forms of cyanosis and should be avoided.

The purpose of including iron(III) in this experiment was not to demonstrate the potential of the HS to increase iron(III) absorption so that it may be used to combat iron deficiency, but rather to demonstrate the potential of the HS to increase the aqueous solubility of a compound with extremely poor solubility through chelation.

Trace mineral: Zinc

The presence of CHD-FA and HA were seen to have similar effects on the uptake of zinc. Increases in zinc absorption were recorded in the presence of CHD-FA and HA, ranging between 105-541% and 109-261% respectively.

These results suggest that ingesting medicinal preparations containing these HS together with food or zinc supplements may have an enhanced effect on the mineral's absorption. This improved uptake would be particularly beneficial in certain groups of individuals who are at risk of zinc deficiencies, such as: individuals suffering from GIT disorders, vegetarians, pregnant or lactating

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women and individuals suffering from chronic alcoholism. Furthermore, supplements combining either of the HS together with zinc in a single formulation may be more beneficial on the overall uptake of the mineral than when compared to traditional supplements containing zinc alone.

As the UL for zinc uptake is approximately four times the RDI. The increases in absorption in zinc absorption the presence of HA should not bring about negative symptoms caused by excessive zinc intake, however the presence of CHD-FA is seen to have an even greater effect on zinc uptake and could possibly cause mild negative symptoms of excessive absorption if used for extended periods.

Table 62 displays the intestinal region of the mouse intestine showing the greatest change in absorption in the presence of CHD-FA and HA for each mineral.

Table 57. Intestinal region showing the greatest change in absorption in the presence of CHD-FA and HA

Mineral	Intestinal segment showing greatest change (CHD-FA)	Intestinal segment showing greatest change (HA)
Calcium	Ileum	Jejunum/colon
Magnesium	Duodenum	Jejunum
Iron(II)	Ileum	Ileum
Iron(III)	Ileum	Duodenum
Zinc	Ileum	Jejunum

In the presence of both HS, there were no trends seen whereby a specific region consistently showed enhanced absorption. However, four of the five minerals showed enhanced absorption in the ileum in the presence of CHD-FA.

As the separate regions of the mouse intestine are known to differ slightly in anatomy as well as in the types of transporters present, the transport of the CHD-

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FA-mineral complex may be favoured by a specific type of transport more prevalent in the ileum.

The presence of HA did not show any GIT region as a preferential region of absorption of the tested minerals.

Ensuring adequate intake of each major and trace mineral is achieved, is essential to maintain proper physiological functioning of the body. These adequate levels can be achieved through a balanced diet or by use of mineral supplements. Mineral deficiencies are accompanied by many negative effects and are a common occurrence in poorer regions as well as in individuals suffering from a variety of disorders. Excessive intake of a mineral can also result in unwanted side effects.

There may be potential advantages to combining CHD-FA with calcium, iron(II) and zinc, in order to enhance their overall absorption and to combat the negative symptoms linked to deficiencies in each mineral.

Combining CDH-FA and zinc in a single formulation may result in excessive levels of the mineral to be absorbed.

Combining HA with magnesium in order to enhance their overall absorption may also assist in combatting the negative symptoms linked to low levels. Similarly to CHD-FA, caution must be taken when combining HA and zinc in a single formulation, as it may result in excessive levels of the mineral to be absorbed.
The concurrent ingestion of HA together with calcium and iron(II) should be avoided.

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Chapter 7

7. Concluding discussion

The large majority of the world's population suffers from one or multiple illnesses at a given time. Qualified healthcare professionals commonly prescribe various conventional medications, either on a short-term or chronic basis, in order to address these illnesses. Furthermore, alternative medications are commonly ingested alongside these conventional medications in order to treat the same illness or to help alleviate any side effects brought on by the prescribed medication. The concurrent use of multiple medications increases the risk of direct drug-drug interactions which may result in changes in drug absorption leading to treatment failure or undesired side effects.

Medicinal preparations containing HS such as CHD-FA and HA are claimed to possess a wide variety of medicinal properties and can be easily acquired over the counter without the need to visit a healthcare professional or produce a prescription. These HS are large organic macromolecules found throughout nature and are similar in structure, containing many different functional groups. These functional groups allow the HS to easily bind and form complexes with a variety of compounds. It has been seen that once in complex with a HS, some compounds have shown better uptake potential in plants, animals and humans.
Due to the many known medicinal properties of these HS preparations, as well as the ease through which they can be acquired, it is likely that many individuals will consider taking these preparations on an *ad-hoc* basis, concomitantly with other medications and/or dietary supplements.

In this study, the effect of CHD-FA and HA on the uptake of a variety of commonly ingested drugs, vitamins and minerals was assessed using the everted mouse gut model.

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In order for drugs to elicit a therapeutic effect, a specific amount must be absorbed by the body. If less is absorbed, the therapeutic potential of the drug is not reached and if a greater amount is absorbed there is a risk of adverse side effects. It is essential, especially when dealing with drugs having a narrow therapeutic index, to ensure that the correct concentration of a drug is absorbed.

Six orally administered drugs, belonging to different drug classes, and possessing differing physicochemical properties were chosen (diclofenac, penicillin V, warfarin, rifampicin, valsartan and zidovudine). The presence of CHD-FA and HA was seen to have an effect on the uptake of the all six drugs. Most drugs were seen to show a consistent decrease in the presence of the HS throughout the intestine. This decrease could result in therapeutic failure, especially in that case of warfarin (a drug with a narrow therapeutic index).

No clear relationship between the drug's physicochemical properties (size, LogP and LogD) and the enhancement or inhibition of absorption could be identified. Thus, the changes in absorption in the presence of CHD-FA and HA cannot be used to predict trends in absorption of other drugs based on their physico-chemical properties. A trend was however seen when assessing the pKa of the drugs where a trend that drugs with a pKa > 5 showed decreased absorption in the presence of CHD-FA. In order to properly assess and confirm this trend, a larger range of drugs would need to be evaluated.

Patients and healthcare providers should be made aware of the potential drug-drug interactions when ingesting these drugs concomitantly with medicinal preparations containing HS.

Nutrients, such as vitamins and minerals, are essential in order for the body to maintain normal physiological functioning. Certain amounts of these nutrients are required through a balanced diet or through vitamin and mineral supplements. Taking in less than the required amount over time will result in a deficiency which is accompanied by a range of negative symptoms, depending on

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the deficient nutrient. Higher than average intake may also result in unwanted effects. Thus, similarly to drugs, it is important to assimilate the correct amounts.

One water-soluble (vitamin B_3) and one fat-soluble vitamin (vitamin E) were selected for the study. Vitamin B_3 showed decreased absorption in all four regions of the GIT in the presence of both HS. This decrease over time may result in a mild deficiency of the vitamin or it may exacerbate the symptoms of an already present deficiency due to a poor diet. Certain groups of individuals are more at risk of suffering from a deficiency and should be cautious of taking HS-containing preparations within two hours of eating or with any vitamin B_3 supplements.

The fat-soluble vitamin E could not be successfully tested using the everted mouse gut model due to the insolubility of the highly lipophilic vitamin in the aqueous environment required for the experiment.

Five minerals were assessed in this study: calcium, magnesium, iron(II), iron(III) and zinc. The presence of both HS resulted in significant increases in the minerals absorption, exceptions being calcium and iron(II) in the presence of HA, which is thought to form an insoluble complex upon binding. Iron(III), known to have low solubility in an aqueous environment was seen to have notably increased absorption in the presence of both HS when compared to the control suggesting that other insoluble minerals may also demonstrate enhanced absorption when bound to CHD-FA or HA.

Mineral deficiencies are a common occurrence in poorer countries and in certain population groups. Ingesting HS-containing medicinal preparations along with food and mineral supplements may assist in greater absorption and a possible decrease in symptoms of deficiency. Furthermore, benefits may be seen from combining these HS with minerals in a single supplement in order to promote mineral absorption.

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In general, the majority of drugs show decreased absorption in the presence of the HS whereas the minerals show an increase when compared to the control. This could be attributed to the different forms of binding of the drug and minerals to the HS. Drugs and vitamins bind to the HS via complex formation whereby non-specific binding occurs through electrostatic bonds, hydrogen bonding and weak van der Waals forces without the formation of a ring structure. Minerals bind to the HS by chelation. This type of binding involves a chelating agent (CHD-FA or HA) forming a stable association through more than one coordination bond with a single metal ion resulting in stable compounds. This difference in binding may cause reduced solubility in the case of the drugs and enhanced solubility of the minerals.

These differences in binding may also account for the conflicting effects on absorption seen with two compounds known to be insoluble in an aqueous solution, Vitamin E and iron(III). Vitamin E was insoluble in the KRB solution, even in the presence of CHD-FA and HA and was thus not able to be absorbed by the intestinal tissue. Iron(III) showed the opposite effect with a significant increase in absorption in the presence of both HS. Thus compounds bound to the HS by chelation may have better solubility than those in complex.

In conclusion, the presence CHD-FA and HA are seen to affect the absorption of drug, vitamins and minerals through drug-drug interactions and care must be taken when ingesting these substances concomitantly.

In conclusion, all the aims and objectives of the study were achieved as set and analytical methods were developed for all the drugs and vitamins tested. These methods have limitations as they are not applicable to complex biological matrices and were directed at assessing the concentration of the analytes in a fairly well defined aqueous buffer system, although there were some contaminants that were derived from the active GIT tissue.

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Several parameters were assessed to try identify which physicochemical properties of the analytes could be used to predict whether the absorption would be enhanced in the presence of the two humic substances. This predictive parameter was not determined due to the wide differences in more than one parameter for each chosen test compound and the differences between the effect of the fulvic and humic acids which allowed too many variables to be assessed for the number of tested drugs.

The study also highlighted several shortcomings with the study design that were only identified during the analysis of the samples. These include analyte solubility and the lack of experimentally derived physicochemical parameters such as LogP and LogD values.

It can be claimed that the presence of the fulvic or humic acid do have significant effects on the absorption of the different drugs and vitamins which are generally lower than the controls but enhances the ionic mineral absorption significantly.

Both these outcomes highlight the fact that both health professionals and patients should be made aware of the fact that concomitant administration of prescription drugs and any humic substances will probably lead to altered bioavailability which could affect the clinical outcome of the drugs.

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Constructive criticism of the study

Everted mouse gut model

The everted mouse gut model was chosen as the absorption model for this study due to its good correlation with human intestinal absorption, the ability to assess the different regions of the GIT and due to its very simplicity and reproducibility. There are, however, potential draw backs which were observed during the experiment.

Firstly, in the model, each region of the intestinal tissue is placed in KRB of pH 7.4 (physiological pH) in order to keep the intact intestinal tissue functional for a usable period after excision. The pH of the intestine *in-situ* ranges from pH 5-8, depending on the region. Depending on the pKa of the drug, it will be ionised/ unionised according to the pH of that region. The state of ionisation largely determines the uptake of the drug through the intestinal membrane. Maintaining the pH at 7.4 during the experiment does not accurately mimic the pH of the intestinal environment and may result differences in drug uptake between the experiment and *in-situ*.

Furthermore, another drawback of the absorption model is the inability to properly assess the uptake of highly lipophilic compounds. As the intestinal tissue is required to remain in KRB buffer at pH7.4, highly lipophilic compounds, such as vitamin E, are insoluble and the absorption across the intestinal tissue cannot be assessed.

Number of test compounds

In this study, specific test drugs, vitamins and minerals were tested. A larger number of drugs will need to be assessed in order to identify trends in absorption in the presence of the HS and to draw better conclusions based on the physicochemical properties of the drugs.

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Chapter 8

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Addendum A

Animal Ethics Approval

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