Gastrointestinal region specific insulin absorption enhancement by *Aloe vera* leaf materials

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This dissertation is dedicated to my parents, Roelf and Marlene Pretorius, who have always loved and supported me unconditionally and who never doubted in me.

ABSTRACT

The oral administration route is still the most popular and preferred route for drug administration, but is, however, not suitable for protein and peptide drugs such as insulin, due to their poor bioavailability when administered orally. This can be attributed mainly to the unfavourable physicochemical properties of the protein and peptide drugs and the considerable barriers that the gastrointestinal tract (GIT) poses to drug absorption. In order to overcome this problem, drug absorption enhancers which act to minimise the barriers presented by the epithelial cells can be added to drug delivery systems. These absorption enhancing agents may facilitate the absorption of poorly absorbable hydrophilic and macromolecular drugs via opening tight junctions. Although it has been shown in previous studies that aloe leaf materials improve intestinal drug transport, their effect on the regional gastrointestinal absorption has not yet been investigated.

The aim of this study is to determine whether the intestinal drug absorption enhancement effects of *A. vera* gel and whole leaf materials are region specific and to identify the region in the GIT where maximum absorption enhancement of insulin is achieved.

The transport of insulin across excised pig intestinal tissues from various regions of the GIT (i.e. the duodenum, proximal jejunum, medial jejunum, distal jejunum, ileum and colon) was measured in the absence and presence of *A. vera* gel and whole leaf materials (0.5% w/v) using the Sweetana-Grass diffusion chamber as well as the everted sac technique. Apical-to-basolateral transport of insulin was measured over a period of 2 h at a concentration of 170 µg/ml in the diffusion apparatus, while 17 µg/ml was used in the everted sac technique due to the larger surface area available for absorption. Test solutions were prepared in Krebs Ringer bicarbonate (KRB) buffer at the pH values of 6.8 for the duodenum and 7.4 for the jejunum, ileum and colon. Analysis of the samples (200 µl) withdrawn from the acceptor chambers at 20 min intervals was conducted by means of a validated high performance liquid chromatograpy (HPLC) method.

The results showed that the *A. vera* gel mediated a statistically significant ($p < 0.05$) increase in insulin transport in the duodenum, distal jejunum and colon compared to the control group in the diffusion apparatus. The *A. vera* whole leaf material mediated a statistically significant ($p < 0.05$) increase in insulin transport in the proximal jejunum compared to the control group in the diffusion apparatus. Insulin transport was enhanced statistically significantly ($p < 0.05$) in the ileum and colon by *A. vera* gel in the everted sac technique, while the *A. vera* whole leaf material increased the insulin transport statistically

significantly ($p < 0.05$) only in the proximal jejunum. Insulin transport was reduced in some of the GIT regions by the *A. vera* gel and whole leaf materials.

The increased insulin transport can possibly be explained by the opening of tight junctions as indicated by a reduction in the transepithelial electrical resistance (TEER) values, while the reduction in insulin transport can probably be explained by different interactions of the aloe materials with different intestinal tissues.

The results indicated that the addition of *A. vera* gel and whole leaf materials had a statistically significant effect on the *in vitro* transport of insulin across some of the six GIT regions.

Key words: Oral administration route, insulin, absorption enhancers, region specific gastrointestinal absorption, *A. vera* gel and whole leaf materials, Sweetana-Grass diffusion chamber, everted sac technique, transepithelial electrical resistance (TEER).

UITTREKSEL

Die orale toedieningsroete is die populêrste en mees verkose toedieningsroete vir geneesmiddeltoediening, maar is ongelukkig nie geskik vir proteïen- en peptiedgeneesmiddels soos insulien nie as gevolg van hul swak biobeskikbaarheid na orale toediening. Die swak biobeskikbaarheid van proteïen- en peptiedgeneesmiddels kan grootliks toegeskryf word aan die ongunstige fisies-chemiese eienskappe van hierdie geneesmiddels en ook die uitdagende biologiese skanse van die gastrointestinale kanaal. Hierdie struikelblokke kan oorkom word deur geneesmiddelabsorpsiebevorderaars te kombineer in geneesmiddelafleweringssisteme. Hierdie absorpsiebevorderaars verminder die integriteit van die biologiese skanse en is gevolglik in staat om die absorpsie van swak-geabsorbeerde, hidrofiliese en makromolekulêre geneesmiddels te bevorder deur die oopmaak van sellulêre-aansluitingskanale. Alhoewel daar in vorige studies bevind is dat alwyn blaarmateriale die intestinale absorpsie van insulien kan bevorder, is hul effek op die area-spesifieke absorpsie nog nie ondersoek nie.

Die doel van hierdie studie was om te bepaal of die intestinale geneesmiddelabsorpsiebevorderingspotensiaal van *A. vera* jel en blaarmateriale area-spesifiek is en om die relevante areas in die gastrointestinale kanaal te identifiseer waar die maksimum absorpsiebevordering van insulien plaasvind.

Die absorpsie van insulien oor varkdermweefsel vanuit verskeie areas van die gastrointestinale kanaal (bv. die duodenum, proksimale jejenum, mediale jejenum, distale jejenum, ileum en kolon) is gemeet in die afwesigheid en teenwoordigheid van *A. vera* jel en blaarmateriale (0.5% m/v). In hierdie studie is daar gebruikgemaak van twee metodes om die mate van insulien - absorpsie te bepaal naamlik die Sweetana-Grass diffusietegniek sowel as die omgekeerde intestinale sak tegniek. Die apikale-na-basolaterale absorpsie van insulien is gemeet oor ‘n tydperk van 2 ure. Die getoetsde insulienkonsentrasies was onderskeidelik 170 µg/ml vir die diffusietegniek en 17 µg/ml vir die omgekeerde intestinale sak tegniek. Die laer konsentrasie in die laasgenoemde tegniek is as gevolg van die groter oppervlakarea wat beskikbaar is vir absorpsie in die spesifieke *in vitro* model. Toetsoplossings was voorberei in Krebs Ringer bikarbonaat (KRB) buffer by pH waardes van 6.8 vir die duodenum en 7.4 vir die jejenum, ileum en kolon. Monsters van 200 µl was elke 20 min onttrek vanuit die ontvangerkamers. Die monsters is toe ontleed met behulp van ‘n gevalideerde hoëdrukvloeistofchromatografiemetode.

Die resultate het getoon dat die *A. vera* jel ‘n statisties betekenisvolle ($p < 0.05$) verhoging in insulienabsorpsie bewerkstellig het in die duodenum, distale jejenum en kolon indien dit met

die kontrolegroep se absorpsiewaardes vanuit die diffusietegniek vergelyk word. Die *A. vera* blaarmateriale het ‘n statisties betekenisvolle ($p < 0.05$) verhoging in insulien - absorpsie bemiddel in die proksimale jejenum teenoor die kontrolegroep se absorpsiewaardes soos verkry vanuit die diffusietegniek. Insulien - absorpsie is statisties betekenisvol ($p < 0.05$) verhoog in die ileum en kolon deur die *A. vera* jel in die in die omgekeerde intestinale sak tegniek, terwyl *A. vera* blaarmateriale die insulien - absorpsie statisties betekenisvol ($p < 0.05$) verhoog het in slegs die proksimale jejenum. Die mate van insulienabsorpsie is verlaag in sommige van die gastrointestinale areas deur beide die *A. vera* jel en blaarmateriale.

Die verhoging in insulienabsorpsie kan moontlik toegeskryf word aan die oopmaak van sellulêre-aansluitingskanale soos aangedui deur die verlaging in die transepiteliale elektriese weerstand (TEEW) waardes, terwyl die verlaging in insulienabsorpsie moontlik verduidelik kan word deur verskeie interaksies van die aloe materiale met die verskillende intestinale-weefseltipes.

Die resultate het aangedui dat die toevoeging van *A. vera* jel en blaarmateriale ‘n statisties betekenisvolle effek op die *in vitro* absorpsie van insulien gehad het in sommige van die getoetsde gastrointestinale areas.

Sleutelwoorde: Orale toedieningsroete, insulien, absorpsiebevorderaars, area-afhanklike gastrointestinale absorpsie, *A. vera* jel en blaarmateriale, Sweetana-Grass diffusieapparaat, omgekeerde intestinale sak-tegniek, transepiteliale elektriese weerstand (TEEW).

CONFERENCE PROCEEDINGS

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LIST OF ABBREVIATIONS

% RSD	Percentage relative standard deviation
3 R’s	Reduce, Replace, Refine
ABC	ATP-binding cassette
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AVG	*Aloe vera* gel
AVWL	*Aloe vera* whole leaf
Caco-2	Human Caucasian colon adenocarcinoma
CYP 3A4	Cytochrome P450 3A4
GIT	Gastrointestinal tract
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
ICH	International Conference of Harmonisation
JAM	Junctional adhesion molecule
KRB	Krebs-Ringer bicarbonate
KRB	Krebs-Ringer bikarbonaat
LOD	Limit of detection
LOQ	Limit of quantitation
MDCK II	Madin-Darby canine kidney II
MDR	Multidrug resistance protein
MW	Molecular weight

NaOH	Sodium hydroxide
PAMPA	Parallel artificial membrane permeability assay
P_{app}	Apparent permeability coefficient
P-gp	P-glycoprotein
R^2	Correlation coefficient
RSD	Relative standard deviation
SD	Standard deviation
TEER	Transepithelial electrical resistance
TEEW	Transepiteliale elektriese weerstand
USP	United States Pharmacopoeia
ZO	Zonula occludins

CHAPTER 1: INTRODUCTION

1.1 Background and justification

1.1.1 Gastrointestinal absorption

Epithelial cells in the gastrointestinal tract (GIT) act as barriers and thereby maintain distinct compartments, which separate the lumen of the intestinal tract from the rest of the body. Molecules can cross the intestinal epithelium by means of three main pathways, namely paracellular passive diffusion, transcellular passive diffusion and carrier mediated transport (Gomez-Orellana, 2005).

Some drugs exhibit poor absorption across the intestinal epithelium after oral administration. This can be attributed to unfavourable physicochemical properties of the drug molecule including large molecular weight and hydrophilicity (Zhang & Tizard, 1996) as well as low lipophilicity or efflux by P-glycoprotein (P-gp) (Adachi *et al.,* 2003). Pre-systemic metabolism, degradation by chemical and enzymatic reactions and low aqueous solubility also contribute to decreased gastrointestinal absorption across the intestinal mucosa (Crowley & Martini, 2004).

The intestinal mucosa is characterised by the presence of villi on the apical side of the epithelial cells, which constitute a large anatomical and functional unit for nutrient and drug absorption (Peters & Jansen, 1988). The intestinal mucosa consists of an epithelial layer, lamina propria and muscularis mucosa. A drug must cross the epithelial barrier of the intestinal mucosa for it to be transported from the lumen of the GIT into the systemic circulation after which its biological actions can be exerted. Drugs administered by the oral route encounter several anatomical and biological barriers such as the aqueous stagnant layer and the apical and basolateral epithelial cell membranes in penetrating the epithelial mucosa (Brange & Langkjoer, 1993; Myung *et al.,* 2009).

Membranes that form the boundary of cells consist of lipid bilayers containing proteins such as receptors and carrier molecules (Myung *et al.,* 2009). Due to the hydrophilic nature of protein macromolecular drugs such as insulin, they cannot diffuse through the lipid bilayer cell membranes and therefore cannot cross the epithelial cells. This failure of insulin to be absorbed from the GIT results in an oral bioavailability of less than 1%, being insufficient for therapeutic action (Yogendraji *et al.,* 2011).

P-gp is an energy dependent transmembrane protein drug efflux pump, located in the apical membrane of intestinal epithelial cells. It is a member of the group of the ATP-binding cassette (ABC) transporters. It has a molecular weight of 170 kDa and has 1280 amino acid residues (Juliano & Ling, 1976). It may limit the bioavailability of many orally administered drugs by transporting them back into the intestinal lumen following absorption by the enterocytes (Paine *et al.,* 1996). There are several similarities between the tissue distribution and gene regulation of CYP3A4 and P-gp, and they have overlapping specificities for substrate inhibitors and inducers (Benet & Cummins, 2001).

1.2 Oral delivery of insulin

Insulin is a hydrophilic protein, which is composed of 51 amino acids contained in two peptide chains (A and B), which are linked by two disulfide bonds. This protein is naturally produced in the Islets of Langerhans in the pancreas (Brange & Langkjoer, 1993). Insulin's structure varies slightly between different animal species, but it is well known that pig insulin is structurally similar to human insulin, the only difference being the peptide sequence of a single amino acid (Chance *et al.,* 1981). Insulin is poorly absorbed from the GIT due to extensive proteolytic degradation and inactivation by intestinal enzymes such as pepsin, trypsin and chymotrypsin as well as insufficient membrane permeability as result of its large molecular size and hydrophilicity (Grabovac *et al,* 2008).

Pepsinogen is an inactive pro-enzyme that is secreted by the chief cells in the pancreas. Within the gastric lumen, the acid present converts the pepsinogen to pepsin, an active proteolytic enzyme. Pepsin breaks down proteins in the stomach contents and functions optimally at pH 2.0. Trypsin is present in the small intestine. Its optimal function is at a pH of 7.7, which is weakly basic. The trypsin then activates chymotrypsin, a pro-enzyme secreted by pancreatic cells, in the lower small intestine, which optimally functions at pH 7.0-8.0 (Martini, 2006).

1.3 Drug absorption enhancers

Drug absorption enhancers are compounds that reversibly remove the barrier function of the intestinal lining and thereby allow drugs to penetrate across the epithelium into the blood circulation (Muranishi, 1990; Salama *et al.,* 2006). These substances may facilitate absorption of poorly absorbable drugs by means of targeting transport proteins, opening tight junctions or by changing the membrane structure (Whitehead *et al.,* 2008). Although there are many structurally diverse compounds which have shown the ability to increase drug transport across the intestinal epithelium after oral administration, very few absorption

enhancers are currently incorporated into products available on the market due to their long term adverse effects, toxicity and concerns regarding their safety and efficacy (Hamman *et al.,* 2005). Tight junctions between epithelial cells are dynamic structures, which can be modulated by compounds in order to enlarge the pores, thereby allowing enhanced paracellular absorption of hydrophilic macromolecules (Gomez-Orellana, 2005). Compounds that selectively open the intestinal epithelial tight junctions by means of various mechanisms have shown potential as novel absorption enhancing excipients in advanced drug delivery systems (Lodish *et al.,* 2000). Co-administration of polymeric absorption enhancers with the poorly absorbable drug can potentially enhance its low gastrointestinal epithelial permeability and thereby promote its oral bioavailability (Gomez-Orellana, 2005).

Transepithelial electrical resistance (TEER) is a measure of tight junction integrity between adjacent intestinal epithelial cells (Johnson *et al.,* 2008). If the size of the openings of tight junctions increases in the presence of a paracellular permeability enhancer, the TEER of the intestinal epithelium will be reduced due to the increased flow of ions through the opened tight junctions and intercellular spaces (Gomez-Orellana, 2005). The reduction in TEER will indicate the ability of the absorption enhancer to open tight junctions between epithelial cells, allowing a potentially larger fraction of orally administered drug to be transported across intestinal tissue (Beneke *et al.,* 2012). Since tight junctions are usually a rate limiting factor in the intestinal epithelial drug absorption process, TEER can be used as an indicator of tight junction permeability and integrity (Johnson *et al.,* 2008; Madara, 1998).

Naturally occurring polysaccharides are capable of enhancing intestinal absorption of co-administered drugs by means of a transient opening of tight junctions between adjacent epithelial cells to allow paracellular transport across the intestinal epithelium (Junginger & Verhoef, 1998; Kotzé *et al.,* 1997). Absorption enhancers of natural origin act according to several different mechanisms of action which may affect drug absorption, drug metabolism and site specific drug absorption. Mechanisms of action for these absorption enhancers include a reduction in hydrochloric acid secretion and an increase in gastrointestinal blood supply (Mahato *et al.,* 2003). Modifications in the GIT epithelial cell membrane permeability, inhibition of gastrointestinal transit, variation of gastric emptying time and varied intestinal motility rate can also occur (Bajad *et al.,* 2001).

Some absorption enhancers have the disadvantage of causing damage to mucosal epithelium and cell membranes. Another challenge with some absorption enhancers is the lack of specificity because they allow the complete content of the intestinal tract, including toxins and pathogens, access to the bloodstream (Whitehead *et al.,* 2008). The use of

absorption enhancers is currently limited due to the fact that it also enhances the transport of undesirable molecules present in the GIT, together with that of protein and peptide drugs once the tight junctions are opened (Gordberg & Gomez-Orellana, 2003). An advantage of using polymeric absorption enhancing agents is the fact that their physicochemical characteristics do not favour their own absorption, resulting in a reduced risk of adverse effects and systemic toxicity (Di Colo *et al.,* 2007).

1.3.1 *Aloe vera* as absorption enhancer

Aloe vera is a perennial succulent xerophyte that belongs to the *Asphodelaceae* family. It is a traditional medicinal plant with diverse therapeutic applications (Newton, 2004; Hamman *et al.,* 2008). The distinct parts from the *A. vera* plant that are used for medicinal purposes include the latex, gel and whole leaf extract (Reynolds & Dweck, 1999). Aloe gel is the colourless fraction found in the inner part of fresh leaves (Bozzi *et al.,* 2007) in which water is held in the form of a viscous mucilage (Newton, 2004). This inner gel is composed of large, thin-walled, parenchyma cells filled with gel consisting of mono- and polysaccharides (Rosca-Casian et *al.,* 2007). The polysaccharides in *A. vera* gel consist mainly of linear chains of glucose and mannose molecules of which mannose comprises the larger part of the polysaccharides (Zhang & Tizard, 1996).

Acemannan (aloverose) has been identified as one of the main polysaccharides in *A. vera* gel with biological activity. It has a backbone of β-(1-4)-D-mannosyl residues which is acetylated at the C-2 and C-3 positions and exhibits a mannose monomer (Moreira & Filho, 2008). Acemannan is also used as a major marker for *A. vera* gel. *Aloe vera* inner leaf gel also contains other polysaccharides such as galactan, galactogalacturan, glucogalactomannan, galactoglucoarabinomannan, arabinan, arabinorhammogalactan, peptic substances and glucoronic acid containing polysaccharides (Choi & Chung, 2003; Ni *et al.,* 2004).

Aloe vera gel and whole leaf extracts have shown potential for use as absorption enhancers for drugs with poor oral bioavailability where it acts on the tight junctions between GIT epithelial cells (Chen *et al.,* 2009). Precipitated polysaccharides from *A. vera* gel has the potential to be used as drug absorption enhancement agents due to their proven transport enhancement effect (Beneke *et al.,* 2012; Lebitsa *et al.,* 2012).

1.4 *In vitro* models for drug absorption studies

Different *in vitro* models are available to conduct pre-clinical drug permeation studies, which include parallel artificial membrane assays, cell culture monolayers on transwell membranes

and excised intestinal tissues in diffusion chambers as well as everted sacs (Balimane & Chong, 2005). Although *in vitro* models do not yet reflect *in vivo* situations to such an extent that they can completely replace *in vivo* studies, the controlled conditions under which *in vitro* experiments are performed make them useful in pharmacokinetic studies (Lerche-Langrand & Toutain, 2000). The greatest limitation of using excised tissues in *ex vivo* studies is the sensitivity and short viability time of the intact intestinal tissue due to oxygen deprivation and accompanying preservation issues. The excised tissue should be kept in cold buffer solution (0-5 °C) during preparation and handling of the tissue in order to minimise enzymatic degradation (Rehner *et al.,* 1981). Furthermore, tissue samples should be handled with great care to ensure that only intact membranes are used in the experiments and to ensure that the integrity (as measured by TEER) is maintained for the duration of the study.

The diffusion chamber method is simple and robust and allows bi-directional transport and metabolism studies on animal intestinal tissue at specific intestinal regions/locations (Barthe *et al.,* 1999). These types of studies are also relatively cost effective since only small tissue samples and small drug solution volumes are required (Le Ferrec *et al.,* 2001). Diffusion chambers can also be used to study interspecies variation in drug absorption (Jezyk *et al.,* 1992).

The everted sac method is fast, simple and inexpensive and can be conducted without specialised equipment (Bohets *et al.,* 2001; Versantvoort *et al.,* 2000). The influence of regional differences on drug absorption and transport can also be measured with this *in vitro* technique (Bohets *et al.,* 2001). The everted sac, incubated in tissue culture medium, maintains good tissue viability (long enough for experimental procedures) and renders reliable data, although it is a closed system (Barthe *et al.,* 1999). It has also been found useful to estimate the effect of absorption enhancers (Leppert & Fix, 1994).

The pig GIT has similar physiological and digestive traits compared to that of humans, which render it an appropriate *in vitro* model for drug absorption studies (Swindle & Smith, 1998). Pig intestinal tissue is available as a by-product from the slaughtering of pigs for meat production and the animals are therefore not bred and sacrificed for purposes of research *per se.* It is readily obtainable from the local abattoir and has been frequently used in experimental procedures investigating drug transport and metabolism (Nolte *et al.,* 2000). Compared to other preclinical animal species, pigs appear to be the most analogous to humans concerning their gastrointestinal processes including the pH, surface area and transit time (DeSesso & Williams, 2008).

1.5 Problem statement

Due to the low bioavailability of peptide drugs, administered via the oral route, they are mainly administered by means of injections with associated risks and disadvantages. There is a need for studies concerning the effective delivery of these drugs via the oral route of administration (Shaji & Patole, 2008).

Aloe vera gel and whole leaf materials have shown high potential to be used as intestinal absorption enhancers (Chen *et al.,* 2009). These plant materials act by opening tight junctions between epithelial cells (Beneke *et al.,* 2012; Chen *et al.,* 2009; Lebitsa *et al*., 2012) as well as reducing hydrochloric acid secretion in the GIT. However, the specific region in the GIT where these materials are most effective as absorption enhancers has not been determined yet. Identification of the region in the GIT where these absorption enhancers are the most effective can contribute to development of dosage forms that target these areas for maximised protein drug delivery.

The pig has emerged as a superior non-primate model for use in *in vitro* studies due to its similarities to humans physiologically, anatomically and developmentally. Pigs also have similar nutritional requirements to humans as they are omnivorous and share very similar metabolical processes associated with digestion (Patterson *et al.,* 2008).

1.5.1 General aim

The aim of this study is to determine if the intestinal drug absorption enhancement effects of *A. vera* gel and whole leaf materials are region specific and to identify the area in the GIT where maximum drug absorption enhancement occurs.

1.5.2 Specific objectives

- To conduct a literature review on relevant topics including drug absorption enhancement, region specific absorption and oral delivery of protein and peptide drugs.
- To investigate the drug absorption enhancing effects of *A. vera* gel and whole leaf materials on insulin across various GIT regions of excised pig tissue by using the Sweetana-Grass diffusion chamber as well as the everted sac technique.
- To calculate the apparent permeability coefficient (P_{app}) values for insulin alone, as well as in the presence of *A. vera* gel and whole leaf materials in the apical to basolateral direction across the intestinal tissue

- To statistically analyse the transport results to investigate whether there are any significant differences between the *A. vera* experimental groups and the control groups for each intestinal region.

1.6 Design of the study

This study was based on quantitative research methodology with an experimental design where the experiental conditions were kept constant and the drug absorption enhancement (dependent variable) was manipulated by the addition of different aloe leaf materials. Control groups were included in the study in order to indicate that the measured effect is indeed caused by the addition of the absorption enhancers and not by external factors or interference. All transport studies were done in triplicate and the averages and standard deviations were calculated for each group in order to indicate repeatability.

In the control group, the regional absorption of insulin was measured across the different GIT regions in the absence of aloe leaf materials. The regional absorption enhancement by *A. vera* gel on insulin transport was measured in the second group and in the third group the regional absorption enhancement effect by *A. vera* whole leaf materials on insulin was measured. The TEER measurement were taken prior to each transport study and then at the end of the study (after 120 min) in order to determine whether the intestinal membrane was still viable and intact.

1.7 Ethics

Excised pig intestinal tissue was collected from the Potchefstroom abattoir from pigs solely slaughtered for meat production. Animals were not slaughtered for research purposes; therefore it complies with the 3'R (Reduce, Replace, and Refine) principle. The only aspects which required ethical consideration were the site of tissue collection (the abattoir should be an authorised site that applies disease control on slaughtered animals) and the proper disposal of the animal tissue after the completion of the experimental procedures. The tissue was taken to the Animal Research Centre at the North-West University for incineration according to guidelines applicable to bio-hazardous waste disposal.

An umbrella ethics application was submitted to the Ethics Committee (AnimCare) of the North-West University, which was approved with the number NWU-00025-15-A5.

1.8 Structure of the dissertation

In this dissertation, Chapter 1 outlines the rationale as well as the aim and objectives of the study, followed by Chapter 2 which gives a review of the relevant literature as a background

for the study. The materials used for the study, experimental procedures and statistical methods used are described in Chapter 3. In Chapter 4, the results and discussions are displayed in tables and figures. The final conclusions and recommendations for future studies are summarised in Chapter 5.

CHAPTER 2: REGION SPECIFIC DRUG ABSORPTION

2.1 Introduction

The oral route of administration remains the most popular and preferred route of drug administration for most new chemical entities, despite tremendous advances in drug delivery techniques in the last few decades. Over 60% of conventional small molecule drug products are administered via the oral route (DeVane, 2004). This is due to advantages such as cost-effectiveness, patient acceptability, high patient compliance, ease of administration and the potential for availability to a large patient population when compared to alternative routes (Balimane *et al.,* 2000; Huang *et al.,* 2011; Lennernas, 2007).

Drugs intended for oral administration needs to have adequate aqueous solubility, chemical stability and must be able to cross the intestinal membrane in order to provide adequate bioavailability for therapeutic action (Balimane *et al.,* 2000; Kang *et al.,* 2009). The efficacy of drugs administered by the oral route depends on their ability to cross cell membranes in order to reach their site of action in therapeutic concentrations (Allen & Gardner, 1980; Chan *et al.,* 2004).

Currently, the oral route of administration is not suitable for protein and peptide drug delivery due to their poor bioavailability (Park *et al.,* 2011; Sharma *et al.,* 2005) of less than 1-2% (Pauletti *et al.,* 1996; Verma *et al.,* 2014; Yogendraji *et al.,* 2011). This poor bioavailability can be attributed to the physicochemical properties of protein and peptide drugs which include their hydrophilicity, large molecular size, their susceptibility to enzymatic degradation and metabolism (Chen *et al.,* 2009; Hamman *et al.,* 2005; Morishita & Peppas, 2006). The gastrointestinal tract (GIT) poses considerable barriers to drug absorption, which must be overcome before an orally administered drug can reach the systemic circulation and exert their therapeutic effects (Gabor *et al.,* 2004; Pauletti *et al.,* 1996). These barriers include the physical barriers and biochemical (enzymatic) barriers, which comprise the lipid bilayer membranes of the intestinal epithelium, digestive enzymes present throughout the GIT as well as efflux systems (Daugherty & Mrsny, 1999; Shargel *et al.,* 2012).

Hydrophilic and macromolecular drugs which are not recognised by a carrier molecule cannot partition into the hydrophobic cell membrane and thus have to cross the epithelial barrier by means of the paracellular transport route (Daugherty & Mrsny, 1999; Renukuntla *et al.,* 2013; Ward *et al.,* 2000). Paracellular transport involves the movement of molecules through the aqueous intercellular spaces between adjacent cells. Unfortunately, this pathway of transport is limited by the presence of tight junctions (zonula occludens) at the

limit between their apical surfaces facing the lumen and the basolateral surfaces which face the interior of the GIT (Daugherty & Mrsny, 1999; Gonzalez-Mariscal *et al.,* 2003). This extracellular transport route across the epithelium is drived by gradients derived from differences in electrical potential, concentration and hydrostatic pressure between the apical and basolateral sides of the epithelium (Hildalgo, 2001).

Protein and peptide drugs are poorly absorbed intact and thus require an intervention such as the addition of absorption enhancing agents (Daugherty & Mrsny, 1999). Various strategies have been investigated to overcome the physical and biochemical barriers hindering oral bioavailability of orally administered protein and peptide drugs. These can be categorised into two main strategies, namely chemical modifications and formulation technologies (Ganem-Quintanar *et al.,* 1997; Gomez-Orellana, 2005).

A formulation strategy used to improve the permeability of these protein and peptide drugs is the co-administration of absorption enhancers (Gomez-Orellana, 2005; Ward *et al.,* 2000), which acts by means of different mechanims for example tight junction regulation (Anderson & Van Itallie, 1995; Beneke *et al.,* 2012). Some naturally occurring polysaccharides are capable of enhancing intestinal absorption of co-administered drugs via the transient and reversible opening of tight junctions to allow for paracellular transport across the intestinal epithelium (Junginger & Verhoef, 1998; Kotze *et al.,* 1997; Whitehead & Mitragotri, 2008). *Aloe vera* gel and whole leaf extracts have shown potential for use as absorption enhancers for drugs with poor bioavailability where it acts on the tight junctions between GIT epithelial cells (Chen *et al.,* 2009).

2.2 The gastrointestinal tract (GIT)

The human GIT is a complex muscular tube which begins at the oral cavity and continues to the pharynx, oesophagus, stomach, small intestine (duodenum, jejunum and ileum), large intestine (colon) and ends at the rectum as illustrated in Figure 2.1 below (Martini, 2006). It is approximately 6 m long and has varying diameters throughout its length (Ashford, 2013). The principal function of the GIT is the digestion and absorption of nutrients. Most of the important digestive and absorptive processes occur in the small intestine, where the chemical digestion process is completed and the digested products are absorbed. The small intestine absorbs approximately 90% of all nutrients, while the remaining absorption occurs in the large intestine (Martini, 2006; Renukuntla *et al.,* 2013).

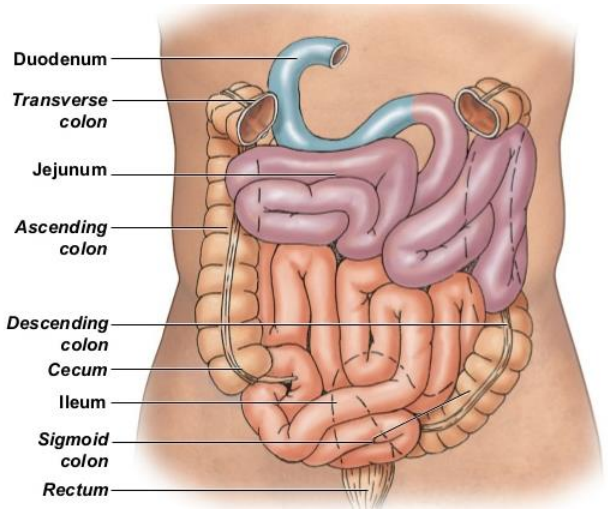


Figure 2.1: Schematic illustration of the human gastro-intestinal tract (SlideShare, 2014)

2.2.1 The intestinal epithelium

The walls of the GIT essentially have the same structure along the entire length, consisting of four principal histological layers: the mucosa, sub-mucosa, muscularis externa and the serosa (Deferme *et al.,* 2008; Rozehnal *et al.,* 2012; Swindle & Smith, 1998), as illustrated in Figure 2.2.

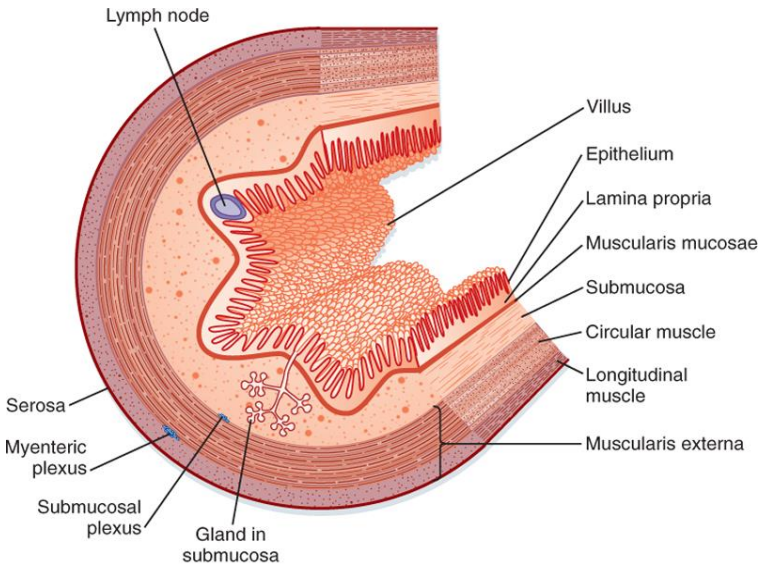


Figure 2.2: Schematic illustration of a cross-section through the human gastro-intestinal wall (Koeppen & Stanton, 2008)

2.2.1.1 Mucosa

The mucosa is the innermost layer of the wall of the GIT (Renukuntla *et al.,* 2013), which is in direct contact with the contents of the intestine (Mosentin, 1998). The mucosal layer of the GIT often forms longitudinal folds, which disappear when it fills. Permanent transverse folds of the mucosa (*plicae*) are present, which dramatically increases the surface area available for absorption (Martini, 2006). The mucosal layer is essentially composed of three distinct components, namely the muscularis mucosa, the lamina propria and the epithelium (Ashford 2013).

2.2.1.1.1 Muscularis mucosa

The muscularis mucosa forms a thin continuous sheet of smooth muscle, and thereby separating the mucosa from the sub-mucosa (Mosentin, 1998; Rozehnal *et al.,* 2012). The smooth muscle consists of two concentric layers of smooth muscle cells, the inner layer (also known as the circular muscle) encircles the lumen and the outer layer (also known as the longitudinal layer) contains muscle cells oriented parallel along the axis of the GIT. Contractions in the muscularis mucosa can alter the shape of the lumen and thereby causes movement of epithelial pleats and folds (Martini, 2006).

2.2.1.1.2 Lamina propria

The lamina propria is the middle part of the mucosal layer, which consists of areolar tissue that contains sensory nerve endings, blood vessels, lymphatic vessels, smooth muscles and scattered areas of lymphoid tissue (Martini, 2006). In most regions of the GIT, the lamina propria contains a narrow band of elastic fibres and smooth muscle cells. In essence, the lamina propria is a highly vascular layer of loose connective tissue, which underlies and supports the intestinal epithelium (Mosentin, 1998).

2.2.1.1.3 Epithelium

The epithelium, which forms the innermost layer of the mucosa, is in direct contact with the luminal contents of the GIT. This superficial monolayer sheet consists of a mixture of cells including enterocytes (or columnar epithelial cells), endocrine cells, crypt cells, absorptive cells and goblet cells, which secrete mucin. The mucus lines the surface of the epithelium, which include the crypts and the villi (Mosentin, 1998). A continuous basement membrane separates the epithelium from the underlying lamina propria (Trier & Madam, 1981).

2.2.2 Sub-mucosa

The sub-mucosa is located between the muscularis mucosa and the muscularis externa (Figure 2.2). It consists of a layer of dense irregular connective tissue containing large blood vessels and lymphatic vessels and a network of intrinsic nerve fibers namely, the sub-mucous plexus or the Meissner's plexus (Ashford, 2013; Martini, 2006; Rozehnal *et al.,* 2012). In some regions of the GIT it also contains exocrine glands, which secrete enzymes and buffers into the lumen of the GIT (Martini, 2006).

2.2.3 Muscularis externa

The smooth muscle tissue of the muscularis externa is arranged in a circular inner layer (consisting of two muscle layers with circular orientation) and an outer longitudinal layer (consisting of a single thin muscle layer with longitudinal orientation) (Ashford, 2013; Martini, 2006). Contractions of the smooth muscles in the muscularis externa provide movement of the luminal content and physical breakdown of food (Ashford, 2013).

2.2.4 Serosa

The serosa is the most outer layer of the GIT wall and is continuous with the peritoneum which covers the muscularis externa (Ashford, 2013; Rozehnal *et al.,* 2012). This serous layer consists of a monolayer of flattened mesothelial cells, which basically overlies loose connective tissues and forms an external protective coat (Renukuntla *et al.,* 2013).

2.3 Anatomy and physiology of the small and large intestine

2.3.1 Small intestine

The small intestine, which consists of three regions namely the duodenum, jejunum and ileum, is the longest (4-6 m) and most convoluted part of the GIT. It starts at the pyloric sphincter distal to the stomach and stretches to the ileocaecal junction proximal to the colon (Ashford, 2013; Chan *et al.,* 2004). The diameter of the duodenum directly distal to the stomach is approximately 40 mm, while it is approximately 25 mm in diameter at the junction with the colon (Martini, 2006). It is suspended within the peritoneal cavity by a thin, broad-based mesentery which is attached to the posterior abdominal wall and that allows the small intestine free movement within the abdominal cavity without getting entangled with the other parts of the GIT (Swindle & Smith, 1998).

The small intestine is the principal digestive-absorptive organ in the human body (Mosentin, 1998) due to the large surface area available for absorption, containing mucosal folds, villi, microvilli as well as organic anion and cation transporters (Sadee *et al.,* 1995).

Macroscopic sub-mucosal folds are present in the small intestine, which encircle the intestinal lumen and increases the surface area of the intestine at least three fold (Balimane & Chong, 2005). Mucosal folds, called plicae circularis, are most numerous in the proximal jejunum and decreases in number down the length of the digestive tract, which also contributes to an increase in the surface area (Swindle & Smith, 1998).

Furthermore, the luminal surface of the small intestinal epithelial cells is covered by finger-like projections called villi (Swindle & Smith, 1998). Villi length ranges from 0.5-1.5 mm and their diameters are approximately 0.1 mm (Ashford, 2013). Each villus is well supplied with blood vessels and contains an arteriole, a venule and a blind-ending lymphatic vessel called a lacteal (Ashford, 2013). The villi increase the luminal surface area of the GIT by at least 30 - fold (Balimane & Chong, 2005).

The brush-like structures called microvilli provide the largest increase in the epithelial surface area of at about 60 - fold (Balimane & Chong, 2005). Each villus is covered by 600-1000 of microvilli (approximately 1 µm long and 0.1 µm in diameter), which are covered by a fibrous glycoprotein known as the glycocalyx (Ashford, 2013).

In addition to the absorptive function of the small intestine, it also has digestive functions. The brush border membrane contains a wide variety of enzymes responsible for the final digestion of carbohydrates and proteins. Cytochrome P450 enzymes are also present in the epithelial cells, which mediate the metabolism of drugs via phase 1 and phase 2 reactions (Ilett *et al.,* 1990). Furthermore, special carrier proteins that facilitate the absorption of various nutrients such as minerals, sugars and amino acids are present on the surface of the microvilli (Mosentin, 1998).

Discrete, organized aggregates of lymphoid follicles, called Peyer's patches, are distributed within the lamina propria and sub-mucosa. Unlike the absorptive epithelium, they are covered with a specialised follicle-associated epithelium (Torres-Medina, 1981). Although the number and location of Peyer's patches vary between different mammal species, the basic morphological structure is similar. The number and size of the Peyer's patches generally increase with age, body weight and length of the small intestine. The terminal ileum contains the most and largest Peyer's patches, while the jejunum has fewer and smaller Peyer's patches (Swindle & Smith, 1998).

2.3.1.1 Regions of the small intestine

2.3.1.1.1 Duodenum

The duodenum is the shortest, widest and most proximal region of the small intestine directly distal to the stomach. It begins at the duodenal bulb and extend around the head of the pancreas in the retro-peritoneal space where it terminates at the ligament of Treiz into the jejunum (Swindle & Smith, 1998). The duodenum is approximately 25-35 cm long and 5 cm in diameter (DeRouchy, 2009) with a surface area of approximately 1.9 m^2 and a pH of 5.5-7.0 (Van de Graaff, 1986) due to the presence of hydrochloric acid secreted from the stomach. The duodenum's primary function is to receive the chyme from the stomach and neutralise its acidic pH before it damages the absorptive surfaces of the small intestine (Martini, 2006).

In this region, the mucosal folds are particularly well developed (Ashford, 2013), although they are few in number (Martini, 2006). Absorption in the duodenum occurs mainly via passive diffusion, active transport and facilitated transport (DeRouchy, 2009). The major digestive enzymes present in the duodenum are secreted by the pancreas and include trypsin, chymotrypsin and carboxypeptidases (Chawia *et al.,* 2003). These enzymes break down (hydrolyse) proteins, fats and carbohydrates in the chyme. Sodium bicarbonate, secreted by the pancreas, serves the role of alkalising chyme before it passes into the next region of the GIT, namely the jejunum. Bile is secreted into the duodenum after its production by hepatocytes in the liver. Its main function is to promote the efficient absorption of fatty acids and cholesterol via emulsification and micellar solubilisation (Ashford, 2013).

2.3.1.1.2 Jejunum

The jejunum is the major drug absorbing region of drugs in most mammals primarily due to its large surface area (Cao *et al.*, 2006; Kasim *et al.,* 2004; Lennernas *et al.,* 1992; Sun *et al.,* 2002). The high capacity for absorption in the jejunum is due to its well-developed mucosal folds (Martini, 2006), large surface area (approximately 184.0 m^2) as well as the abundant villi present on the luminal surface (Balimane & Chong, 2005; Cao *et al.,* 2006).

The jejunum is approximately 2.5 m long with a diameter of 5 cm (Martini, 2006) and accounts for the proximal 40% of the small intestine (Rozehnal *et al.,* 2012). The proximal half of the jejunum has very prominent plicae and villi, which gradually decrease in size down the length of the jejunum. This reduction parallels a reduction in the absorptive capacity. Most of the nutrient absorption occurs before the chyme reaches the ileum (Martini, 2006). The major metabolic constituents of the jejunum include amylase, maltase,

lactase, sucrose and CYP3A5, where absorption mechanisms include passive diffusion, active transport and facilitated transport (Chawia *et al.,* 2003).

2.3.1.1.3 Ileum

The ileum forms the most distal region of the small intestine comprising the last 300-420 cm (Balimane & Chong, 2005; Mosentin, 1998; Rozehnal *et al.,* 2012). Distinctive masses of aggregated lymphoid nodules, called Peyer's patches, are present in the ileum, especially the distal ileum (Martini, 2006).

The proximal part of the ileum contains abundant villi, and this contributes to absorption (Chawia *et al.,* 2003) via a relatively large surface area of approximately 276.0 m^2 (Balimane & Chong, 2005). Digestive enzymes present in the ileum include nuclease, nucleotidase and enterokinase (Chawia *et al.,* 2003). Major absorption processes include passive diffusion, convective transport, active transport, facilitated transport, ion pairing and pinocytosis (Chawia *et al.,* 2003). A continuous Peyer's patch is present along the length of the terminal ileum (Rothkotter *et al.,* 1990).

2.3.2 Large intestine (colon)

The colon forms the final major region (90-150 cm) of the GIT where it stretches from the ileocecal junction to the rectum. It has a relatively small surface area of 1.3 m^2, and has a pH ranging from 7.0 to 7.5 (Balimane & Chong, 2005). The colon consists of the cecum (approximately 85 mm), the ascending colon (approximately 200 mm), the hepatic flexure, the transverse colon (usually longer than 450 mm), the splenic flexure, the descending colon (approximately 300 mm) and the sigmoid colon (approximately 400 mm) (Ashford, 2013). Its main functions include water and electrolyte re-absorption as well as excretion of metabolic waste products and toxic substances (Mosentin, 1998).

The colon has a larger diameter (approximately 5 cm) and a thinner wall than the small intestine (Chawia *et al.,* 2003). The wall of the colon forms a series of pouches (haustra), which permits elongation and expansion of the colon (Martini, 2006).

Unlike the small intestine, the colon has no specialised villi (however, it does contain microvilli), thus its absorptive capacity is less (Swindle & Smith, 1998). The mucosal folds are formed by the creases in the intestinal lumen between the haustra which produces a series of internal folds (Martini, 2006). The large intestine has numerous goblet cells and an increased number of lymphatic nodules as well as the characteristic crypts of Lieberkühn, which is predominantly associated with goblet cells and a few absorptive endocrine cells (Swindle & Smith, 1998).

An extensive number (approximately 10^{12} per gram of contents) and variety of bacteria permanently colonise the colon and is capable of performing several metabolic reactions, including the reduction of inactive conjugated drugs to their active form and the hydrolysis of fatty acid esters (Ashford, 2013).

Undigested polysaccharides in the diet and the carbohydrate components of secretions such as mucous, provides the bacteria with their energy and carbon sources and is important for the maintenance of the functional and morphological integrity of the colonic epithelium (Mosentin, 1998). The bacteria degrade the polysaccharides to provide short chain fatty acids (acetic, propionic and butyric acids) which lower the luminal pH and to produce gasses such as hydrogen, methane and carbon dioxide (Ashford, 2013).

2.4 Transport pathways of molecules across plasma membranes

Drug absorption across the intestinal epithelial cell membranes is a complex and multi-pathway process. It depends on the membrane properties as well as the drug's physicochemical characteristics (Salama *et al.,* 2006). The physicochemical properties of a drug will mainly determine by which pathway it will cross the epithelial membrane (Daugherty & Mrsny, 1999).

The rate and extent to which a compound is absorbed by the intestinal epithelium is a critical factor in determining its overall bioavailability (Chan *et al.,* 2004) since the absorption, distribution, biotransformation and excretion of a drug involves its passage across cell membranes (Benet *et al.,* 1996). Absorption of dissolved molecules from the lumen of the GIT begins with transport across the apical membrane of the epithelial cells lining the mucosal surface. This is followed by passage across the epithelial cells and entry into the blood or lymphatic system (Mosentin, 1998).

Drug transport across the intestinal epithelium can occur via two main transport pathways: firstly the transcellular transport pathway (i.e. across the lipid-bilayer membrane through the epithelial cell), and secondly the paracellular transport pathway (through the intercellular spaces between cells) (Balimane *et al.,* 2006; Daugherty & Mrsny, 1999). Generally, lipophilic and small amphiphatic drugs are transported transcellularly across the intestinal epithelia by partitioning into and out of the lipid bilayer, while the paracellular pathway is mainly available for very small hydrophilic drugs (Salama *et al.,* 2006). Proteins and peptides are poorly absorbed intact from the GIT and require the addition of enhancing agents or special uptake mechanisms (Daugherty & Mrsnsy, 1999).

The transcellular transport pathway can occur by means of different mechanisms of action namely passive diffusion, carrier-mediated transport (which may be active transport or facilitated diffusion) and endocytosis (Ashford, 2013). Figure 2.3 illustrates the transcellular and paracellular transport pathways.

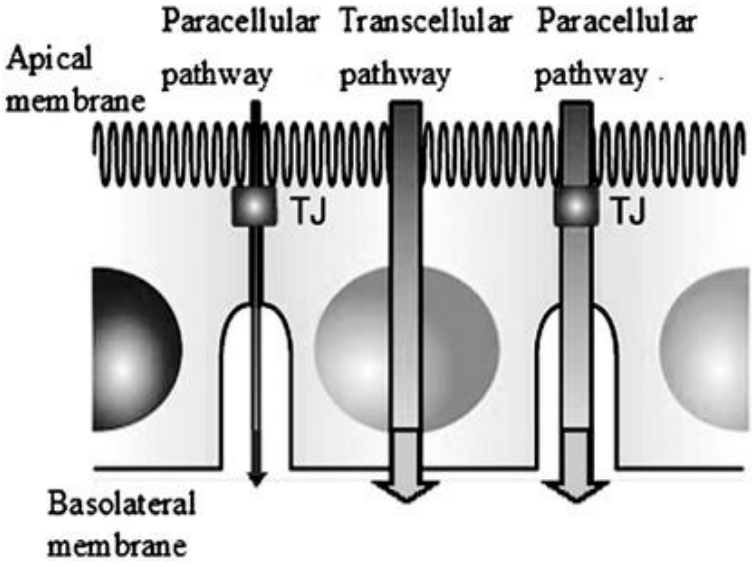


Figure 2.3: Schematic illustration of the transcellular and paracellular transport pathways of molecules across the intestinal epithelium (Hossain & Hirata, 2008)

2.4.1 Transcellular transport

As mentioned before, the transcellular pathway of absorption involves the transport of molecules across the epithelial membrane by means of different mechanisms of action. The transcellular route of absorption is ideal for lipophilic drugs with a relatively high affinity for the cell membrane's lipid bilayer (Renukuntla *et al.,* 2013). Passive diffusion, for example, requires the compound to partition into the membrane before it partitions out of the membrane on the other side. This pathway of absorption is generally only accessible to relatively hydrophobic molecules with a molecular weight (MW) below 500 Da, because larger molecules generally experience difficulty crossing the epithelial membrane by passive diffusion (Hochman *et al.,* 1994; Lipinski *et al.,* 2001; Lipinski, 2004).

2.4.1.1 Passive diffusion

During passive transport, drug molecules pass across the lipid membrane by means of passive diffusion from a region of high concentration (e.g. in the lumen) to a region of low concentration (e.g. in the blood) along the concentration gradient (Ashford, 2013; Shargel & Yu, 1999). It is an energy-independent, non-specific process, using the difference in

concentration and charge across the membrane as driving force for molecule transport. Drug transport via passive diffusion utilising the transcellular pathway is considered to be the most important transport pathway for drug molecules across the intestinal epithelium because the cell membrane presents a surface area more than 1000 times larger than that available for the paracellular pathway (Artursson *et al.,* 1993; Deferme *et al.,* 2008; Fasano, 1998).

For a drug to be transported by passive diffusion via the transcellular pathway, it needs to exhibit sufficient solubility in the membrane, after which it should exhibit sufficient solubility in the blood in order to partition readily out of the membrane phase and into the blood (Ashford, 2013).

2.4.1.2 Carrier-mediated transport (active and facilitated transport)

Some are absorbed transcellularly by a carrier-mediated transport system, which involves specific membrane-carrier transporters. Amino acids, di- and tripeptides, monosaccharides, water soluble vitamins and nucleosides are examples of molecules that are absorbed via this pathway (Daugherty & Mrsny, 1999).

Carrier-mediated transport can be classified into two main groups, i.e. facilitated transport and active transport.

2.4.1.2.1 Active transport

Active transport requires the active participation by the cell membrane in the transport process, thus requiring energy as a driving force (Ashford, 2013). The energy required for this transport to occur against a concentration gradient (from a region of low concentration to a region of high concentration) is derived from ATP-hydrolysis by Na+, K+-ATPase (Smith, 2005).

This specialised transport pathway requires a carrier/membrane transporter to bind to a drug molecule and to carry it across the membrane. The drug molecule forms a complex with the transporter on the surface of the apical cell membrane of the columnar absorptive epithelial cell, after which the drug-carrier complex moves across the membrane and releases the drug on the other side of the membrane. The drug transporter then returns to the initial position in the apical cell membrane in order to transport another molecule (Ashford, 2013). This specialised transport process is selective, saturable and energy-dependent (Siccardi *et al.,* 2005). Active transport occurs at a rate proportional to the drug concentration only at concentrations where the transporter is not saturated (Ashford, 2013).

A large number of carrier-mediated active transport systems are present in the small intestine, such as the vitamin transporters, sugar transporters, peptide transporters, nucleoside transporters, bile acid transporters, amino acid transporters and organic anion transporters. Generally, each carrier system is concentrated in a specific region of the GIT, such as the bile acid transporters which are only found in the ileum. The active transporter substrate to be transported will thus preferentially be absorbed in the region of highest carrier density (Ashford, 2013).

2.4.1.2.2 Facilitated transport

Facilitated transport is an energy independent transport process, which occurs with a concentration gradient, i.e. from a region of high concentration to a region of low concentration. Unlike active transport, it cannot transport molecules against a concentration gradient. Since active transporter carriers are utilised, this process can be saturated and it is susceptible to inhibition by competitive inhibitors. Facilitated transport plays a minor role in drug absorption even though it enables a faster rate of transport than passive diffusion (Ashford, 2013).

2.4.2 Paracellular transport

The paracellular transport pathway involves movement of molecules through the aqueous intercellular spaces between adjacent epithelial cells and through pores in the tight junctions (zonula occludens that are protein structures responsible for joining the cells to each other at their apical membranes) (Daugherty & Mrsny, 1999). Hydrophilic or polar molecules with molecular weights higher than 200 Da (Ashford, 2013) and larger than 11.5 Å are generally restricted from the paracellular pathway due to the small pore sizes of the tight junctions (i.e. 0.8 nm in the human jejunum and 0.3 nm in the ileum and colon) (Hamalainen & Frostell-Karlsson, 2004; Lennernas, 1998; Norris *et al.,* 1998).

The intercellular spaces only occupy about 0.1% of the total surface area of the epithelium (Artursson *et al.,* 1993; Deferme *et al.,* 2008; Fasano, 1998; Madara & Pappenheimer, 1987). Furthermore, the different epithelia of the body have tight junctions differing in tightness. Tight junctions in the absorptive epithelia such as that of the small intestine tend to be leakier than other epithelia. As the number and size of the pores between the epithelial cells in the GIT decrease, the importance of the paracellular transport pathway also decreases (Ashford, 2013; Hamalainen & Frostell-Karlsson, 2004; Lennernas, 1998; Norris *et al.,* 1998).

The driving forces for this aqueous extracellular transport pathway across the epithelium are the electrochemical potential gradients derived from differences in concentration, electrical potential and hydrostatic pressure between the two sides of the epithelium (Hidalgo, 2001).

2.5 Barriers to absorption of drugs

Successful oral delivery of a drug requires its absorption across the intestinal epithelium. The GIT is responsible for the absorption of nutrients, while simultaneously preventing the absorption of potentially harmful substances. The gastrointestinal epithelium is a tightly bound collection of a single layer of columnar cells, which separates the lumen of the GIT from the systemic circulation. The intestinal epithelium allows permeation of certain molecules and substances, while others are prevented from entering the systemic circulation (Aijaz *et al.,* 2006; Norris *et al.,* 1998). This selective permeability is accomplished through physical and biochemical barriers, which can also affect drug absorption and the intact absorption of protein and peptide drugs (Hamman *et al.,* 2005).

2.5.1 Physical barrier

The intestinal mucosa is the major physical barrier to the absorption of orally administered drugs. The epithelial cell membrane has a bilayer structure and is composed of polysaccharides, proteins, lipids and lipoproteins. The physical barrier layers to the passage of drug molecules from the GIT lumen consist of a mucus layer, the glycocalyx and the cell membrane, while the intercellular spaces are sealed by occluding junctions known as tight junctions (Siccardi *et al.,* 2005). Tight junctions (zonula occludins) are dynamic structures, which form the most apical component of the intercellular junctional complex system between adjacent intestinal epithelial cells (Chiba *et al.,* 2008; Madara, 1998) and act as a semipermeable barrier to paracellular transport (Chiba *et al.,* 2008). These complex systems also include the underlying adherens junctions (zonula adherens) and the most basally located desmosomes (macula adherens) and gap junctions in addition to the tight junctions (Ward *et al.,* 2000) as illustrated in Figure 2.4.

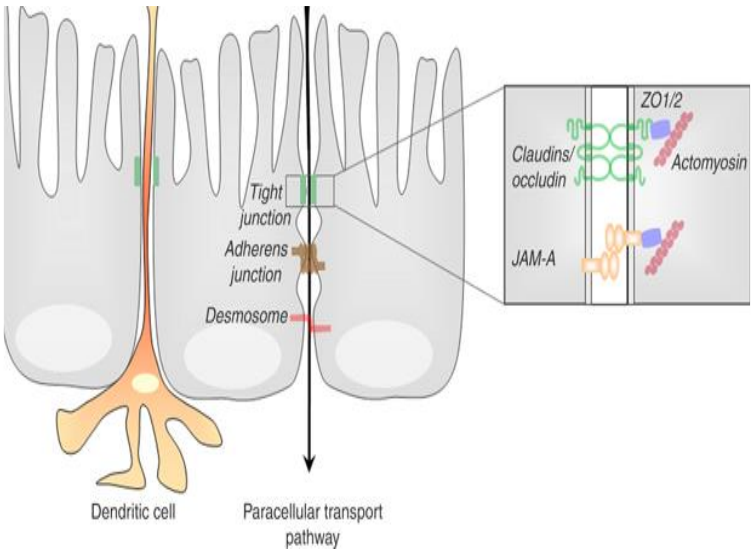


Figure 2.4: The tight junction as a component of the intestinal epithelial membrane (Menard *et al*., 2010)

Tight junctions form a selective, regulated barrier at the aqueous extracellular spaces between the adjacent epithelial cells where they restrict the movement of nutrients, fluids and molecules between intestinal lumen and the sub-mucosa (Chiba *et al.,* 2008; Hollander, 1992), while also separating the apical and basolateral cell surface domains (Salama *et al.,* 2006). These tight junction barriers form the major rate-limiting barrier for the paracellular transport of molecules, ions and solutes (Madara, 1998).

The adherens junctions and the desmosomes maintain cellular proximity by forming strong adhesive bonds, while also providing a site of intercellular communication. Loss of adherens junctions causes the disruption of cell to matrix and cell to cell contacts, ineffective epithelial cell polarisation and polarisation (Turner, 2009).

Tight junctions consist of transmembrane proteins, which include occludin, claudins and the junctional adhesion molecules (JAM). Occludin regulates the barrier function of the tight junction, provides structural integrity by its N-terminal and extracellular domains (Ward *et al.,* 2000) and regulates the permeation selectivity of the junction (Mullin *et al.,* 2005). Occludin has also been associated with neutrophil transmigration (Johnson, 2005). The peripheral membrane proteins, zonula occludins 1 (ZO-1) and ZO-2 are essential for tight junction assembly and maintenance, due to the fact that they have multiple domains for interaction with other proteins including claudins, occludin and actin (Turner, 2009). Claudins are the major molecules regulating tight junction permeability and the major protein of the tight junction fibril (Mullin *et al.,* 2005), with its four transmembrane domains forming the major

structural components of tight junction strands (Ward *et al.,* 2000). JAM is structurally distinct from occludin and claudin.

Transepithelial electrical resistance (TEER) is a measure of tight junction integrity, which reflects the electrical resistance provided by the tight junction between the apical and basolateral sides of an epithelial cell monolayer. Any change in the tight junction complex properties can result in a major effect on the TEER (Johnson *et al.,* 2008).

2.5.2 Biochemical barrier

Protein molecules administered orally, face another barrier to absorption: the enzymatic barrier. The instability of proteins in the acidic environment and their metabolism by luminal digestive enzymes may cause their degradation in the GIT. Protein digestion by proteases starts in the stomach and is continued by the different enzymes along the entire GIT (Hamman *et al.,* 2005).

Pepsins are located in the stomach and trypsin, chymotrypsin and carboxypeptidases from the pancreas are located in the small intestinal lumen (Guyton, 1992). The pancreatic enzymes are responsible for only 20% of the enzymatic degradation of ingested proteins (Pauletti *et al.,* 1996). The remainder of the degradation occurs at the brush-border membrane (by various peptidases) or within the enterocytes of the intestinal tract. There also exists a specific cytosolic enzyme called insulin-degrading enzyme which specifically degrades insulin (Bai, 1995).

2.6 Problems associated with protein and peptide drug delivery

Protein and peptide drugs have poor oral bioavailability of less than 1-2% (Pauletti *et al.,* 1996; Verma *et al.,* 2014; Yogendraji *et al.,* 2011) due to their large molecular size, susceptibility to enzymatic degradation and their hydrophilicity (Hamman *et al.,* 2005; Morishita & Peppas, 2006). The small intestine has the ability to metabolise drugs by numerous pathways after oral administration (Krishna & Klotz, 1994). Apart from these intrinsic physicochemical properties of peptide and protein drugs, the GIT contributes to their low bioavailability through the presence of various physical and biochemical barriers (Daugherty & Mrsny, 1999).

Intact proteins must first be broken down into their constituent free amino acids before they can be absorbed by the intestinal epithelial tract. Unlike low molecular weight drugs, proteins have a complex structure that defines their biological activity. Protein molecules can be deactivated by even the slightest changes in the environment, resulting in the disruption of its primary amino acid sequence, its secondary two-dimensional structure, its

tertiary folding or the quaternary structure (combination of peptide sub-units) (Carino & Mathiowitz, 1999).

Due to their poor absorption and insufficient bioavailability, most therapeutic proteins and peptides have been restricted to injectable dosage forms. Although absorption enhancing agents exist that can improve the intestinal absorption of some of these drugs, they are not used clinically due to their toxicity and lack of effectiveness (Hamman *et al.,* 2005).

Drug efflux also poses a significant barrier to oral administered drugs, which may include protein and peptide drugs. Apical efflux transporters include adenosine triphosphate-binding cassette (ABC) proteins such as P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP) and multidrug resistance protein (MDR) (Chan *et al.,* 2004). P-gp is an energy dependent transmembrane protein drug efflux pump, located in the apical membrane of intestinal epithelial cells. It has a molecular weight of 170 kDa and has 1280 amino acid residues (Juliano & Ling, 1976). It may limit the bioavailability of many orally administered drugs by transporting it back into the intestinal lumen following absorption by the enterocytes (DeSesso & Jacobson, 2001; Paine *et al.,* 1996).

2.7 Strategies for effective oral delivery of protein drugs

Several strategies have been employed to improve the oral bioavailability of protein drugs. These strategies focus on minimising or potentially eliminating one of the barriers to intestinal drug absorption. Absorption or penetration enhancers are commonly used to improve permeation through the epithelial layer, for example by opening the tight junctions. Two main strategies are followed to improve drug absorption, namely chemical formulations and formulation strategies (Gomez-Orellana, 2005; Legen *et al.,* 2005).

2.7.1 Chemical modifications

Various chemical modifications have been used in order to allow recognition of protein and peptide drugs by membrane transporters, to increase their enzymatic stability and increase their membrane permeation capability (Hamman *et al.,* 2005; Mahato *et al.,* 2003). Structural manipulation or chemical modification of protein and peptide drugs can improve their pharmacodynamic properties and their pharmacokinetic profile (Hamman *et al.,* 2005).

2.7.2 Formulation strategies

2.7.2.1 Drug absorption enhancing agents

Ideally, absorption enhancing agents should reversibly remove or temporarily disrupt the intestinal barrier without damaging the tissue or exhibit toxic effects, thus allowing drug molecules to permeate the intestinal epithelial cells and allowing them to enter the blood circulation (Hamman *et al.,* 2005; Mahato *et al.,* 2003; Muranishi & Yamamoto, 1994). Furthermore, an ideal absorption enhancing agent should also have the following properties: it should have a predictable duration of action, compatibility with the drug to be delivered, have a rapid response, specificity for the drug to be delivered, its effect should result in therapeutic drug plasma levels and its effect shoud be completely reversible (Fix, 1996; Junginger & Verhoef, 1998).

Although intestinal drug absorption enhancement has been associated with acute epithelial damage in some cases, there is evidence that certain drug absorption enhancing agents can increase intestinal drug absorption in a reversible way without causing damage or exerting toxic effects (Whitehead *et al.,* 2008). It is of utmost importance to find safe and effective drug absorption enhancers in order to ensure successful oral delivery of poorly absorbable drugs (Vinson *et al.,* 2005).

Absorption enhancers can be grouped into several classes such as chelating agents, surfactants, bile salts, salicylates, fatty acids, toxin and venom extracts, complexation agents and non-surfactants (Lee & Yamamoto, 1990; Mahato *et al.,* 2003), as summarised in Table 2.1. These absorption enhancers have different mechanisms of action which improves the permeation of therapeutic agents at either the apical cell membrane (transcellular pathway) or the tight junctions between cells (paracellular pathway), or both (Lee & Yamamoto, 1990). These mechanisms include a change in membrane fluidity, alteration of mucus rheology, leakage of proteins through the membrane, and increasing paracellular transport by opening the tight junctions (Mahato *et al.,* 2003; Hamman *et al.,* 2005).

Table 2.1: Classes of drug absorption enhancers (Hamman *et al*., 2005; Lee, 2000; Mahato *et al*., 2003)

Absorption enhancer	Examples	Mechanism of action	References
Chelating agents	Ethylene diamine tetraacetic acid,	Acts on paracellular and transcellular pathways, Complexation of calcium	(Raiman *et al.,* 2003)

	Ethylene glycol tetraacetic acid	and magnesium (tight junction opening)	
	Polyacrylates	Opening of tight junctions, increase paracellular transport	(Raiman *et al.,* 2003)
Bile salts	Na-taurocholate, Na-taurodeoxycholate, Na-taurodihydrofusidate	Disrupts membrane integrity by phospholipid solubilisation and cytolytic effects, reduces mucus viscosity	(Yamashita *et al.,* 1990)
	Na-deoxycholate	Reduces mucus viscosity, increases paracellular transport	(Junginger & Verhoef, 1998)
	Na-glycocholate	Peptidase inhibition, increases paracellular transport	(Junginger & Verhoef, 1998)
Surfactants	Na-dodecyl sulphate, Na-dioctyl sulfosuccinate, Nonylphenoxy polyoxyethylene	Acts on paracellular pathway Membrane damage by extracting membrane proteins or lipids, phospholipid acyl chain perturbation Reduces membrane viscosity, inhibits peptidase	(Anderberg *et al.,* 1992)
Complexation agents	α-, β- and γ- cyclodextrin, Methylated β- cyclodextrin	Acts on paracellular and transcellular pathways. Inclusion of membrane compounds, increase transcellular transport	(Junginger & Verhoef, 1998)
Toxin and venom	Zonula occludins toxin (ZOT)	Interaction with the zonulin surface receptor	(Su *et al*., 2001)

extracts		induces actin polymerisation (tight junction opening)	(Liu *et al*., 1999)
	Melittin (bee venom extract)	α-helix ion channel formation, bilayer micellisation and fusion	(Cox *et al.,* 2002) (Fasano, 1998) (Fasano & Nataro, 2004)
Salicylates	Na-salicylate Salicylate ion	Increase cell membrane fluidity, decrease concentration of non-protein thiols, prevent protein aggregation or self-association	(Muranishi & Yamamoto, 1994) (Kajii *et al.,* 1986)
Fatty acids	Short chain fatty acids e.g. Oleic acid	Act on paracellular and transcellular pathways. Phospholipid acyl chain perturbation, increase paracellular transport	(Junginger & Verhoef, 1998)
	Medium chain glycerides e.g. Na-caprate , cyclodextrins	Acts on paracellular and transcellular pathways. Paracellular: Na-caprate dilates tight junctions. Transcellular: epithelial cell damage or disruption of cell membranes	(Hochman *et al.,* 1994) (Ye *et al.,* 1995)
	Long chain fatty acid esters e.g. palmitoylcarnitine , Hexadecylphosphocholine		

2.7.3 Mechanisms of action of drug absorption enhancers

2.7.3.1 Opening of tight junctions

Due to the dynamic nature of tight junctions, several strategies have been developed in order to enhance the paracellular absorption of some hydrophilic drugs (Gomez-Orellana, 2005; Hunter & Hirst, 1997; Ward *et al.,* 2000). Some naturally occurring polysaccharides are capable of enhancing intestinal absorption of co-administered drugs by the transient

opening of tight junctions between adjacent epithelial cells to allow paracellular transport (Kotze *et al.,* 1997; Junginger & Verhoef, 1998; Johnson, 2005).

The controlled opening of tight junctions is an attractive approach to increase the paracellular absorption of hydrophilic drugs like proteins and peptides because this transport pathway avoids the intracellular enzymatic degradation that is present in the cytosolic contents of the epithelial cells (Pauletti *et al.,* 1996; Salama *et al.,* 2006; Ward *et al.,* 2000).

Due to its net positive charge, cationic polysaccharides such as the chitosan can interact with the anionic components of the glycoproteins on the intestinal epithelial cell membrane surface. Enhanced transport through the paracellular pathway is achieved by the interaction of the polysaccharides with the cell membrane, which results in the structural re-organisation of the tight junction associated proteins (Schipper *et al.,* 1997). Tight junction opening can also be achieved by a combination of ionic interactions with the negatively charged groups of the glycocalyx and mucoadhesion with the cell membrane (Junginger & Verhoef, 1998; Kotze *et al.,* 1997; Kotze & De Boer, 1998; Thanou *et al.,* 2000). Anionic polymers, like the poly (acrylic acid) derivatives can also mediate tight junction opening by means of the combination of enzyme inhibition and extracellular calcium depletion (Borchard *et al.,* 1996; Junginger & Verhoef, 1998).

Polysaccharides of plant origin, such as those found in *Aloe vera* gel and whole leaf, have also shown the ability to open tight junctions between intestinal epithelial cells. This was indicated by a reduction in the TEER measurements across epithelial cell monolayers (i.e. Caco-2). The transport of macromolecular model compounds was also shown to be increased across the intestinal epithelium when the aloe leaf materials were co-applied (Beneke *et al.,* 2012; Chen *et al.,* 2009; Lebitsa *et al.,* 2012).

2.7.3.2 Efflux transporter inhibitors

Efflux transporters such as P-glycoprotein (P-gp) can be inhibited in the intestinal epithelial layer. This can increase the oral bioavailability of drugs that are substrates for these efflux transporters. By blocking the drug-binding site, altering the integrity of cell membrane lipids and interfering with ATP hydrolysis, P-gp inhibition can occur (Varma *et al.,* 2003). The ideal P-gp inhibitor should exhibit minimal systemic effects while exhibiting near maximum inhibition locally at the intestine (Bansal *et al.,* 2009).

Three generations of efflux transporter inhibitors exist based on their specificity and affinity. First generation P-gp inhibitors are pharmacologically active drug substances and these compounds have limited use due to the relatively high concentrations required for P-gp

inhibition. Second-generation P-gp inhibitors lack pharmacological activity and exhibit higher P-gp affinity. Third-generation P-gp inhibitors are highly potent and selective inhibitors of P-gp (Varma *et al.,* 2003). Co-administration of these inhibitors provides the possibility to maximise the oral bioavailability of drugs that are substrates for both efflux and metabolising systems (Hunter & Hirst, 1997).

2.7.3.3 Muco-adhesive systems

Bio-adhesion is the process whereby attachment of a synthetic or biological macromolecule adheres to a biological surface. When the drug delivery device adheres specifically to the mucus layer and forms bonds with it, it is called muco-adhesion, and if it adheres to the cells, it is referred to as cyto-adhesion (Vasir *et al.,* 2003).

The mucous layer which covers the mucosal cell surface can be seen as an unstirred layer, consists of water, electrolytes, proteins, nucleic acids and glycoprotein. Muco-adhesive delivery systems have several advantages for drug delivery such as a prolonged gastrointestinal residence time, an intimate contact of the delivery system with the mucosal membrane to provide a high concentration gradient as a driving force for passive drug absorption as well as minimum luminal drug degradation, localisation of the delivery system at the absorption window of the particular drug and providing a basis for functioning of drug absorption enhancers and enzyme inhibitors (Bernkop-Schnurch, 2005).

Unfortunately, gastrointestinal muco-adhesive systems have not succeeded in retaining or localising drug delivery systems at a specific region. This inability to remain at the site of adhesion for extended periods of time is due to the relatively high natural turnover rate of the mucus and sloughing of epithelial cells (Kompella & Lee, 2001).

2.8 Methods for determining transport of drugs

2.8.1 Current models

Different types of methods/techniques have been established for investigating the principal transport/absorption mechanisms in animals, which can involve the whole animal (*in vivo*), an isolate organ as part of the animal (*in situ*) and excised tissues removed completely from the animal (*in vitro* or *ex vivo*).

As an alternative to human intestinal tissue, animal tissues displaying similar physiological and anatomical characteristics of those in humans can be used (Balimane *et al.,* 2000). Excised animal tissue models have been used for the last eight decades in order to investigate the various mechanisms underlying the absorption of nutrients from the intestine

(Balimane *et al.,* 2000). Techniques utilised for these excised tissue based on *in vitro* models include isolated membrane vesicles, the Ussing chamber and the everted sac technique.

2.8.1.1 *In situ* perfusion models

In situ perfusion models of small intestinal segments where the intestine is still part of the anesthetised animal are commonly used for studying drug absorption and metabolism. This type of model mimics the *in vivo* conditions closely due to an intact nervous system, blood supply and full expression of membrane transporters and metabolizing enzymes. This leads to good absorption and metabolism prediction, which provides a good correlation between animal perfusion test and the human effective permeability of several drugs. Input of the drug compound can be closely controlled in terms of concentration, pH, osmolality, intestinal region and flow rate (Lennernas, 1998). *In situ* methods can be used for GIT regional absorption studies and observing the gross effects of drug absorption enhancers, but give no information about events at the cellular or membrane level (Park, 1992).

This model, however, is not practical for high-throughput screening. Due to the fact that the calculated permeability is based on the disappearance of a compound from the luminal side, this may lead to overestimation of the intestinal absorption for drugs undergoing gut metabolism or extensive accumulation in intestinal tissues (Antunes *et al.,* 2013). *In situ* models do not require the drug to pass through the stomach and therefore prevent the precipitation of acidic compounds, so dissolution rates do not confuse intestinal drug concentrations and therefore plasma levels (Le Ferrec, 2001; Balimane *et al.,* 2006).

A drawback of this model is that the anaesthesia can influence intestinal drug absorption, which complicates the choice of anaesthetic (Le Ferrec, 2001; Balimane *et al.,* 2006). *In situ* techniques are expensive to perform and difficult to handle and it can therefore not be used routinely during drug development (Acra, 1991).

2.8.1.2 *In vivo* animal models

In vivo models using whole animals are widely used to predict intestinal absorption and metabolism. Because the gastrointestinal anatomy of some mammals demonstrate functional similarity with that of humans, the drug absorption characteristics in animals are mostly sufficient as a reliable predictor of the biological factors that can influence the intestinal absorption of drugs in humans (Hildalgo, 2001; Westerhout *et al.,* 2014).

When using *in vivo* models, drugs are orally administered in order to measure their absorption from the gut to the blood and tissue compartments of the body. Samples are withdrawn from the systemic circulation at pre-determined time intervals after oral administration followed by sample analysis to determine the drug plasma concentration at each time point (Hildalgo, 2001).

The main advantage of *in vivo* models is the occurrence of the dynamic interplay between the intact components of the mesenteric blood circulation, the mucous layer and all the other physiological factors that can influence drug dissolution and absorption (Kararli, 1995).

Disadvantages of *in vivo* models include the relatively large amounts of drug required, the complex and invasive nature of the analytical models necessary for plasma analysis, the limited insight provided into the mechanisms of drug absorption and the fact that the variables inherent to the absorption process cannot be separated, thus not allowing identification of individual rate limiting factors (Le Ferrec, 2001; Hildalgo, 2001). The method is also time consuming, labour intensive and requires large amounts of resources (Balimane *et al.,* 2000).

2.8.1.3 *In vitro* models

In vitro models for the assessment of intestinal absorption have been developed due to the fact that *in vivo* animal studies cannot be used as a high volume screening tool in the early stages of drug development due to ethical considerations and time constraints (Deferme, 2008). Various *in vitro* methods are available to determine the intestinal absorption potential of drug candidates. Each method has several advantages and disadvantages that must be taken into account upon deciding which method will suit the outcome of the current experiment the best. Compared to *in vivo* models, *in vitro* techniques for the assessment of permeability are less labour - intensive and more cost - effective (Balimane *et al,* 2000).

For *in vitro* drug permeation or transport experiments, several barriers may be used, including artificial membranes (parallel artificial membrane permeability assay [PAMPA]), cell culture monolayers (Caco-2 cells [Human colon adenocarcinoma] and MDCK cells [Mardin-Darby canine kidney cells]), isolated mucosal cells and intact tissue techniques (human or animal intestinal tissue).

When selecting an *in vitro* model, the following criteria should be considered:

- Simplicity,
- Reproducibility,

- Rapid turnaround time,

- Predictability of *in vivo* absorption in humans (Habucky, 1995).

Numerous *in vitro* methods have been used in the selection process for assessing the intestinal absorption potential of drug candidates. Although it is very difficult to develop a single *in vitro* system that can simulate all the conditions existing in the human intestine, various *in vitro* models are used routinely as decision making tools in the drug discovery process (Balimane *et al.,* 2000).

One of the major concerns with all *in vitro* systems is that the effects of physiological factors such as gastric emptying, gastrointestinal transit rate, and gastrointestinal pH cannot be integrated in the data analysis. The successful application of *in vitro* models to predict the drug absorption across the intestinal mucosa depends on the extent to which the *in vitro* model mimics the characteristics of the *in vivo* intestinal epithelium (Balimane *et al.,* 2000). Another disadvantage of *in vitro* models is the limited viability of the excised intestinal tissue used for transport experiments. The tissue only remains viable for 2-3 hours after the death and isolation of the tissue from the animal. TEER measurement across the intestinal membranes can be used to monitor the integrity of the tissue (Bohets *et al.,* 2001).

2.9 *In vitro* techniques for testing drug transport across excised intestinal membranes

2.9.1 Sweetana-Grass diffusion chambers

Ussing type diffusion chambers were initially introduced in 1951 for studying the active (vectorial ion) transport of sodium as a source of electric current in short-circuited, isolated frog skin (Ussing & Zehran, 1951). It is now widely used to study ion transport over a diverse range of different membranes (Le Ferrec, 2001; Rozehnal *et al.,* 2012). These chambers can provide a physiologically relevant system for measuring the transport of ions, nutrients and drugs across various excised intestinal epithelial tissues (He *et al.,* 2013). The technique provides a physiologically relevant method that enables the precise measurement of electrical and transport parameters of intact intestinal epithelium (Clarke, 2009; He *et al.,* 2013; Hug, 2002).

Grass and Sweetana optimised the technique for drug absorption studies (Grass & Sweetana, 1988). They developed an adaptation of the Ussing chambers (referred to as Sweetana-Grass diffusion chambers), which can measure tissue permeability of compounds. The design allows the cell to be manufactured in a wide range of sizes in order to allow optimisation of surface area to volume ratio for a variety of tissues of different types (i.e. cell

cultures or excised intestinal tissues). The apparatus can also be used for the evaluation of transport of compounds across mucosal/epithelial barriers from different origins namely gastrointestinal epithelium, buccal epithelium or sublingual epithelium (Grass & Sweetana, 1988).

In this technique, intestinal mucosal sheets are clamped between a donor (apical/mucosal) chamber and an acceptor (basolateral/serosal) chamber. The apical chamber is filled with the test solution including the compound to be tested, while the basolateral chamber is filled with a cell culture medium such as Krebs Ringer Bicarbonate buffer solution (in the case of apical to basolateral transport determination). The holding block, which contains the six cells clamped together, is continuously supplied with an O_2:CO_2 (95:5) mixture. The test compound is added to either the mucosal or serosal side of the tissue to study transport in the absorptive or secretory direction, respectively. Apparent permeability coefficients (P_{app}) are calculated as for Caco-2 transport experiments from the appearance rate of the compound in the basolateral compartment (Bohets *et al.,* 2001).

Although the Ussing chamber technique is an attractive in vitro system for studying drug transport due to its simplicity (Le Ferrec *et al,* 2001; Rombeau, 2012; Tang *et al.,* 2012), the excised animal intestinal tissue only has a limited viability. Plumb *et al.* (1987) reported that intestinal oedema and disruption of the villus can be observed after only 20 minutes of incubation. However, many studies have demonstrated active glucose transport for at least 120 mininutes after the death of the animal (Grass & Sweetana, 1988; Söderholm, *et al.,* 1998).

2.9.2 Everted sac technique

The everted sac technique was introduced by Wilson and Wiseman in 1954 (Wilson & Wiseman, 1954) for studying the transport of sugars and amino acids from the apical to the basolateral side of intestinal membranes. Since then, the technique has been widely used to study intestinal drug transport (Barthe *et al.,* 1999) as well as the role of efflux transporters such as of P-gp in the intestinal absorption of drugs (Balimane *et al.,* 2000). This *in vitro* model is simple to establish, and gives excellent absorption kinetics. The technique can be used in all kinds of drug absorption studies, especially in biopharmaceutical investigations studying drug absorption enhancement strategies (Barthe *et al.,* 1998).

Since the initial development, several changes have been made to the technique in order to improve the reliability of the data generated by it. The eversion of the intestine exposes the highly active mucosa to the well-oxygenated medium, while the distension increases the surface area of the exposed apical side and reduces the thickness of the wall. Both active

and passive transport can be analysed, which makes it an ideal model to study nutrient absorption holistically. However, the eversion procedure can possibly damage the tissue and lead to loss of membrane integrity. The relatively small amount of fluid inside the cavity allows a rapid rise in concentration of transported nutrients (Balimane *et al.,* 2000).

Advantages of the technique are that it is fast and inexpensive, while regional differences in drug absorption can be studied. Also, the small volume inside the sac results in a relatively rapid increase in concentration of the inner compartment (serosal) which is an advantage from an analytical standpoint, but may be a disadvantage for well absorbed compounds (loss of sink conditions). The major disadvantage of the everted sac technique is the fact that compounds have to cross all the layers (including muscle) of the small intestine (instead of just the intestinal mucosa). The model can be used to screen for P-gp substrates by comparing transport in the absence and presence of inhibitors (Barthe *et al.,* 1998). Intestinal transport mechanisms can also be studied. For example, carrier-mediated transport will be saturable and subject to substrate competition. Finally, the technique offers the possibility to study drug transport in combination with intestinal metabolism (Barthe *et al.,* 1998).

2.10 *Aloe vera*

Aloe vera (L.) Burm.f. (*Aloe* barbadensis Miller) is a perennial succulent xerophyte that belongs to the *Asphodelaceae* family. It is a traditional medicinal plant with diverse therapeutic applications (Newton, 2004; Hamman *et al.,* 2008). The distinct parts from the *A. vera* plant that are used for medicinal purposes include the latex, gel and whole leaf extract (Reynolds & Dweck, 1999).

The intact inner fleshy part of the leaf which includes the cell walls and organelles consists of approximately 98.5% water, while the gel consists of approximately 99.5% water (Ni & Tizard, 2004). The 0.5-1% solid material remaining consists of several compounds such as minerals, polysaccharides, phenolic compounds, fat and water soluble vitamins and organic acids (Boudreau & Beland, 2006).

The thick epidermis of the leaf is mainly composed of lignified cell walls and accounts for 20-30% of the whole leaf weight. The parenchymatous fillet is formed by big rounded cells with primary walls, which accounts for approximately 65-80% of the weight of the leaf. The cell wall strength is increased by the presence of secondary lignified walls, which causes cross-linking (Femenia *et al.,* 1999).

Figure 2.5: The leaves of the *Aloe vera* plant (Aloe Vera.com, 2014)

2.10.1 Composition of *Aloe vera*

Aloe vera gel is the colourless fraction found in the inner part of fresh leaves (Bozzi *et al.,* 2007) in which water is held in the form of a viscous mucilage (Newton, 2004). This inner fleshy part of the leaf is composed of large, thin-walled, parenchyma cells filled with gel consisting of mono- and polysaccharides (Rosca-Casian *et al.,* 2007). The polysaccharides in *A. vera* gel consist mainly of linear chains of glucose and mannose molecules of which mannose comprises the larger part of the polysaccharides (Zhang & Tizard, 1996).

Acemannan (aloverose) has been identified as one of the main polysaccharides with biological activity in *A. vera* gel. It has a backbone of β-(1-4)-D-mannosyl residues which is acetylated at the C-2 and C-3 positions and exhibits a mannose monomer (Moreira & Filho, 2008). Acemannan is also used as a major marker for *A. vera* gel. *Aloe vera* inner leaf gel also contains other polysaccharides such as galactan, galactogalacturan, glucogalactomannan, galactoglucoarabinomannan, arabinan, arabinorhammogalactan, peptic substances and glucuronic acid containing polysaccharides (Choi & Chung, 2003; Ni *et al.,* 2004).

The aloe gel contains emolin, emodin and barbalon which are converted to salicylic acid. This can possibly explain the anti-inflammatory activity of aloe gel (Robson *et al.,* 1982). *A. vera* whole leaf materials are produced by grinding the rind of the leaf and the parenchyma together and then removing the aloin via charcoal filtration in order to retain as many polysaccharides as possible, since they are particularly responsible for the therapeutic efficacy of *A. vera* (Davis *et al.,* 1994). These biological activities should, however, be

assigned to a synergistic action of the compounds contained in the leaf parenchymatous tissue rather than to a single substance (Dagne *et al.,* 2000).

The thick, mucilage like properties of the raw aloe gel is mainly due to the acetylated poly (glucomannan) molecules. The molecular weights of these molecules generally range from 30-40 kDa or more and can be as high as high as 1000 kDa in fresh aloe leaf materials (Femenia *et al.,* 1999).

2.10.2 *Aloe vera* as absorption enhancer

Absorption enhancers of natural origin act according to different mechanisms of action, which may affect drug absorption, drug metabolism and site specific drug absorption. Modifications in the GIT epithelial cell membrane permeability, inhibition of gastrointestinal transit, variation of gastric emptying time and varied intestinal motility rate can also occur (Bajad *et al.,* 2001).

Aloe vera gel and whole leaf extracts have shown potential for use as drug absorption enhancers for drugs with poor oral bioavailability where it acts on the tight junctions between GIT epithelial cells (Chen *et al.,* 2009; Lebitsa *et al.,* 2012). Furthermore, the precipitated polysaccharides from *A. vera* gel have shown the potential to be used as drug absorption enhancement agents due to increased paracellular drug diffusion together with potential efflux inhibition (Beneke *et al.,* 2012).

2.11 Summary

The oral route of administration remains the most popular and preferred route of drug administration for most new chemical entities (Balimane *et al.,* 2006). Unfortunately, protein and peptide drugs have insufficient bioavailability via this route due to unfavourable physicochemical properties, barriers of the GIT and enzymatic degradation. Before protein and peptide drugs can be administered effectively by the oral route of administration, the barriers of absorption must first be overcome (Bernkop-Schnurch, 2005).

Several approaches have been explored to increase the oral bioavailability of these drugs, which include chemical modification of the drug and formulation technologies such as efflux inhibition and the addition of drug absorption enhancers. Although these strategies have been employed, only limited success has been achieved in the oral absorption of protein and peptide drugs. Most commonly encountered problems with the use of the absorption enhancing agents include long term toxic effects and insufficient bioavailability of the co-administered drugs (Aungst, 2000; Fasano, 1998).

Various models have been developed and employed to enable researchers to develop the necessary techniques in order to investigate these strategies in a cost - effective, predictable and repeatable way. The Sweetana-Grass diffusion apparatus and the everted sac technique have proven invaluable for use as *in vitro* models for drug absorption research, especially with excised animal tissues. Due to the multi-variable processes in the intestine, it is still difficult to use only a single *in vitro* model to accurately predict a drug's *in vivo* permeability characteristics (Balimane *et al.,* 2000).

Absorption enhancers acting on the controlled and reversible opening of tight junctions have proved successful in the improvement of the low bioavailability of protein and peptide drugs. *Aloe vera* is a perennial succulent xerophyte, which contains a variety of polysaccharides facilitating tight junction regulation and thus minimising the epithelial cell membrane barrier by enhancing transport via the paracellular pathway. Paracellular transport enhancers present an attractive way to improve the transport of hydrophilic drugs through the tight junctions and intercellular spaces, which may contribute to effective oral delivery of protein and peptide drugs (Ward *et al.,* 2000).

CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction

Aloe vera gel and whole leaf materials have shown potential as drug absorption enhancers in previous studies; however, the specific region in the GIT where they exhibit the highest effect on drug absorption has not yet been determined. Two *in vitro* techniques namely everted sac and Sweetana-Grass diffusion chambers were used to investigate the effect of *A. vera* gel and whole leaf materials on the transport of insulin across excised epithelial tissue from different regions of the pig intestine.

Insulin is poorly absorbed intact from the GIT due to extensive proteolytic degradation as well as insufficient membrane permeability as result of its large molecular size and hydrophilicity (Grabovac *et al,* 2008). Since insulin has such a low oral bioavailability, it can be considered as an ideal drug model for *in vitro* drug absorption enhancement studies.

A veterinary surgeon was consulted in order to help identify the anatomically distinguishable regions of the pig GIT as well as to advise on proper dissection methods for the collection of viable intestinal tissue. All transport studies were performed in the apical (donor; representing the intestinal lumen) to basolateral (receiver; representing the blood circulation) direction. A control group was included in this study (i.e. insulin alone) to serve as a reference against which the degree of insulin transport was compared when applied in the presence of the aloe leaf materials.

For the *in vitro* permeability studies, the pig GIT was divided into four major anatomical regions namely the duodenum, jejunum, ileum and colon. The jejunum was divided into three sub-regions namely the proximal jejunum, medial jejunum and distal jejunum.

All transport experiments were performed in triplicate. A validated high performance liquid chromatography (HPLC) method was used to analyse samples for their insulin content.

3.2 Materials

Pieces of intestinal tissue were excised from the GITs of slaughtered pigs at the Potchefstroom abattoir. Although these animals are slaughtered for meat production and the GITs are by-products, ethics approval was obtained for use of animal tissues in research (NWU-00025-15-A5). Dehydrated *A. vera* gel (AVG) (Daltonmax 700® gel) and *A. vera* whole leaf (AVWL) (Daltonmax700® whole leaf extract) materials were obtained from

Improve USA, Inc. Sodium bicarbonate powder and Krebs Ringer Bicarbonate (KRB) buffer and insulin were purchased from Sigma-Aldrich (Johannesburg, South Africa).

3.3 Methods

3.3.1 Buffer preparation for transport studies

The KRB buffer powder mixture (Sigma Aldrich, Johannesburg, South Africa) was added to approximately 500 ml distilled water in a volumetric flask after which it was stirred with a magnetic stirrer. Sodium bicarbonate powder (2.52 g) was added to the solution and made up to the desired volume of 1000 ml with distilled water. After stirring for approximately 5 minutes until all the powder had been dissolved, the pH was measured and recorded. For transport studies across the duodenum tissue, the pH of the KRB buffer was adjusted to 6.8 using 0.1 M HCl and for the proximal jejunum, medial jejunum, distal jejunum, ileum and colon, the pH of the KRB buffer was kept at 7.4.

3.3.2 Collection and preparation of excised pig intestinal tissue

Pig intestinal tissue was collected on the day of the transport experiment from the local abattoir (Potch Abattoir, Potchefstroom, South Africa). Immediately after slaughtering of the pigs, a piece of the specific intestinal region was identified and excised from a pig GIT.

Segments of approximately 120 cm in length were excised for each experiment from the specific GIT region of interest. The inside of the intestinal segment was rinsed several times with ice-cold KRB buffer (pH 6.8 for duodenum and pH 7.4 for ileum, jejunum and colon) in order to remove the intestinal contents (Barthe *et al.,* 1999). The excised intestinal segment was then immediately submerged and kept in ice-cold KRB buffer in a cooler box during transportation to the laboratory. The process of obtaining the intestinal tissue, dissecting out the region of interest and transporting it to the laboratory did not exceed 30 minutes from the time that the pig was slaughtered.

In the laboratory, the pig intestinal segment was removed from the cooler box and immediately used for experimental procedures. The intestinal segment was carefully mounted over a wetted glass test tube with a rounded end as shown in Figure 3.1.

Figure 3.1: Image illustrating the excised intestinal segment after it has been mounted over a wetted glass rod

The lumen was in contact with the test tube while the serosa was gently removed using blunt dissection as illustrated in Figure 3.2 (Legen *et al.,* 2005).

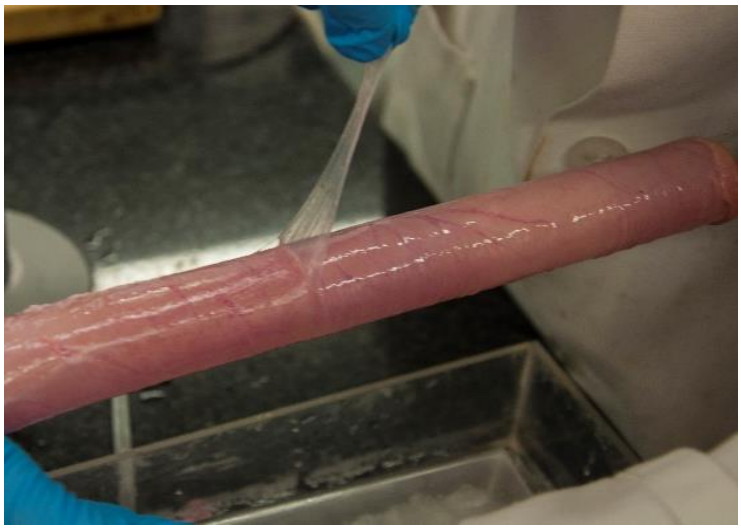


Figure 3.2: Image illustrating the removal of the serosa from the mounted intestinal segment

After completion of the general preparation of the excised intestinal tissue as described above, two different methods were followed depending on the technique that was used for the transport study namely the Sweetana-Grass diffusion chamber technique or the everted sac technique as described below.

3.3.3 Transepithelial electrical resistance (TEER)

The transepithelial electrical resistance (TEER) is obtained from the potential difference between the apical and the basolateral sides of a cell monolayer. It is inversely related to the ion flow across the cell monolayer (Powell, 1987). The TEER value gives an indication of the viability of intestinal mucosa mounted in Sweetana-Grass diffusion chambers (Wallon *et al.,* 2005), but a decrease in the TEER value can also be considered as an indication of the opening of tight junctions (Borchard *et al.,* 1996; Schipper *et al.,* 1997).

The TEER was measured in each diffusion chamber across the mounted intestinal tissue in the Sweetana-Grass diffusion chamber using a Dual Channel Epithelial Voltage Clamp (Warner Instruments, Hamden, Connecticut, USA). The TEER was measured and recorded before the start of the transport study (after an equilibrium period of 15 minutes) and after completion of the insulin transport study. This was done for each respective chamber to assess the effect of the *A. vera* gel and *A. vera* whole leaf materials on the opening of tight junctions, which may lead to absorption enhancement of insulin.

3.3.4 Insulin transport across excised pig intestinal tissue using the Sweetana-Grass diffusion chamber technique

The apical-to-basolateral transport of insulin (170 µg/ml) (Chen *et al.,* 2009) was investigated across excised pig intestinal tissue in Sweetana-Grass diffusion chambers in the presence (test groups) and absence (control group) of *A. vera* leaf materials (0.5% w/v).

After initial preparation of the intestinal tissue (as described in section 3.3.2), the excised pig intestinal tissue was cut along the mesenteric border on the glass rod with the aid of a surgical scalpel blade. The resultant intestinal tissue sheet (Figure 3.3) was gently washed from the glass test tube with buffer solution onto a sheet of heavy duty filter paper in a clean glass dish to ensure that a smooth surface was maintained. The process rendered the apical side of the intestinal membrane facing upwards. Throughout the preparation procedure, the tissue was kept moist with KRB buffer.

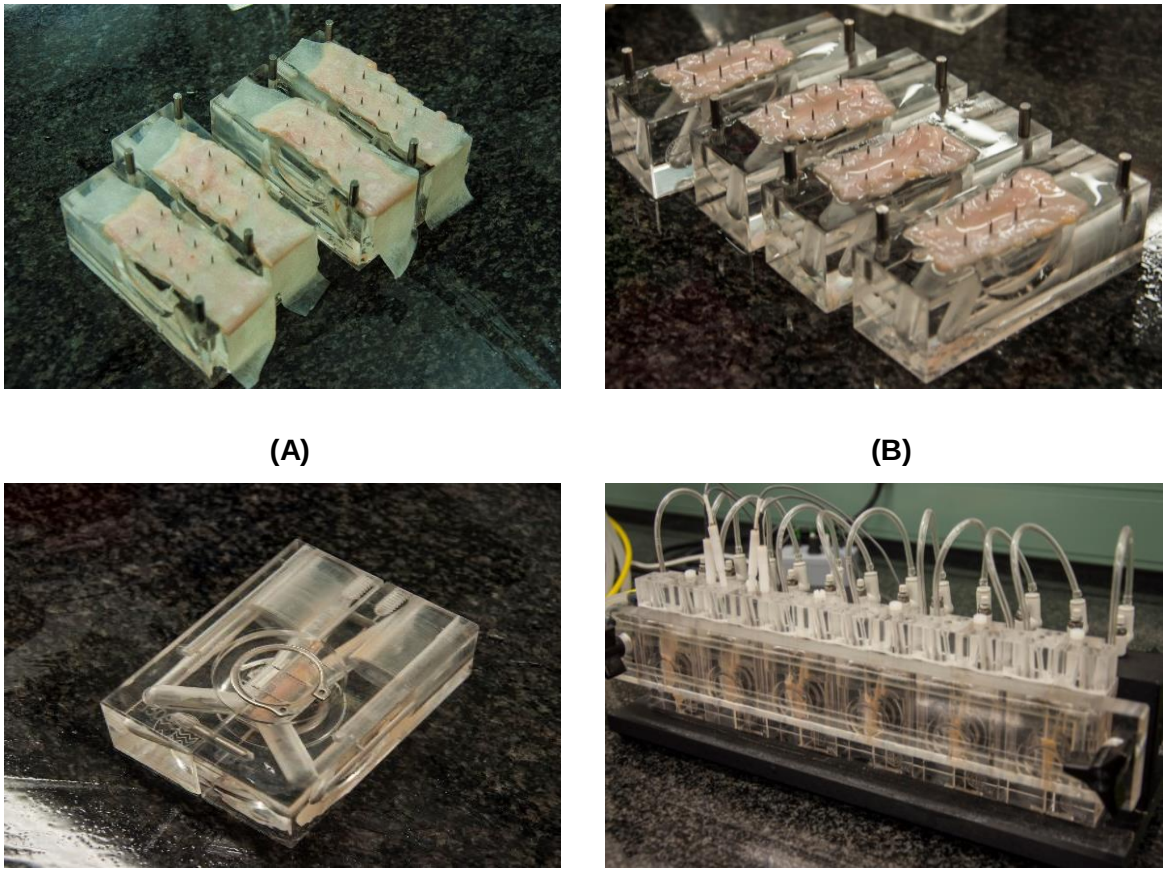
Figure 3.3: Image of the intestinal sheet obtained after cutting segment along mesenteric border

Smaller pieces of intestinal tissue (approximately 6 cm^2) that are suitable for use in the diffusion chambers were cut from the large flat sheet of tissue on the filter paper with scissors (Figure 3.4). The filter paper helped to provide structure to the tissue, preventing it from being stretched. This approach facilitated easier handling, thereby minimising damage to the tissue sample.

Figure 3.4: Image of the smaller intestinal tissue sheets mounted between the half cells for transport studies in the Sweetana-Grass diffusion chamber apparatus

These smaller pieces of tissue were mounted and clamped into half-cells of the Sweetana-Grass diffusion chamber containing needle-like pins. Sheets that contained macroscopically visible Peyer's patches were discarded as variation in the transport rate might occur across

these patches due to altered morphology and thickness of the epithelial layer in these areas (Daugherty *et al.,* 1999; Norris *et al.,* 1998). After placing the small intestinal tissue sheets on the half-cells, the filter paper faced upwards and the apical side of the intestinal tissue faced downwards. The filter paper was removed carefully and the matching half-cells were clamped together with metal rings. The assembled six chambers were inserted into the heating block (Figure 3.5). Each chamber section was filled with 7 ml pre-heated (37 ºC) KRB buffer. Each half-cell in each chamber was supplied with gas (95% O_2: 5% CO_2) at a flow rate of 15 to 20 ml/min to ensure circulation of the buffer inside the half-cells and to supply the tissue with oxygen.



(A) **(B)**

(C) **(D)**

Figure 3.5: **Images illustrating the (A) mounting of the excised intestinal tissue sheets onto the Sweetana-Grass diffusion chamber half cells containing pins with the filter paper facing upward, (B) half-cells with intestinal tissue sheets after filter paper was gently removed, (C) the assembled cell block for insertion into the Sweetana-Grass diffusion chamber apparatus and (D) the assembled Sweetana-Grass diffusion apparatus used for transport studies**

The KRB buffer was removed from the donor (apical) chamber by aspiration with an Integra Vacusafe vacuum system (Vacusafe®, Hudson, USA) and immediately replaced with 7 ml of pre-heated (37°C) test solution, containing insulin in the presence and/or absence of *A. vera* components. Samples of 200 µl were taken at 20 minute intervals from the acceptor (basolateral) chamber over a total period of 2 hours. The sample volume was immediately replaced with an equal volume of KRB buffer after each withdrawal. The pH of the replacement buffer was adjusted to 6.8 or 7.4 respectively, prior to heating in a water bath. The concentration of insulin in each sample was determined by means of a validated HPLC method.

3.3.5 Insulin transport across excised pig intestinal tissue using the everted sac technique

After the pig intestinal tissue had initially been prepared (as described in section 3.3.2), the excised intestinal segment was everted, so that the muscular membrane faced the inner compartment. The intestinal tissue was then cut into smaller segments (approximately 5 cm long) and rinsed with 20 ml ice-cold KRB buffer (Barthe *et al.,* 1999). Each everted segment was mounted onto a glass apparatus specifically designed for everted sac permeation studies (Dixit *et al.,* 2012). Both ends of each tissue segment were secured to the glass apparatus with thread in order to prevent leakages (Figure.3.6).

Figure 3.6: Image illustrating the mounted intestinal segment on the glass apparatus for use in the everted sac technique

The glass apparatus with the everted sacs (mucosal side outside) were then submerged in a 500 ml glass beaker containing insulin (17 µg/ml) in the presence and/ or absence of the *A. vera* materials (0.5% m/v) in KRB buffer. The serosal side of the intestine (inner sac) was filled with 40 ml freshly prepared, pre-heated (37°C) and pH adjusted (depending on the region of the intestinal tissue) KRB buffer.

The beaker containing the assembly (everted intestinal sacs tied to the glass apparatus) and test solution were then placed on a magnetic stirrer at a constant temperature of 37°C (Dixit *et al.,* 2012). Each apparatus was supplied with gas flow (95% O_2: 5% CO_2) at a flow rate of 15 to 20 ml/min to supply the tissue with oxygen and aid circulation of the buffer inside the beakers. Samples of 200 µl were collected from the acceptor chamber (inner compartment of the sac) at 20 min intervals and the same volume was immediately replaced with an equal volume of pre-heated KRB buffer. The total duration of the experiment was 2 hours. Samples taken were transferred into HPLC vials containing glass inserts, which were then analysed by means of a validated HPLC analytical method to determine the insulin content.

3.4 High-performance liquid chromatography analysis of insulin

3.4.1 Introduction

Chromatography is an analytical technique that separates molecules in a mixture on the basis of their molecular structure and composition. This involves a stationary phase (a solid, or a liquid supported on a solid) and a mobile phase (a liquid or a gas). The mobile phase flows through the stationary phase which carries the mixture with it. Sample components that

display stronger affinities and interactions with the stationary phase will move more slowly through the column than the components with weaker affinities and interaction, resulting in different separation rates (Kupiec, 2004). A pump moves the mobile phase(s) through the column and a detector that shows the retention times of the specific molecules to be investigated (Liu & Lee, 2006). Gradient elution can also be used to separate molecules, which involves separation of the molecules with variation of the mobile phase composition during analysis (Abidi, 1991).

High-performance liquid chromatography (HPLC) is a specific form of liquid chromatography used to separate and quantify the specific active compounds in a solution (Martin & Guiochon, 2002). HPLC is currently the most widely used quantitative and qualitative analysis technique in the pharmaceutical industry and in pharmaceutical analysis laboratories (Kupiec, 2004). It is the safest, fastest and most versatile chromatographic technique for the quality control of drug components (Malviya *et al.,* 2010).

The validation of a HPLC method involves the use of specific laboratory investigations to prove that the method's performance characteristics are suitable and reliable for the intended analytical applications (ICH, 2005). The acceptability of analytical data corresponds directly to the criteria used to validate the method. The fundamental parameters for a bio-analytical validation include accuracy, precision, selectivity, sensitivity, reproducibility and stability (ICH, 2005).

The procedure and HPLC analytical method described in this section was developed under the supervision of Professor J.L. du Preez in the Analytical Technology Laboratory (ATL) of the North-West University (NWU), Potchefstroom, South Africa.

3.4.2 Chromatographic conditions

The HPLC system used as well as the parameters are summarised in Table 3.1.

Table 3.1: Analytical instrument and chromatographic conditions used to analyse insulin in the transport samples

Analytical conditions	**Description**
Analytical instrument	Agilent 1100 series HPLC equipped with a gradient pump, autosampler, UV detector and Chemstation Rev. A.08.03 data acquisition and analysis software.

Column	Venusil ASB C18, 250 x 4.6 mm, 5 µm, 300 Å pore size (Agela Technologies, Inc., Newark, Delaware), or Jupiter C18-column, 250 x 4.6 mm, 5µm spherical particles, 300 Å pore size, 13.3% carbon load, endcapped (Phenomenex, Torrance, CA)
Mobile phase	Phase A: Acetonitrile Phase B: Mixture of degassed HLPC grade water with 0.1% v/v orthophosphoric acid (H_3PO_4)
Flow rate	1.0 ml/min
Sample volume injection	50 µl
Detection wavelength	UV absorbance at 210 nm
Retention time	5.86 min
Stop time	12 min
Solvent	Krebs-Ringer bicarbonate buffer

The mobile phase consisted of two components (acetonitrile and water mixed with 0.1% v/v orthophosphoric acid) and was applied during analysis via a gradient as shown in Table 3.2.

Table 3.2: The gradient elution schedule as used in the high-performance liquid chromatography (HPLC) analysis method

Time (min)	% Mobile Phase A	% Mobile Phase B
0	20	80
6	60	40
8	60	40
8.2	20	80
12	20	80

3.4.3 Standard solution preparation

In order to define the correlation between instrument response and the concentration of the analyte, a series of insulin solutions were prepared. This concentration series was used to generate a standard curve to test for linearity.

A solution was made by dissolving approximately 15 mg human recombinant insulin in 100 ml of the solvent (KRB buffer). A 5 ml volume of this sample was then diluted to 50 ml with the KRB buffer and transferred to a HPLC vial to be analysed. This procedure was repeated taking 5 ml of the diluted sample and filling it to 50 ml with KRB buffer, after which the respective solutions were also transferred to HPLC vials for analysis. Three solutions of insulin were obtained with known concentrations namely solution 1 (150 µg/ml), solution 2 (15 µg/ml) and solution 3 (1.5 µg/ml). Volumes of 10, 20, 30, 40 and 50 µl of these solutions were injected in duplicate into the HPLC system.

The standard solution prepared in this section was used to determine insulin's accuracy and inter- and intra-day precision.

3.4.4 Validation parameters of the HPLC analytical method

In order to establish whether the performance characteristics of the HPLC method used to analyse the transport samples met the requirements for the intended analytical application, the validation of the analytical method was conducted according to the prescribed validation parameters (USP, 2014).

3.4.4.1 Specificity

The specificity of the analytical procedure can be defined as the ability of the procedure to analyse the analyte in the presence of components expected to be present in the sample solution such as impurities, degradation products and matrix components (ICH, 2005). The method complies with specificity when no intrusive peaks with the same retention time as the model drug is identified.

As described before for standard solution preparation, insulin was dissolved in KRB buffer and a sample was diluted 1:1 with water, 0.1 M hydrochloric acid, 0.1 M sodium hydroxide and 10% hydrogen peroxide, respectively, in order to produce degradation products. Solutions containing insulin with the *Aloe vera* gel and whole leaf materials, respectively, were also prepared. These solutions represented the solutions used in the *in vitro* transport studies. All these solutions were analysed in duplicate with the HPLC.

3.4.4.2 Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results (e.g. a response such as UV absorbance), which are directly proportional to the concentration of an analyte (USP, 2014).

Standard solutions with concentrations ranging from 0.1 µg/ml to 169 µg/ml were injected at different injection volumes in duplicate into the chromatograph. The linearity of insulin was determined by linear regression analysis of the plot of the peak areas as a function of the concentration (µg/ml) of the standard solutions. The correlation coefficient (R^2) value was calculated by using Microsoft Excel software. To be acceptable, the analytical method requires an R^2 value of 0.99 or higher.

3.4.4.3 Limit of quantification and limit of detection

The limit of quantification (LOQ) is defined as the lowest concentration of analyte in a sample that can be determined with suitable precision and accuracy using the particular analytical method under the stated experimental conditions (USP, 2014). The LOQ was determined by injecting six replicates of standards containing insulin at low concentration (17 µg/ml). The LOQ value was accepted as the lowest concentration of insulin that can be detected with a relative standard deviation (RSD) of ≤ 15% for the six replicates.

The limit of detection (LOD) can be defined as the lowest known concentration of analyte that can be distinguished from the baseline noise, but not always quantitated as an exact value (USP, 2014). It was taken as a peak equal to approximately three times the average baseline noise.

3.4.4.4 Accuracy

The accuracy of an analytical procedure expresses the closeness of the test result obtained by that procedure to the true value, which is established across a concentration range (ICH, 2005; USP, 2014).

The insulin solution was prepared, as described in section 3.3.5.3, after which 6 ml of the solution was added to each of ten 20 ml volumetric flasks and filled to volume with KRB buffer. The accuracy of the analytical method was determined by analysing spiked samples of 50 µl containing insulin in known concentrations in triplicate, which were in the low, medium and high regions of the linear range. The spiked insulin concentrations were prepared by using a solution with exactly 46.38 µg/ml insulin for the range of low concentration, 92.76 µg/ml for the medium range and 154.6 µg/ml for the high range. An

HPLC method is considered accurate when the average of the analyte recovery is between 98-102% (USP, 2014).

3.4.4.5 Precision

Precision of an analytical procedure expresses the degree of agreement (in other words repeatability) among individual test results within a series of measurements obtained from multiple sampling of the same solution under the prescribed conditions (USP, 2014). The results of this procedure are expressed as the relative standard deviation (RSD) or standard deviation (SD) of a series of measurements (USP, 2014). Precision can be divided into two categories namely intra-day and inter-day precision.

Intra-day precision (repeatability)

This is the precision under the same operating conditions over a relatively short period of time (ICH, 2005; USP, 2014). The intra-day precision was determined by analysing the same insulin solutions as in section 3.3.5.3, with samples containing insulin concentrations of 46.38 µg/ml, 92.76 µg/ml and 154.60 µg/ml directly after each other. The samples were injected in triplicate into the HPLC for analysis. Repeatability must be less than 2%, (n=9) (USP, 2014).

Inter-day precision

The same insulin solutions were analysed as in section 3.3.5.3, with samples containing 46.38 µg/ml, 92.76 µg/ml and 154.60 µg/ml respectively. These concentrations were analysed on two different days in order to determine the inter-day variability of the analysis method. The samples were kept at a stable temperature in the auto sampler (5°C) in order to assure stability. The inter-day precision must be < 5%, (n=9).

3.4.4.6 Ruggedness

Stability of the sample solutions

A standard solution of insulin in KRB buffer was prepared and injected into the chromatograph for analysis. The sample was re-analysed hourly for up to 24 hours in order to determine the stability of insulin in the solution.

System repeatability

A standard solution of insulin was consecutively analysed six times in order to test the repeatability of the retention time and the peak area. The retention time and the peak areas should have a RSD of ≤ 2%.

3.4.5 Data processing and statistical analysis

3.4.5.1 Apparent permeability coefficient (P_{app})

The insulin concentrations obtained in the transport samples were corrected for dilution and expressed as cumulative drug transport (percentage of initial dose) and the apparent permeability coefficient (P_{app}) values for the test compound from samples withdrawn from the receiver (basolateral) compartment, were calculated with the aid of Equation 3.1 (Thanou *et al.,* 2000).

$$P_{app} = \frac{dc}{dt}\left(\frac{1}{A.60.C_0}\right) \qquad \text{(Eq 3.1)}$$

Where P_{app} is the apparent permeability coefficient ($cm.s^{-1}$), $\frac{dc}{dt}$ is the permeability rate (amount of insulin permeated per minute), A is the diffusion area of the excised tissue (cm^2) and C_0 is the initial concentration (µg/ml) of the insulin.

Permeation-enhancement ratios (R) were calculated from the P_{app} values with the aid of equation 3.2.

$$R = \frac{P_{app}\text{test}}{P_{app}\text{control}} \qquad \text{(Eq. 3.2)}$$

Where R is the permeation-enhancement ratio, P_{app} test is the apparent permeability coefficient for the test solution, and P_{app} control is the apparent permeability coefficient ($cm.s^{-1}$) for the control group (i.e. insulin alone).

3.4.5.2 Statistical analysis of data

The results obtained from the transport were statistically analysed by using software from Statistica (Statsoft®, 2007).

The Papp values obtained from this study were analysed by means of one-way repeated analysis of variance (ANOVA) to determine if the differences between the control groups and the *A. vera* gel and the *A. vera* whole leaf materials, respectively, were statistically

significant. Differences were considered statistically significant if $p < 0.05$. This was done for each region and for both *in vitro* techniques.

Levene's test and Dunnett's test were performed as post-hoc tests to determine which of the mean P_{app} values of the test formulations with the *A. vera* gel and the *A. vera* whole leaf materials differed significantly from the mean P_{app} values of the control for each respective region.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Introduction

This *in vitro* transport study was based on quantitative research methodology with an experimental design, with its rationale based on reports of increased bioavailability of vitamins C and E after intake with *Aloe vera* gel and whole leaf extract (Vinson *et al.,* 2005). Beneke *et al* (2012) have also reported that the polysaccharides contained in *Aloe vera* leaf materials may potentially be used as absorption enhancing agents, which act by opening paracellular tight junctions.

In this study, *Aloe vera* gel and whole leaf materials were investigated for their transport enhancing effects of insulin across excised pig intestinal tissues from different gastrointestinal tract regions. The transport studies for each of the six regions were performed in triplicate using both the Sweetana-Grass diffusion chamber technique and the everted sac technique. The samples from the *in vitro* transport studies were analysed using a validated HPLC method. This analytical method was validated to ensure that it meets the specified standards of reliability and accuracy by validating parameters such as linearity, accuracy, precision, robustness and system suitability.

The TEER was measured prior to the start of each experiment and after its conclusion in order to determine if the membrane integrity was maintained throughout the experimental procedure. The TEER values also gave an indication of the ability of the *Aloe vera* leaf materials to open tight junctions to allow paracellular transport to take place. The insulin transport studies included a control group (insulin alone) and experimental groups consisting of insulin dissolved together with *Aloe vera* gel and whole leaf materials in (KRB) buffer, respectively.

The cumulative insulin transport was measured as a function of time (% of initial dose) in the apical-to-basolateral direction across excised pig intestinal tissue. The *in vitro* transport data were used to calculate apparent permeability coefficient (P_{app}) values. The average P_{app} values and corresponding standard deviation (SD) values were also calculated.

4.2 Validation of a high-performance liquid chromatography (HPLC) analytical method for insulin

The following results were obtained during the validation of a HPLC analytical method for insulin.

4.2.1 Specificity

Figure 4.1 illustrates the HPLC chromatogram of the model compound, insulin, dissolved in KRB buffer. The chromatograms in Figures 4.2 to 4.5 show the respective peaks of insulin, in the presence of 0.1 M hydrochloric acid (HCl) at 40°C over 24 hours, in the presence of 0.1 M sodium hydroxide (NaOH) at 40°C for 24 hours and in the presence of 10% hydrogen peroxide (H_2O_2) at 40°C for 24 hours. Figure 4.6 shows a HPLC chromatogram illustrating an insulin peak in the presence of *Aloe vera* gel and Figure 4.7 shows the HPLC chromatogram illustrating an insulin peak in the presence of *A. vera* whole leaf materials.

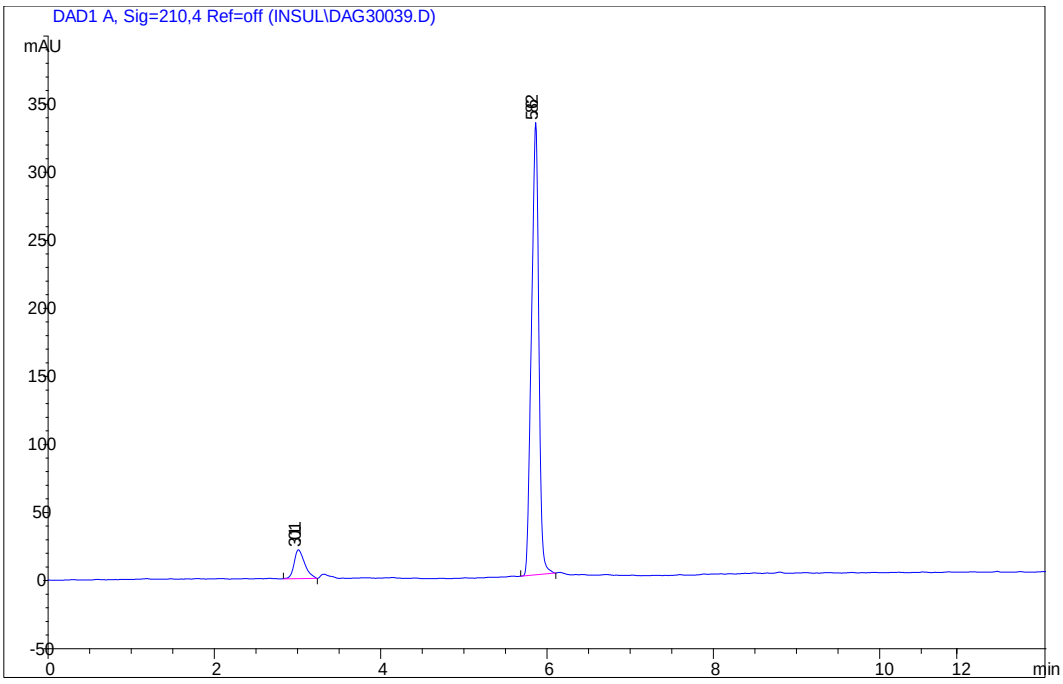


Figure 4.1: HPLC chromatogram illustrating an insulin peak in KRB buffer at a retention time of 5.862 min

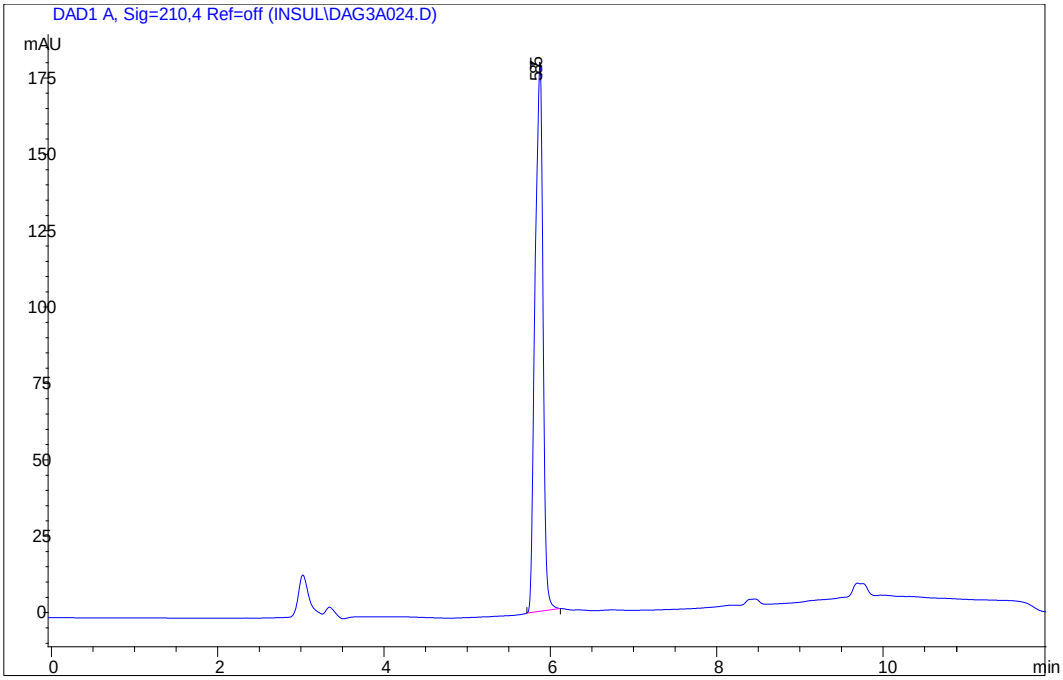


Figure 4.2: **HPLC chromatogram illustrating an insulin peak (at a retention time of 5.875 min) when the sample was kept in water at 40°C for 24 h**

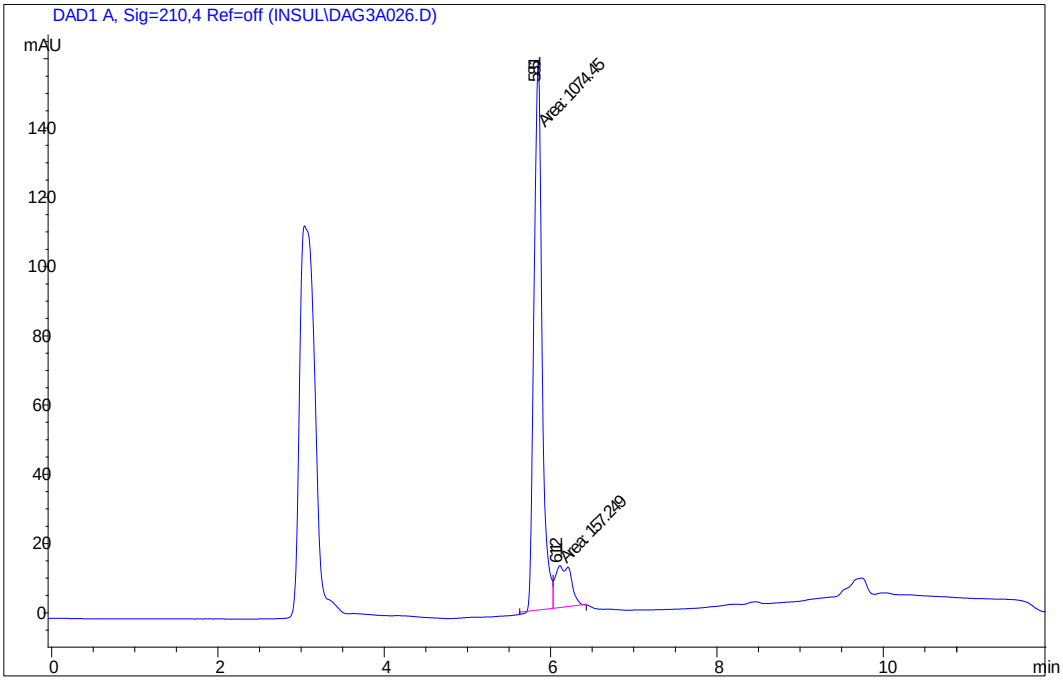


Figure 4.3: **HPLC chromatogram illustrating an insulin peak (at a retention time of 5.851 min) when the sample was kept in 0.1 M HCl at 40°C for 24 h**

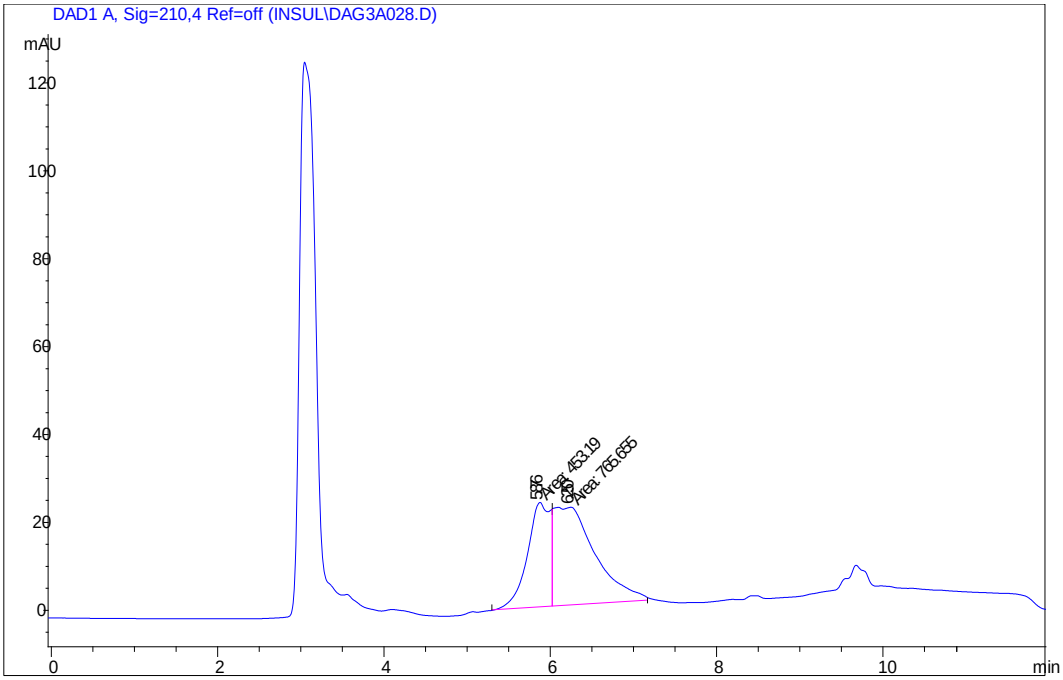


Figure 4.4: **HPLC chromatogram illustrating an insulin peak (at a retention time of 5.876 min), kept in 0.1 M NaOH at 40°C for 24 h**

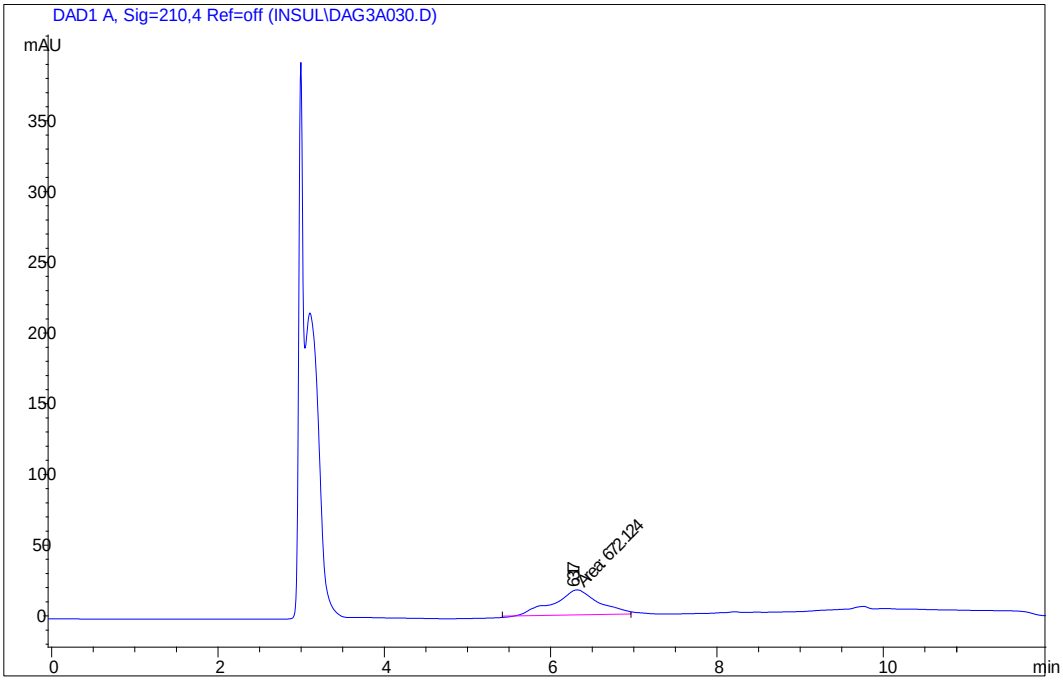


Figure 4.5: **HPLC chromatogram illustrating an insulin peak (at a retention time of 6.317 min) kept in 10% H_2O_2 at 40°C for 24 h**

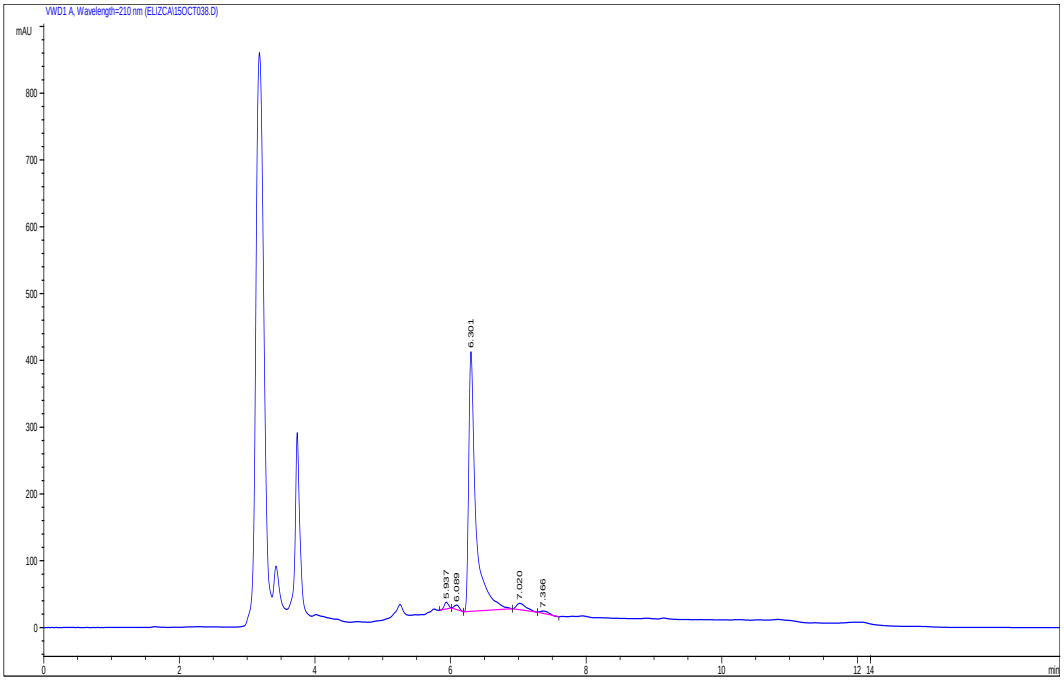


Figure 4.6: HPLC chromatogram illustrating an insulin peak (at a retention time of 6.301 min) in the presence of *Aloe vera* gel

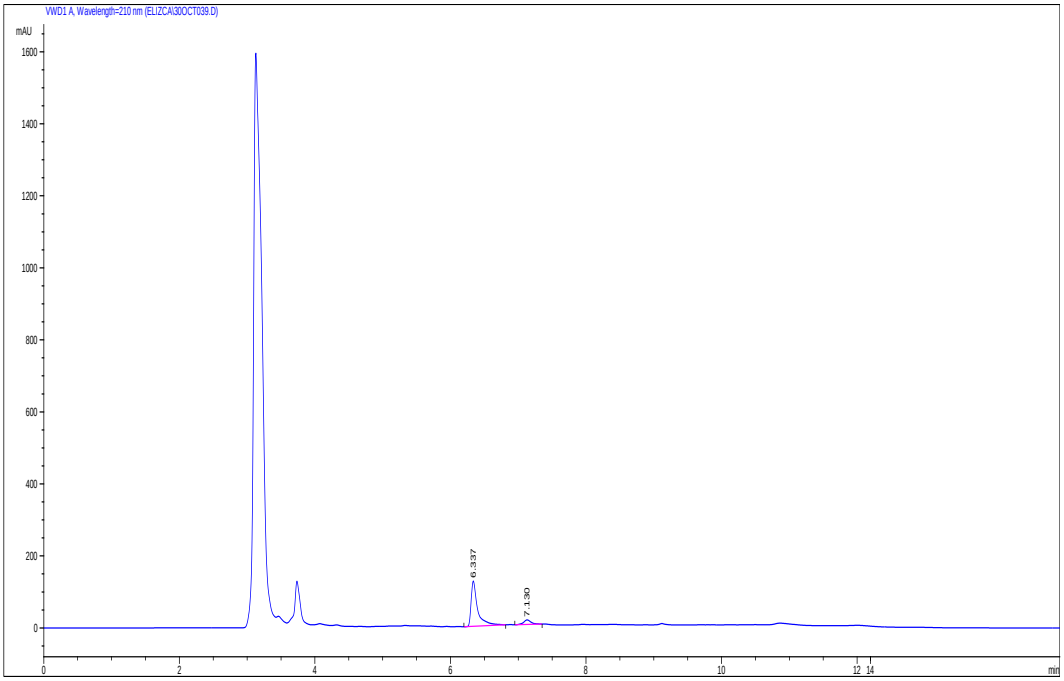


Figure 4.7: HPLC chromatogram illustrating an insulin peak (at a retention time of 6.337 min) in the presence of *Aloe vera* whole leaf material

The addition of *A. vera* leaf materials did not interfere with the insulin peak on the HPLC chromatogram when KRB buffer was used as solvent. Potential overlaps of degradation

products did, however, occur in extreme conditions where insulin was incubated with either 0.1 M HCl or 0.1 M NaOH at 40°C.

4.2.2 Linearity

Figure 4.8 illustrates a graph of linear regression where the peak area was plotted as a function of insulin concentration. A high degree of linearity was achieved which was confirmed by the regression value (R^2 = 0.999), which indicates a high degree of correlation between the HPLC response and insulin concentration. The HPLC method of analysis for insulin complied with the requirements of linearity, which is a R^2 value of 0.99 (USP, 2014). The linearity of this method was therefore confirmed over an insulin concentration range of 0.3 - 170 µg/ml (Table 4.1).

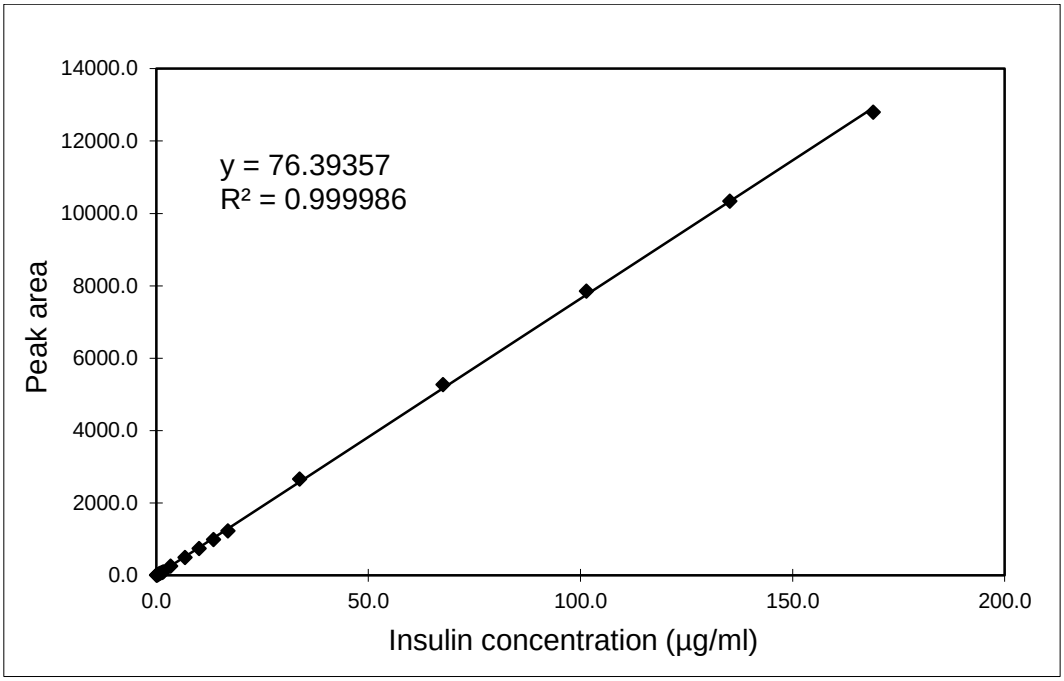


Figure 4.8: Linear regression graph obtained for insulin peak area plotted as a function of insulin concentration

Table 4.1: HPLC chromatogram peak areas for each insulin concentration and linear regression of the graph over the specified concentration range

Concentration (µg/ml)	Mean peak area
0.1	3.5
0.2	8.7
0.3	19.2
0.7	39.7
1.0	57.2
1.4	77.1
1.7	97.3
3.4	250.8
6.8	494.6
10.1	738.7
13.5	985.6
16.9	1229.5
33.8	2660.5
67.6	5272.8
101.4	7850.2
135.2	10340.6
169.0	12793.2
R^2	0.9998575
y-intercept	0
Slope	76.394

4.2.3 Limit of quantification (LOQ) and limit of detection (LOD)

LOQ is the lowest concentration of the analyte (insulin), which can be detected with a relatively standard deviation (RSD) value of ≤ 15% for the six replicates. The LOQ for insulin using the HPLC method was determined to be 0.3 µg/ml.

The limit of detection (LOD) is the lowest known peak concentration of the analyte (insulin) that is discernible from the baseline noise, which is usually taken as a peak which is equal to approximately three times the average baseline noise (USP, 2014). The LOD for insulin using the HPLC method was 0.05 µg/ml.

4.2.4 Accuracy and precision

4.2.4.1 Accuracy

Table 4.2 shows that the HPLC method yielded a mean recovery of 98.7% with a percentage relative standard deviation value (%RSD) of 0.7%. These % recovery values for the spiked samples are, with the exception of two values, within the acceptable limits for accuracy as stipulated by the ICH (ICH, 2005), which states that the recovery needs to be between 98 to 102%.

Table 4.2: Insulin recovery from spiked samples

Concentration spiked (µg/ml)	Peak area			Recovery	
	Area 1	Area 2	Mean	(µg/ml)	%
46.38	3160.3	3160.0	3160.2	45.48	98.06
46.38	3167.8	3143.8	3155.8	45.42	97.92
46.38	3148.2	3132.1	3140.2	45.19	97.44
92.76	6390.6	6388.7	6389.7	91.96	99.14
92.76	6393.0	6418.0	6405.5	92.19	99.38
92.76	6393.4	6408.8	6401.1	92.12	99.31
154.6	10610.9	10594.4	10602.7	152.59	98.70
154.6	10549.1	10724.7	10636.9	153.08	99.02
154.6	10711.8	10670.1	10691.0	153.86	99.52
				Mean recovery (µg/ml)	98.7
				SD (µg/ml)	0.7
				% RSD	0.7

4.2.4.2 Precision

Since the insulin analysis method does not include any sample preparation/manipulation, the accuracy data can be used to calculate intermediate precision by determining the inter-day and intra-day precision, as described in the sections below.

4.2.4.2.1 Inter- and intra-day precision

Table 4.3 shows that acceptable inter-day precision was achieved with a %RSD value of 1.23%. This complies with the requirements for precision (repeatability) as stated in the

USP (USP, 2014), according to which a %RSD value of 5% for inter-day precision and 2% for intra-day precision is required.

As seen in Table 4.3, the inter-day precision of the HPLC method of analysis for insulin was acceptable since there were no statistically significant differences between the values obtained ($p < 0.5$). The repeatability for the insulin was within acceptable limits.

Table 4.3: Inter-day precision of the HPLC method of analysis for insulin

Days	%Recovery			Mean	SD	%RSD
Day 1	99.1	99.4	99.3	99.28	0.10	0.10
Day 2	98.1	98.0	98.7	98.25	0.29	0.29
Day 3	101.7	101.1	100.9	101.20	0.34	0.33
		Between days		**99.58**	**1.22**	**1.23**

The ANOVA single factor statistics of insulin for the inter-day precision parameters are illustrated in Table 4.4 and the results of the inter- and intra-day precision ANOVA statistics are shown in Table 4.5.

Table 4.4: ANOVA single factor statistics for the inter-day precision parameters

Groups	Count	Sum	Mean	Variance
Day 1	3	297.83	99.28	0.02
Day 2	3	294.76	98.25	0.12
Day 3	3	303.61	101.20	0.17

Table 4.5: Inter- and intra-day precision ANOVA statistics

ANOVA summary					
Source of variation	**Sum of squares**	**Degrees of freedom**	**Mean squares**	**F-ratio**	**p-value**
Inter-day	13.47	2.00	6.73	65.09	8.55E-05
Intra-day	0.62	6.00	0.10		
Total	**14.09**	**8.0**			

4.2.5 Ruggedness

4.2.5.1 Stability of insulin in solution

Table 4.6 shows that insulin was stable in solution over a 24 hour period with only about 2% degradation over the entire test period.

Table 4.6: Stability of insulin in solution over 24 hours

Time (h)	Peak area	% Recovery
0	2080.07	100.00
1	2092.50	100.60
2	2064.45	99.20
3	2051.82	98.60
4	2053.02	98.70
5	2051.43	98.60
6	2039.45	98.00
7	2040.79	98.10
8	2053.41	98.70
9	2009.65	96.60
10	2035.69	97.90
11	2042.49	98.20
12	2044.11	98.30
13	2035.59	97.90
14	2039.45	98.00
15	2036.64	97.90
16	2030.15	97.60
17	2026.27	97.40
18	2026.57	97.40
19	2033.57	97.80
20	2040.50	98.10
21	2025.89	97.40
22	2029.77	97.60
23	2026.96	97.40
24	2034.12	97.80

4.2.5.2 Repeatability

The %RSD for repeated injections of the same sample was acceptable with a value of 0.51% for the peak area and a value of 0.32% for the retention time as shown in Table 4.7. This complies with the criterion for system repeatability that the %RSD should be ≤ 2%.

Table 4.7: %RSD for the peak areas and retention times of repeated injections of insulin

Injection sample	Peak area	Retention time (min)
1	6747.90	5.893
2	6713.30	5.859
3	6730.50	5.853
4	6788.50	5.869
5	6705.90	5.888
6	6679.90	5.906
Mean	**6727.7**	**5.878**
SD	**34.35**	**0.019**
%RSD	**0.51**	**0.324**

4.2.5.3 Robustness

The HPLC method for insulin was able to tolerate chromatographic condition changes and it could be accepted that the method should perform well under normal operating conditions (Table 4.8).

Table 4.8: Changes in chromatographic operating parameters within specified ranges

Chromatographic condition	Range
Flow rate	0.9 – 1.1 ml/min
Injection volume	2.5, 5, 10, 20, 30, 40 and 50 µl
Wavelength	UV at 208 – 212 nm
Mobile phase composition	A: A degassed and filtered mixture of HPLC grade water and 0.1% orthophosphoric acid was prepared. B: Acetonitrile Starting concentration could be 18 to 22% acetonitrile and the gradient end time could

	be between 5.8 to 6.2 minutes without any ill effect. The retention time of the insulin peak will vary under different conditions, but this will not affect the method's performance.

4.2.6 Conclusion

The HPLC method of analysis for insulin was sensitive and sufficiently selective for the determination of insulin concentrations in *in vitro* transport samples. The criteria for the validation parameters were met as described above.

4.3 Transport studies

Insulin transport in the absence (control group) and presence of *A. vera* gel and whole leaf materials across excised tissues from different regions of the pig GIT were determined. The different regions investigated included the duodenum, proximal jejunum, medial jejunum, distal jejunum, ileum and colon. The transport studies were conducted using two *in vitro* models namely the Sweetana-Grass diffusion chamber technique and the everted sac technique.

The results obtained from the *in vitro* transport studies were used to calculate P_{app} values which were statistically analysed and the results were compared. The P_{app} values of the control group were compared with the P_{app} values of the test groups by means of a statistical analysis (ANOVA and post-hoc tests). A p-value of < 0.05 was considered to indicate a statistically significant difference in insulin transport.

4.3.1 Duodenum

In the duodenum, the *A. vera* gel mediated a statistically significant ($p < 0.05$) increase in insulin transport in both the diffusion chamber technique and the everted sac technique as illustrated in Figures 4.9 and 4.10. The increase in the cumulative percentage transport of insulin (P_{app}) in the presence of *A. vera* gel over 120 min in the diffusion chamber technique was 1.63 fold and 2.77 fold in the everted sac technique. The increased insulin transport is also reflected by the higher average P_{app} values as depicted in Table 4.10 and 4.11. The *A. vera* whole leaf materials mediated a slight increase in insulin transport in both of the *in vitro* techniques, but was not statistically significant ($p > 0.05$).

Figure 4.9 shows that insulin transport was significantly increased by *A. vera* gel and this increase correlates well with the 24.1% TEER reduction which was obtained by measuring

TEER at the beginning and end of the transport study. This result suggests that *A. vera* gel enhanced the paracellular transport of insulin by means of a transient opening of tight junctions in the duodenum. This correlates well with TEER reduction results from previous *in vitro* transport experiments across Caco-2 cell monolayers (Chen *et al.,* 2009).

A possible explanation for the higher percentage insulin transport in the presence of *A. vera* gel using the everted sac technique compared to that of the diffusion chamber technique, is the larger surface area available for absorption. The surface area available for absorption in the diffusion chambers was 1.78 cm^2 and the surface area available for absorption in the everted sacs was 35.00 cm^2.

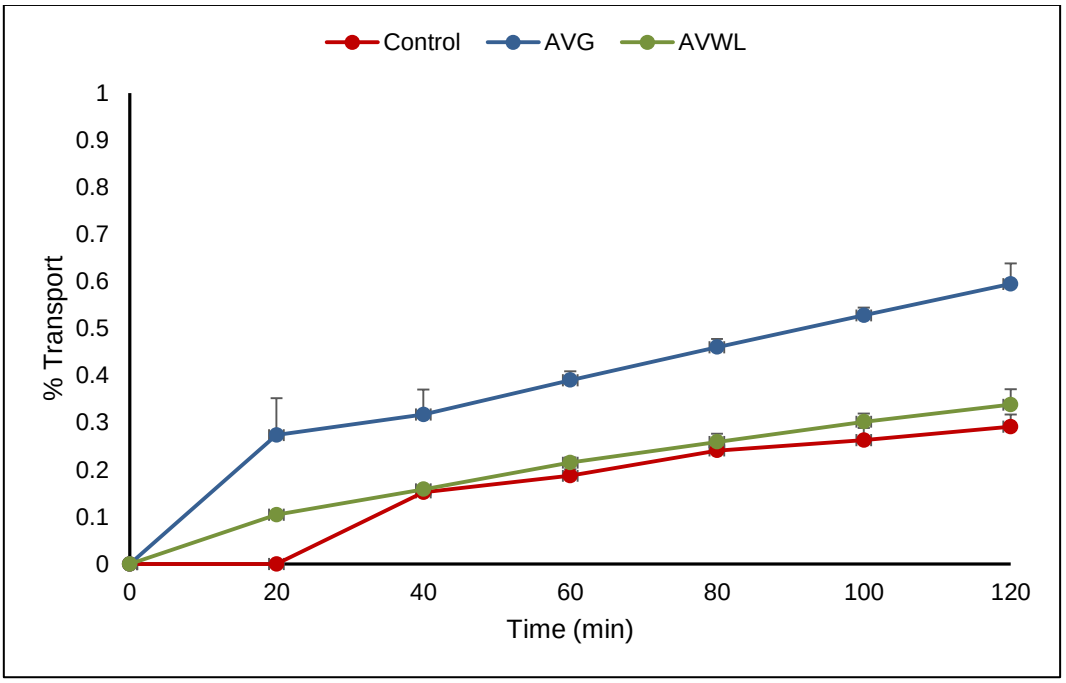


Figure 4.9: **Insulin transport across excised pig duodenum tissue using the Sweetana-Grass diffusion chamber technique. (AVG = *Aloe vera* gel, AVWL = *Aloe vera* whole leaf)**

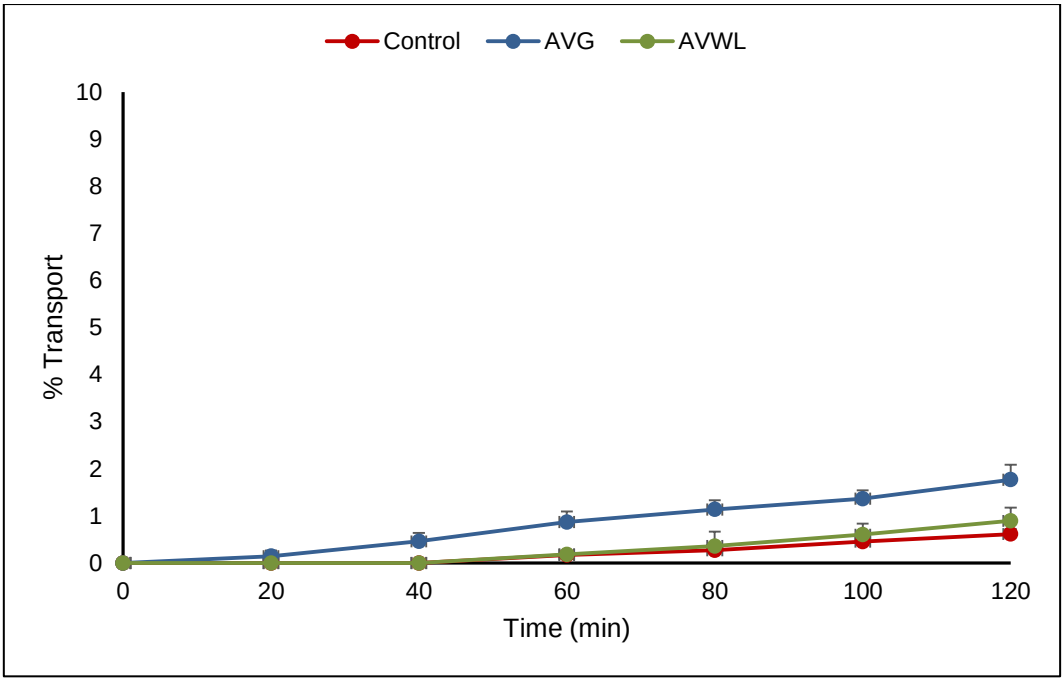


Figure 4.10: **Insulin transport across excised pig duodenum tissue using the everted sac technique. (AVG = *Aloe vera* gel, AVWL = *Aloe vera* whole leaf)**

The standard deviation (SD) of the transport data was relatively low and is illustrated by the error bars in Figures 4.9 and 4.10. This represents good repeatability of insulin transport across the duodenum in the presence and absence of *A. vera* leaf materials.

The P_{app} values for each experimental group obtained from each of the *in vitro* techniques were calculated and are shown in Tables 4.9 and 4.10.

Table 4.9: **P_{app} values for insulin in the Sweetana-Grass diffusion chamber technique across excised pig duodenum tissue**

Sweetana-Grass diffusion chamber technique			
Repeat	**Control ($\times 10^{-7}$)**	***A. vera* gel ($\times 10^{-7}$)**	***A. vera* whole leaf ($\times 10^{-7}$)**
1	2.58	4.72	2.74
2	2.22	3.53	2.89
3	2.67	3.96	2.51
Average P_{app}	2.49	4.07	2.72
SD	0.19	0.49	0.16

Table 4.10: P_{app} values for insulin in the everted sac technique across excised pig duodenum tissue

Everted sac technique			
Repeat	**Control ($\times 10^{-7}$)**	***A. vera* gel ($\times 10^{-7}$)**	***A. vera* whole leaf ($\times 10^{-7}$)**
1	0.31	0.65	0.39
2	0.23	0.84	0.49
3	0.23	0.66	0.21
Average P_{app}	0.26	0.72	0.45
SD	0.04	0.09	0.11

Although the duodenum has only a surface area of approximately 1.9 m^2 (Balimane & Chong, 2005) and is the shortest region of the small intestine with a length of only 20-25 cm (Swindle & Smith, 1998), it still has some capacity for insulin absorption. The low pH in the duodenum of between 5.5 and 7.0 (Van de Graaff, 1986) also creates an unfavourable environment for protein and peptide drugs in which they tend to denature and are deactivated which can attribute to the low bioavailability.

4.3.2 Jejunum

The jejunum has a very high capacity for absorption due to well - developed mucosal folds (Martini, 2006) with an abundance of villi and microvilli on the luminal surface and a subsequent large surface area of approximately 184.0 m^2 (Balimane & Chong, 2005; Cao *et al.,* 2006). The jejunum and ileum, which forms the lower regions of the small intestine, are considered to be the major sites of drug absorption, due to the tight junctions being more permeable in these regions (Ward *et al.,* 2000) and the four-fold increase in the jejunum surface area and the two-fold surface area in crease in the ileum by the abundant villous structures which is an important factor in drug absorption (Masaoka *et al.,* 2006). The absorptive capacity, however, decreases from the proximal jejunum down the length of the jejunum due to the decrease in size of the villi and *plicae* which is most prominent in the proximal jejunum. In general, most of the drug absorption usually occurs before the jejunum terminates into the ileum (Martini, 2006).

Previous studies on regional absorption have been conducted in rats, but never before in pigs (Fetih *et al.,* 2005; Sakuma *et al.,* 2007). Due to the fact that the majority of drug and nutrient absorption occurs in the jejunum (Ward *et al.,* 2000), the jejunum was divided into proximal, medial and distal regions in order to investigate this region in more detail. This

was done in order to evaluate the regional differences in the drug absorption capacity of the small intestine including the different regions of the jejunum.

4.3.2.1 Proximal jejunum

As illustrated in Figures 4.11 and 4.12, the *A. vera* whole leaf materials mediated a statistically significant ($p < 0.05$) increase in insulin transport (P_{app}) in the duodenum when using the Sweetana-Grass diffusion chamber technique and also when using the everted sac technique. For the diffusion chamber technique, the whole leaf materials mediated a 3.97 - fold increase in the cumulative insulin transport was achieved compared to the control group (insulin only). A 3.28 - fold increase in the cumulative insulin transport (P_{app}) was achieved in the everted sac technique when compared to the control group.

Tables 4.11 and 4.12 illustrate the P_{app} values of insulin in the presence of *A. vera* gel and whole leaf materials. The aloe leaf materials mediated a reduction in TEER in the diffusion chambers, which corresponds with the increased insulin transport and gives an indication of the possible paracellular tight junction opening capacity of the *A. vera* whole leaf and gel materials.

The *A. vera* gel mediated a statistically significant ($p < 0.05$) increase in the cumulative percentage transport of insulin in the everted sac technique of 1.84 fold as compared to the control group. This high percentage transport, compared to that of the diffusion chamber technique may be explained by the larger surface area available for insulin transport (as discussed in Section 4.4.1).

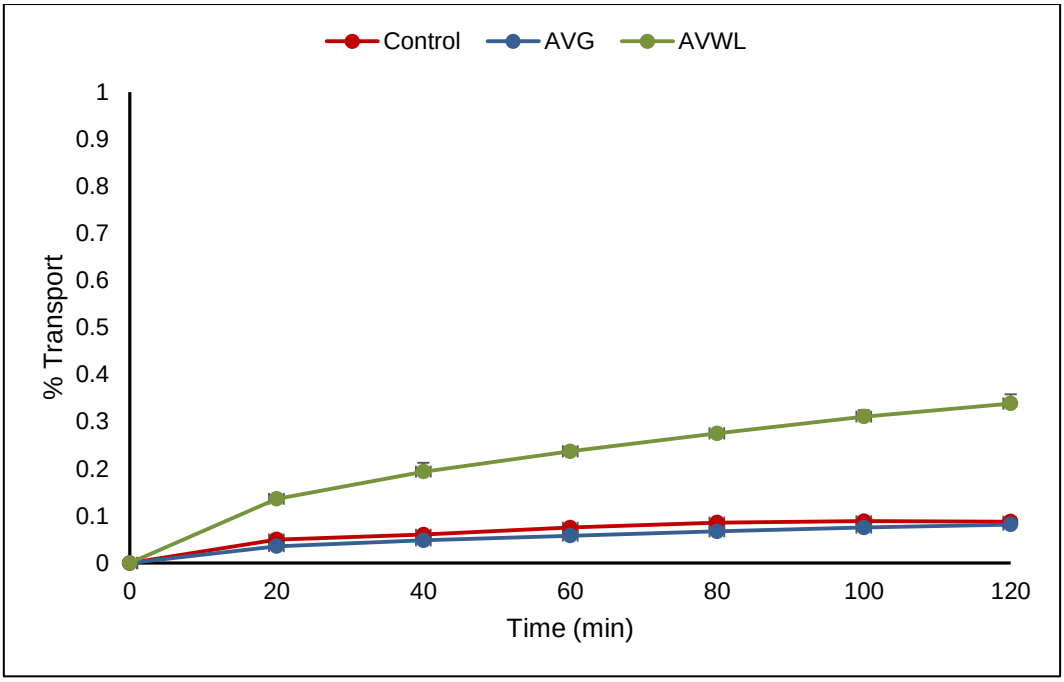


Figure 4.11: **Insulin transport across excised tissue from pig jejunum (proximal) using the Sweetana-Grass diffusion chamber technique. (AVG = *Aloe vera* gel, AVWL = *Aloe vera* whole leaf)**

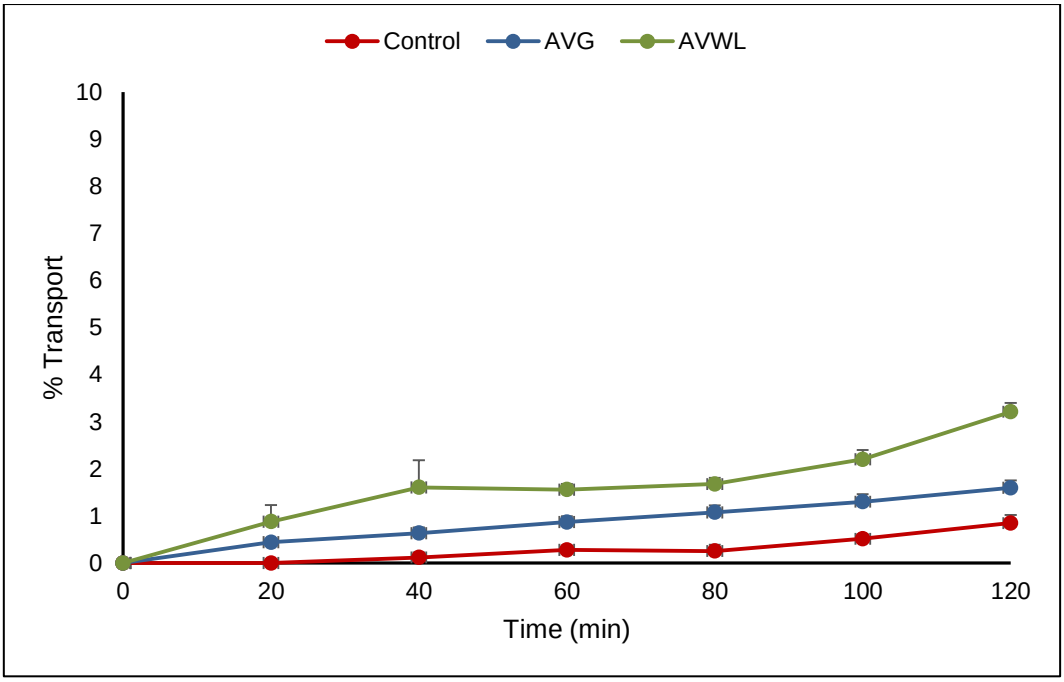


Figure 4.12: **Insulin transport across excised pig jejunum (proximal) using the everted sac technique. (AVG = *Aloe vera* gel, AVWL = *Aloe vera* whole leaf)**

The relatively small error bars (or SD values) shown in Figures 4.11 and 4.12 indicate a high degree of repeatability. The P_{app} values for each experimental group in each of the *in vitro* techniques were calculated and are shown in Tables 4.11 and 4.12.

Table 4.11: P_{app} values for insulin in the Sweetana-Grass diffusion chamber technique across excised pig proximal jejunum tissue

Sweetana-Grass diffusion chamber technique			
Repeat	**Control ($\times 10^{-7}$)**	***A. vera* gel ($\times 10^{-7}$)**	***A. vera* whole leaf ($\times 10^{-7}$)**
1	0.71	0.51	2.31
2	0.64	0.62	2.64
3	0.50	0.59	2.31
Average P_{app}	0.61	0.57	2.42
SD	0.09	0.05	0.15

Table 4.12: P_{app} values for insulin in the everted sac technique across excised pig proximal jejunum tissue

Everted sac technique			
Repeat	**Control ($\times 10^{-7}$)**	***A. vera* gel ($\times 10^{-7}$)**	***A. vera* whole leaf ($\times 10^{-7}$)**
1	0.37	0.60	1.06
2	0.29	0.54	1.02
3	0.29	0.64	1.07
Average P_{app}	0.32	0.59	1.05
SD	0.04	0.04	0.02

4.3.2.2 Medial jejunum

The *A. vera* whole leaf materials mediated a statistically significant ($p < 0.05$) decrease of 0.60 fold in the cumulative percentage insulin transport in the diffusion chamber technique when compared to the control group (Figure 4.13 and Table 4.13). The *A. vera* whole leaf material therefore had a detrimental effect on insulin transport in the medial jejunum. TEER reduction occurred to a lesser extent in the whole leaf group when compared to the control group and may explain the observed decrease in insulin transport. In both *in vitro* techniques, the *A. vera* gel mediated a slight increase in insulin transport, but it was not statistically significant. In the everted sac technique, no statistically significant increase or

decrease in the cumulative percentage of insulin transport was observed with the addition of either *A. vera* gel or whole leaf materials.

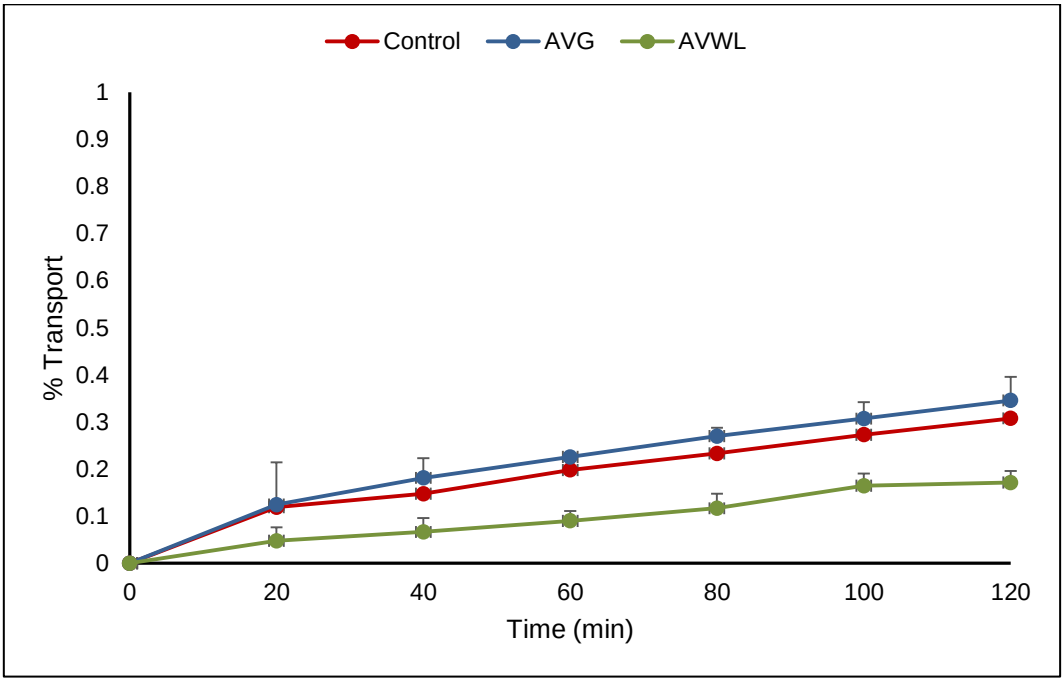


Figure 4.13: **Insulin transport across excised pig jejunum (medial) using the diffusion chamber technique. (AVG = *Aloe vera* gel, AVWL = *Aloe vera* whole leaf)**

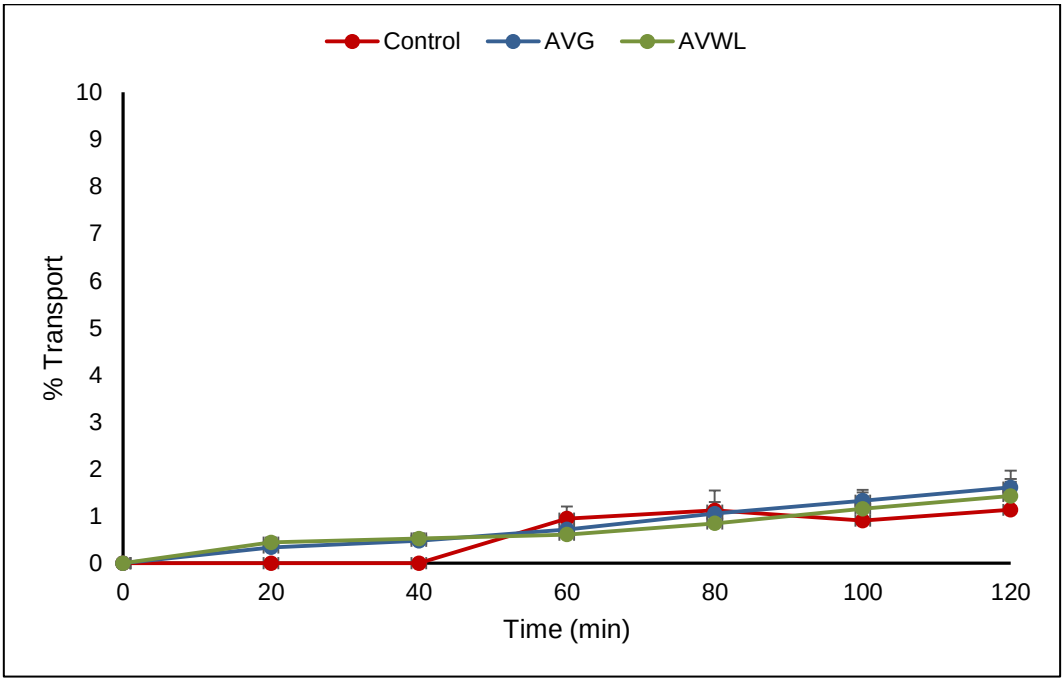


Figure 4.14: **Insulin transport across excised pig jejunum (medial) using the everted sac technique. (AVG = *Aloe vera* gel, AVWL = *Aloe vera* whole leaf)**

The average P_{app} values for each group in each of the *in vitro* techniques as well as the SD values were calculated and are shown in Tables 4.13 and 4.14.

Table 4.13: P_{app} values for insulin in the Sweetana-Grass diffusion chamber technique across excised pigmedial jejunum tissue

Sweetana-Grass diffusion chamber technique			
Repeat	**Control ($\times 10^{-7}$)**	***Aloe vera* gel ($\times 10^{-7}$)**	***Aloe vera* whole leaf ($\times 10^{-7}$)**
1	2.13	2.43	1.33
2	2.15	2.55	1.04
3	2.02	2.50	1.63
Average P_{app}	2.20	2.49	1.33
SD	0.09	0.05	0.24

Table 4.14: P_{app} values for insulin in the everted sac technique across excised pig medial jejunum tissue

Everted sac technique			
Repeat	**Control ($\times 10^{-7}$)**	***A. vera* gel ($\times 10^{-7}$)**	***A. vera* whole leaf ($\times 10^{-7}$)**
1	0.56	0.54	0.42
2	0.23	0.68	0.46
3	0.82	0.67	0.65
Average P_{app}	0.54	0.63	0.51
SD	0.24	0.06	0.11

4.3.2.3 Distal jejunum

The *A. vera* gel mediated a statistically significant ($p < 0.05$) increase in insulin transport (P_{app}) in the diffusion chambers across the distal jejunum tissue, whereas the whole leaf materials mediated a statistically significant ($p < 0.05$) decrease in insulin transport in the everted sac technique. The *A. vera* gel mediated a 2.17 - fold increase in the cumulative percentage insulin transport (P_{app}) in the diffusion chamber technique and the whole leaf materials in the everted sac technique mediated a statistically significant decrease of 0.40 - fold in the cumulative percentage insulin transport as compared to the control group.

The TEER measurements for the distal jejunum in the diffusion chamber technique showed a reduction of 9.7% which indicates that the *A. vera* gel mediated an increase in insulin

transport by acting on the tight junctions and opening them, as reflected by the reduction in TEER.

The occurrence of the respective increase and decrease in the cumulative percentage insulin transport in this region with the addition of *A. vera* leaf materials can possibly be explained by the presence of Peyer's patches which caused unpredictable insulin transport, although avoidance of these patches was attempted. It was not possible to completely avoid the Peyer's patches in the everted sac technique, due to the large number present in the distal jejunum. Although the area available for transport in the everted sac technique was significantly larger (see Section 4.4.1), interaction of the *A. vera* leaf materials with the tissue and/or the model compound might have occurred which hindered insulin transport across the membrane. These interactions may involve movement of molecules into the intercellular spaces and blocking the fenestrae of the tight junctions and thereby decreasing transport (it is known that the fenestrae sizes differ between gastrointestinal tract regions).

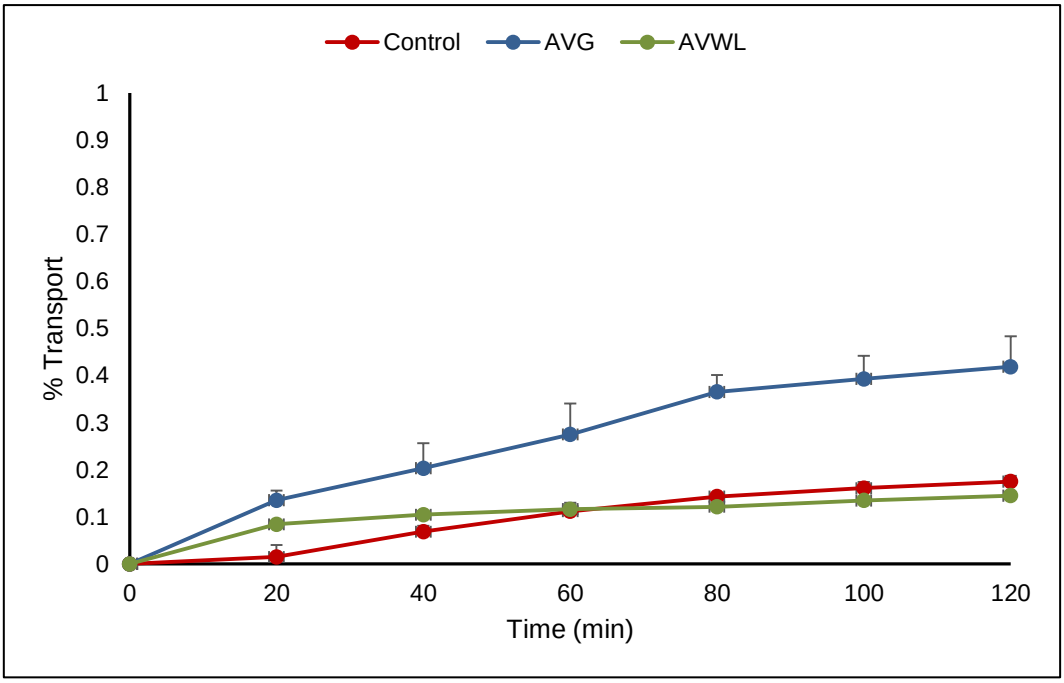


Figure 4.15: **Insulin transport across excised pig jejunum (distal) using the diffusion chamber technique. (AVG = *Aloe vera* gel, AVWL = *Aloe vera* whole leaf)**

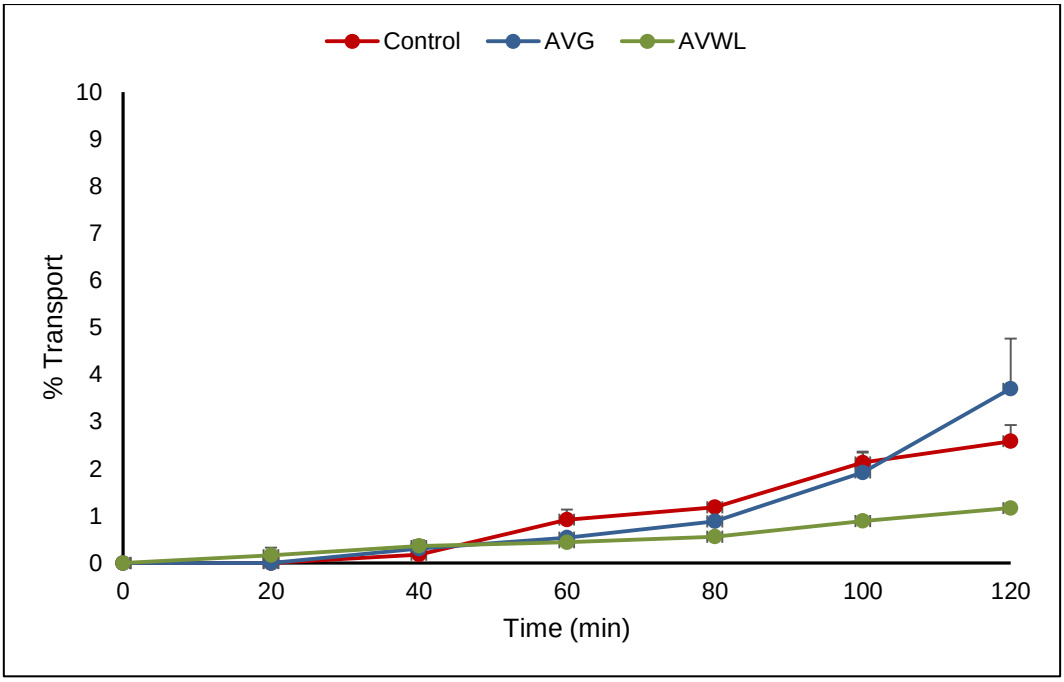


Figure 4.16: **Insulin transport across excised pig jejunum (distal) using the everted sac technique. (AVG = *Aloe vera* gel, AVWL = *Aloe vera* whole leaf)**

The SD and P_{app} values for insulin transport in the *in vitro* techniques were calculated and are shown in Tables 4.15 and 4.16.

Table 4.15: **P_{app} values for insulin in the Sweetana-Grass diffusion chamber technique across excised pig distal jejunum tissue**

Sweetana-Grass diffusion chamber technique			
Repeat	**Control ($\times 10^{-7}$)**	***Aloe vera* gel ($\times 10^{-7}$)**	***Aloe vera* whole leaf ($\times 10^{-7}$)**
1	1.53	3.56	0.91
2	1.67	2.71	0.95
3	1.27	3.43	0.92
Average P_{app}	1.49	3.23	0.92
SD	0.16	0.37	0.02

Table 4.16: P_{app} values for insulin in the everted sac technique across excised pig distal jejunum tissue

Everted sac technique			
Repeat	**Control ($\times 10^{-7}$)**	***A. vera* gel ($\times 10^{-7}$)**	***A. vera* whole leaf ($\times 10^{-7}$)**
1	1.01	1.01	0.45
2	1.11	1.25	0.43
3	1.20	1.70	0.43
Average P_{app}	1.11	1.32	0.44
SD	0.08	0.03	0.01

4.3.3 Ileum

The *A. vera* whole leaf materials mediated a statistically significant ($p < 0.05$) decrease in the cumulative percentage insulin transport (P_{app}) of 0.19 - fold in the diffusion chamber technique as compared to the control group. The TEER values for the ileum in the diffusion chamber technique only decreased slightly, which indicated that the membrane was still intact, but also indicates that the whole leaf materials did not mediate prominent tight junction opening. A possible explanation for the decrease in insulin transport, as depicted by the average transport values in Figure 4.18, is the interaction of the whole leaf material with the intestinal tissue as described earlier.

The *A. vera* gel mediated an increase in insulin transport (P_{app}) which was statistically significant ($p < 0.05$) when using the everted sac technique. A 5.45 - fold increase in the cumulative percentage insulin transport (P_{app}) was observed as compared to the control group. Although the *A. vera* gel mediated an increase in insulin transport in the diffusion chamber technique, it was not statistically significant.

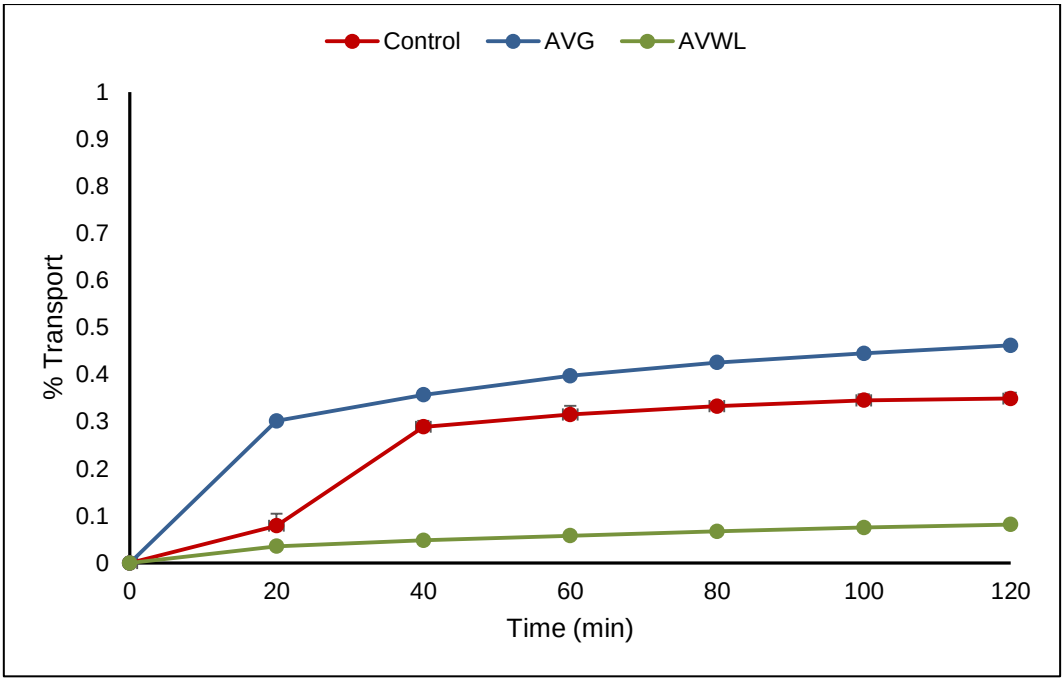


Figure 4.17: **Insulin transport across excised pig ileum using the diffusion chamber technique. (AVG = *Aloe vera* gel, AVWL = *Aloe vera* whole leaf)**

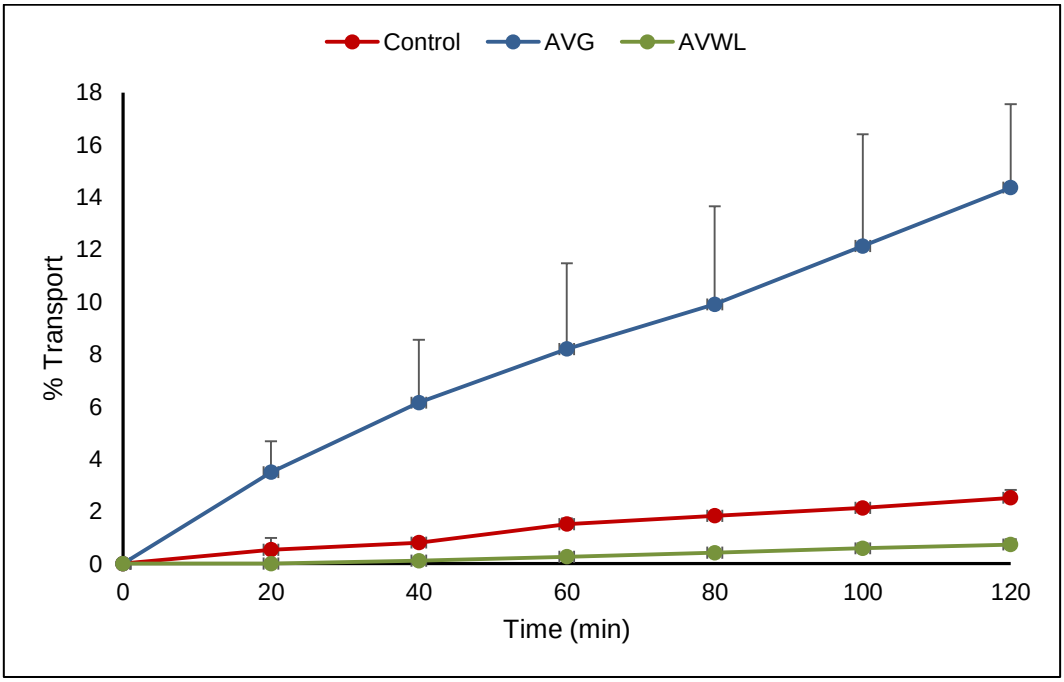


Figure 4.18: **Insulin transport across excised pig ileum using the everted sac technique. (AVG = *Aloe vera* gel, AVWL = *Aloe vera* whole leaf)**

The small SD values represented by the small error bars, as depicted in Figure 4.17 for the Sweetana-Grass diffusion chamber technique, indicated a high degree of repeatability. The SD values of the *A. vera* gel group using the everted sac technique were large, indicating

variable transport enhancement. This poor repeatability has been mentioned as a reason for the low rate at which chemical drug absorption enhancers are translated into commercial products (Brayden & Maher, 2010).

The P_{app} values for insulin in each group in each of the in vitro techniques were calculated and are shown in Tables 4.17 and 4.18.

Table 4.17: P_{app} values for insulin in the Sweetana-Grass diffusion chambers across excised pig ileum tissue

Sweetana-Grass diffusion chamber technique			
Repeat	**Control ($\times 10^{-7}$)**	***A. vera* gel ($\times 10^{-7}$)**	***A. vera* whole leaf ($\times 10^{-7}$)**
1	3.26	3.43	0.51
2	3.33	3.64	0.62
3	2.45	3.27	0.59
Average P_{app}	3.01	3.45	0.57
SD	0.40	0.15	0.05

Table 4.18: P_{app} values for insulin in the everted sac technique across excised pig ileum tissue

Everted sac technique			
Repeat	**Control ($\times 10^{-7}$)**	***A. vera* gel ($\times 10^{-7}$)**	***A. vera* whole leaf ($\times 10^{-7}$)**
1	1.04	7.05	0.36
2	1.04	4.27	0.37
3	0.91	5.05	0.21
Average P_{app}	1.00	5.45	0.31
SD	0.06	1.17	0.07

4.3.4 Colon

In both of the *in vitro* techniques, the *A. vera* gel has mediated a statistically significant ($p < 0.05$) increase in insulin transport (P_{app}). The *A. vera* gel increased the cumulative percentage insulin transport (P_{app}) by 14.10 - fold as compared to the control group in the diffusion chamber technique, while mediating a 2.07 - fold increase in the cumulative insulin transport in the everted sac technique as compared to the insulin transport in the control group. The differences in insulin transport between the two *in vitro* techniques can be

explained by the differences in the surface area available for absorption, as described in Section 4.4.1.

The increased transport is illustrated in Figures 4.19 and 4.20 by the blue lines representing the insulin transport and also in Tables 4.19 and 4.20 by the higher P_{app} values. The TEER values decreased slightly for the colon in the diffusion chamber technique, which indicated that the membrane was still intact. It also indicates that the *A. vera* gel did not have a significant effect on tight junction opening. The high insulin transport in the colon for the diffusion chamber technique, in the presence of the *A. vera* gel, can possibly be explained by the thinner walls of the colon (Chawia *et al.,* 2003) which could have enabled more insulin molecules to be transported across the membrane due to the decreased resistance offered by the colon membrane.

The whole leaf materials, however, caused a statistically significant ($p < 0.05$) decrease in insulin transport (P_{app}) in the everted sac technique. A decrease in the cumulative insulin transport of 0.11 - fold occurred in the group containing the *A. vera* whole leaf materials as compared to the insulin transport in the control group. This is possibly due to the interaction of the *A. vera* whole leaf materials with the intestinal membrane.

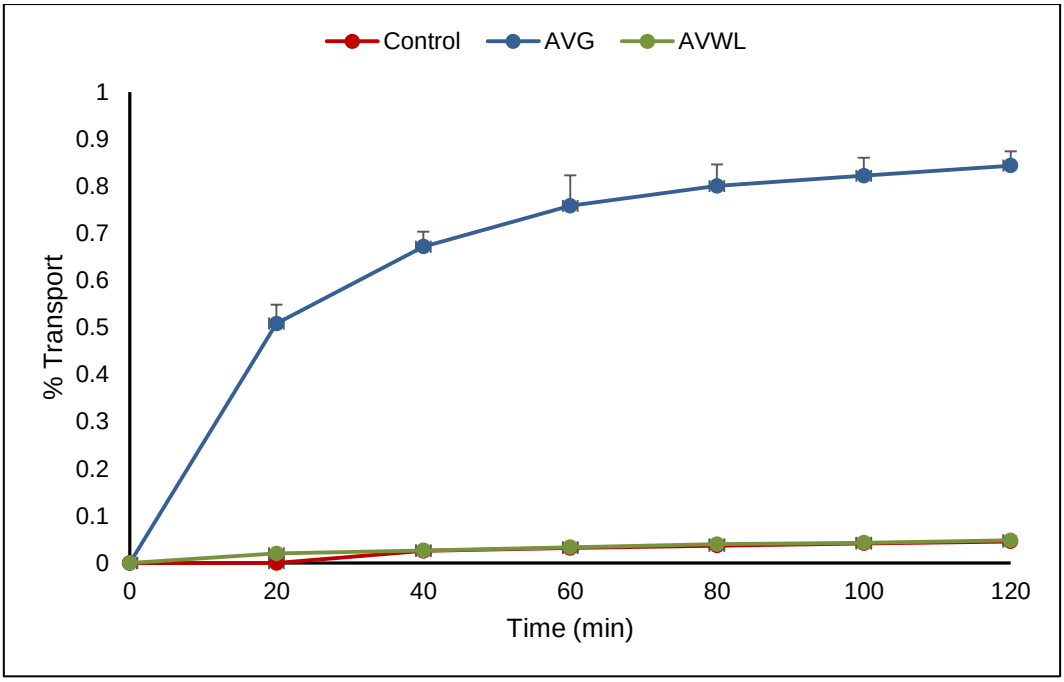


Figure 4.19: **Insulin transport across excised pig colon using the diffusion chamber technique. (AVG = *Aloe vera* gel, AVWL = *Aloe vera* whole leaf)**

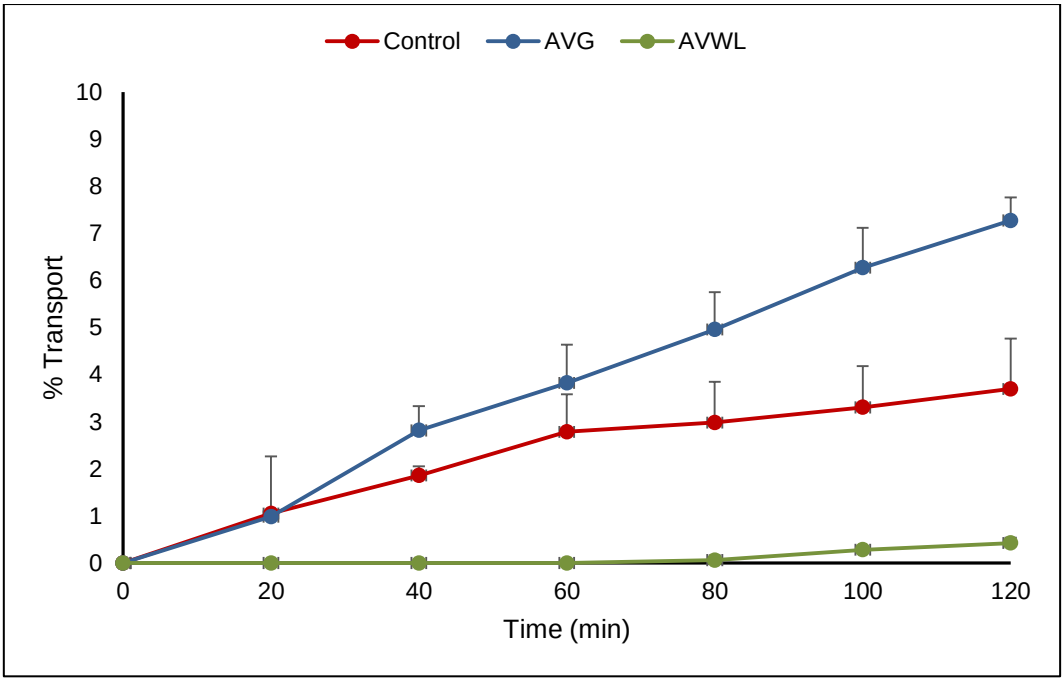


Figure 4.20: **Insulin transport across excised pig colon using the everted sac technique. (AVG = *Aloe vera* gel, AVWL = *Aloe vera* whole leaf)**

The average P_{app} values as well as the SD values for each experimental group have been calculated and are shown in Table 4.19 for the Sweetana-Grass diffusion chamber technique

and in Table 4.20 for the everted sac technique. A high degree of repeatablility is illustrated by the relatively small error bars (representing the SD values) in Figures 4.19 and 4.20.

Table 4.19: P_{app} values for insulin in the Sweetana-Grass diffusion chambers across excised pig colon tissue

Sweetana-Grass diffusion chamber technique			
Repeat	**Control ($\times 10^{-7}$)**	***A. vera* gel ($\times 10^{-7}$)**	***A. vera* whole leaf ($\times 10^{-7}$)**
1	0.45	5.57	0.30
2	0.37	5.24	0.42
3	0.34	5.68	0.30
Average P_{app}	0.39	5.50	0.34
SD	0.05	0.19	0.06

Table 4.20: P_{app} values for insulin in the everted sac technique across excised pig colon tissue

Everted sac technique			
Repeat	**Control ($\times 10^{-7}$)**	***A. vera* gel ($\times 10^{-7}$)**	***A. vera* whole leaf ($\times 10^{-7}$)**
1	1.71	3.42	0.12
2	0.86	2.76	0.14
3	1.70	2.62	0.22
Average P_{app}	1.42	2.94	0.16
SD	0.40	0.35	0.04

4.4 Summary of results

Although the diffusion chamber technique and the everted sac technique both measured the cumulative insulin transport across the intestinal membranes of the different regions of the GIT, they had significantly different surface areas available for absorption as discussed in Section 4.4.1. The comparison of the insulin transport between the two *in vitro* techniques, however, corresponded to a large degree, which indicates that the techniques are accurate in measuring drug transport.

4.4.1 Insulin transport: Sweetana-Grass diffusion chamber technique

The P_{app} values of insulin transported in the absence (control group) and presence of *A. vera* gel and whole leaf materials across excised tissues from different regions are shown in

Figure 4.21. In the bar graph (*) denotes a statistically significant ($p < 0.05$) increase in insulin transport, where (**) denotes a statistically significant ($p < 0.05$) decrease in insulin transport.

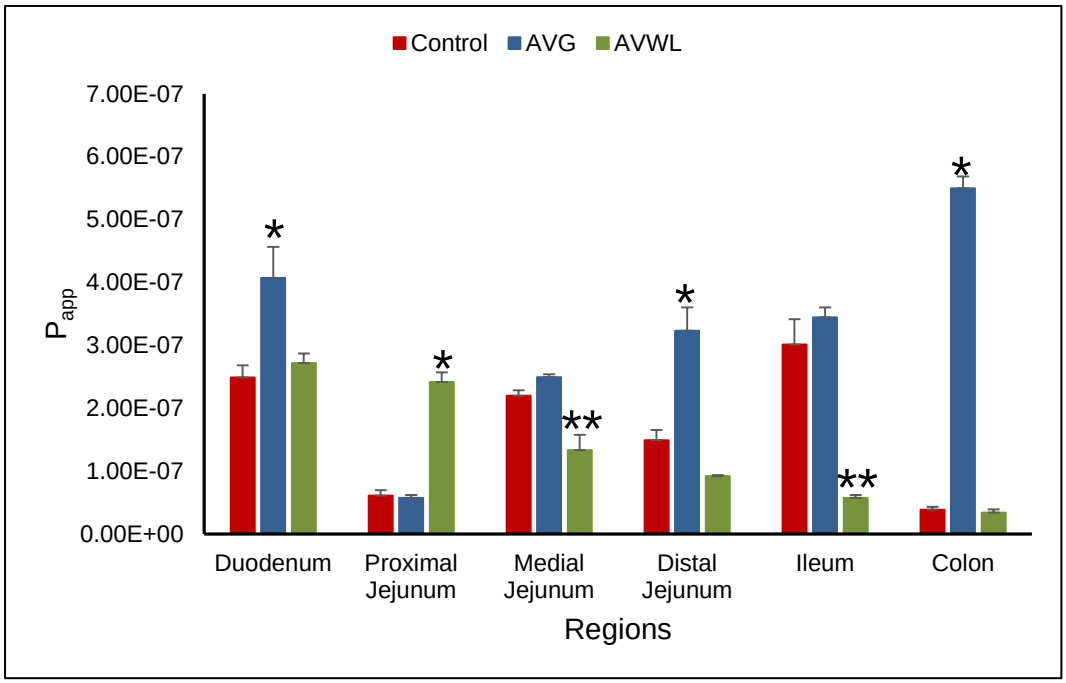


Figure 4.21: Average P_{app} values for insulin across different gastrointestinal regions using the Sweetana-Grass diffusion chamber technique. (AVG = *Aloe vera* gel, AVWL = *Aloe vera* whole leaf)

The *A. vera* gel mediated a statistically significant ($p < 0.05$) increase in insulin transport in the duodenum, distal jejunum and colon compared to the control group in the diffusion chamber technique. The *A. vera* whole leaf material mediated a statistically significant ($p < 0.05$) increase in insulin transport in the proximal jejunum compared to the control group in the diffusion chamber technique, while it mediated a statistically significant decrease in insulin transport in the medial jejunum, distal jejunum, ileum and colon.

4.4.2 Insulin transport: everted sac technique

The average P_{app} values of insulin transported in the absence (control group) and presence of *A. vera* gel and whole leaf materials across excised tissues from different regions are shown in Figure 4.22. In the bar graph, (*) denotes a statistically significant ($p < 0.05$) increase in insulin transport, where (**) denotes a statistically significant ($p < 0.05$) decrease in insulin transport.

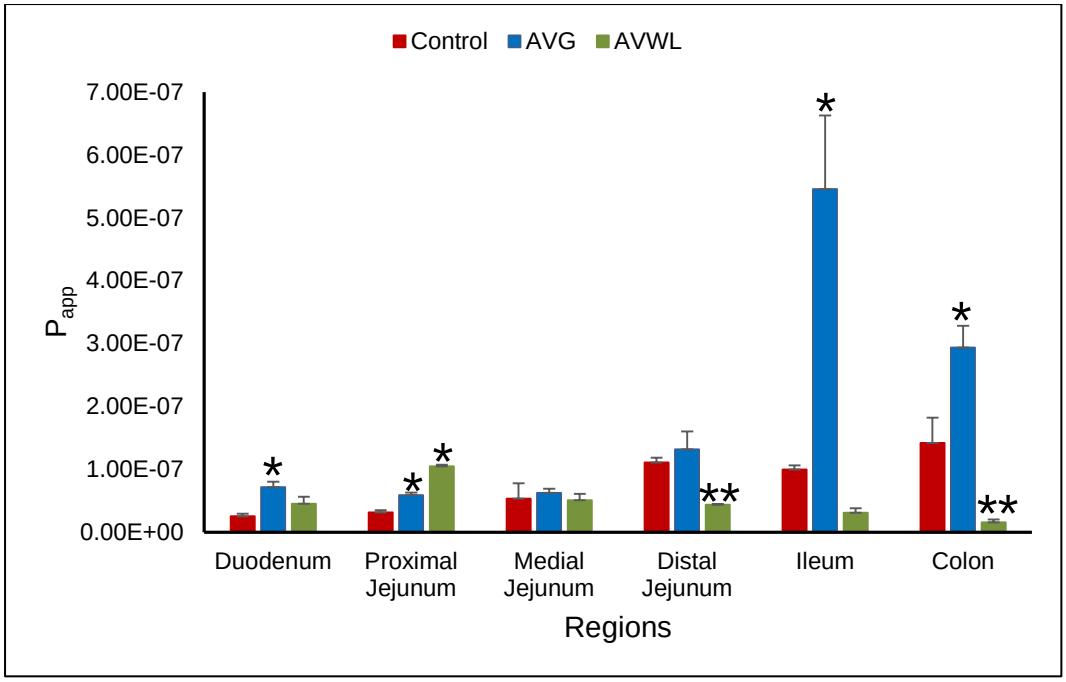


Figure 4.22: Average P_{app} values for insulin across different gastrointestinal regions using the everted sac technique. (AVG = *Aloe vera* gel, AVWL = *Aloe vera* whole leaf)

Insulin transport was enhanced statistically significantly ($p < 0.05$) in the ileum and colon by *A. vera* gel in the everted sac technique, while the *A. vera* whole leaf material increased the insulin transport statistically significantly ($p < 0.05$) only in the proximal jejunum. Similarly to the results obtained in the diffusion chamber technique, insulin transport was statistically significantly reduced by the *A. vera* gel whole leaf materials in the medial jejunum, distal jejunum, ileum and colon.

4.5 Conclusion

The cumulative insulin transport in the presence of the *A. vera* components proved to be higher than with insulin alone. For certain regions, the *A. vera* gel exhibited higher drug absorption enhancing effects than the *A. vera* whole leaf materials, and *vice versa.* The presence of the *A. vera* components showed a reduction in the measured TEER values, which indicated that it opened the tight junctions present in the intestinal tissue. The increased insulin transport can possibly be explained by opening of tight junctions as indicated by a reduction in the TEER values, while the reduction in some instances of insulin transport can probably be explained by different interactions of the aloe materials with different intestinal tissues.

When considering all the transport data and the significant increases and decreases that occurred in the different regions, it can be concluded that insulin transport was regionally dependent throughout the GIT. The effect of the *A. vera* leaf components also showed a regionally dependent effect on the insulin transport across the intestinal tissues.

CHAPTER 5: FINAL CONCLUSIONS AND FUTURE RECOMMENDATIONS

5.1 Final conclusions

The absorption enhancement effects of *A. vera* gel and whole leaf materials on insulin transport across different GIT regions (i.e. duodenum, proximal jejunum, medial jejunum, distal jejunum, ileum and colon) using pig intestinal tissue were determined. It can be concluded that the addition of *A. vera* gel and whole leaf materials had a statistically significant effect on the *in vitro* transport of insulin across some of the six selected GIT regions as described below.

The results showed that the intestinal absorption of insulin was statistically significantly ($p < 0.05$) enhanced by *A. vera* gel across the duodenum, distal jejunum and colon tissues in the diffusion chamber technique, whereas the *A. vera* whole leaf materials mediated a statistically significant ($p < 0.05$) increase in insulin transport only in the proximal jejunum compared to the control group in the diffusion chamber technique. The insulin transport was enhanced statistically significantly ($p < 0.05$) in the ileum and colon by *A. vera* gel in the everted sac technique, while the *A. vera* whole leaf material increased the insulin transport statistically significantly ($p < 0.05$) only in the proximal jejunum in the everted sac technique.

The regional apical to basolateral transport experiments indicated that the highest insulin transport was achieved in the ileum and colon. The colon was identified as the gastrointestinal region in which the highest concentration of insulin could be absorbed with the addition of *A. vera* gel (0.5% w/v). This supports the finding by previous studies (Beneke *et al.,* 2012; Lebitsa *et al.,* 2012) that *A. vera* gel has the potential to modulate drug pharmacokinetics by opening tight junctions.

5.2 Future recommendations

The following aspects are recommended for future research in order to further characterise and investigate the pig intestinal tissue model as well as to investigate the mechanism of action of *A. vera* leaf materials as absorption enhancers acting on the paracellular transport pathway and tight junctions.

- The regional differences in the intestine, such as the thickness of the mucosa, surface area, residence time, pH and enzyme activity should be further investigated,

as these characteristics influence the rate and extent of drug absorption (Kompella & Lee, 2001).

- Various drugs representing the different BCS drug classes should be used in the regional absorption studies in order to investigate the influence of drug physicochemical properties on the absorption in the different regions. This can possibly aid in new formulation strategies developed for gastrointestinal drug delivery as well as for the continuous research towards effective oral delivery systems.

- *In vivo* studies should be conducted to verify the results obtained by *in vitro* studies in order to create an information database which can be used for possible clinical trials.

- Drug absorption studies should be conducted across human intestinal tissue from the different regions in order to determine the impact that the anatomical, biochemical and physiological differences have on drug absorption compared to that of laboratory animals.

- Extensive research should be conducted on regionally specific drug absorption, which will possibly give a more thorough understanding of *in vivo* drug absorption.

- As there is a need for a clinically relevant non-rodent model of the human GIT, pig model should be further investigated, as it is uniquely suited for this purpose.

- Finally, future studies should be conducted on protein and peptide drug absorption enhancement by *A. vera* gel across different alternative routes of administration such as nasal, rectal, buccal and transdermal.

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ADDENDUM A

CONFERENCE PROCEEDINGS

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT
POTCHEFSTROOM CAMPUS

Gastrointestinal region specific insulin absorption enhancement by *Aloe vera* leaf materials

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Purpose: To determine if the intestinal drug absorption enhancement effects of *A. vera* gel and whole leaf materials are region specific and to identify the region in the gastrointestinal tract (GIT) where maximum absorption enhancement of insulin is achieved.

Methods: The transport of insulin across excised pig tissues from various regions of the GIT (i.e. the duodenum, proximal jejunum, medial jejunum, distal jejunum, ileum and colon) was measured in the absence and presence of *A. vera* gel and whole leaf materials (0.5% w/v) using the Sweetana-Grass diffusion as well as the everted sac technique. Apical-to-basolateral transport of insulin was measured over a period of 2 h at a concentration of 170 µg/ml in the diffusion apparatus, while 17 µg/ml was used in the everted sac technique due to the larger surface area available for absorption. Test solutions were prepared in Krebs Ringer bicarbonate (KRB) buffer at the pH values of 6.8. for the duodenum and 7.4 for the ileum, jejunum and colon. Analysis of the samples (200 ul) withdrawn from the acceptor chambers at 20 min intervals was conducted by means of a validated high performance liquid chromatograpy (HPLC) method.

Results: The *A. vera* gel mediated a statistically significant ($p < 0.05$) increase in insulin transport in the duodenum, distal jejunum and colon compared to the control group in the diffusion apparatus. The *A. vera* whole leaf material mediated a statistically significant ($p < 0.05$) increase in insulin transport in the proximal jejunum compared to the control group in the diffusion apparatus. The insulin transport was enhanced statistically significantly ($p < 0.05$) in the ileum and colon by *A. vera* gel in the everted sac technique, while the *A. vera* whole leaf material increased the insulin transport statistically significantly ($p < 0.05$) only in

the proximal jejunum. Insulin transport was reduced in some of the GIT regions by the *A. vera* gel and whole leaf materials. The increased insulin transport can possibly be explained by opening of tight junctions as indicated by reduction in the transepithelial electrical resistance (TEER) values, while the reduction in insulin transport can probably be explained by different interactions of the aloe materials with different intestinal tissues.

Conclusion: The results indicated that the addition of *A. vera* gel and whole leaf materials had a statistically significant effect on the in vitro transport of insulin across some of the six GIT regions.

ADDENDUM B

IN VITRO TRANSPORT OF INSULIN ACROSS EXCISED PIG INTESTINAL TISSUE

B.1 Cumulative percentage insulin transport across excised pig duodenum in the Sweetana-Grass diffusion chamber technique

Table B.1: Average cumulative percentage transport for insulin across excised pig duodenum in the absence of *Aloe vera* leaf materials

Time (min)	%Transport chamber 1	%Transport chamber 2	%Transport chamber 3	Average % transport	SD
0	0	0	0	0	0
20	0	0	0	0	0
40	0.159	0.139	0.158	0.152	0.011
60	0.198	0.172	0.192	0.187	0.014
80	0.243	0.213	0.266	0.241	0.027
100	0.270	0.234	0.285	0.263	0.026
120	0.307	0.261	0.305	0.291	0.026

Table B.2: Average cumulative percentage transport for insulin across excised pig duodenum in the presence of *Aloe vera* gel

Time (min)	%Transport chamber 1	%Transport chamber 2	%Transport chamber 3	Average % transport	SD
0	0	0	0	0	0
20	0.203	0.357	0.261	0.274	0.078
40	0.256	0.352	0.342	0.317	0.053
60	0.372	0.389	0.410	0.390	0.019
80	0.459	0.444	0.478	0.460	0.017
100	0.546	0.515	0.523	0.528	0.016
120	0.645	0.569	0.569	0.595	0.044

Table B.3: Average cumulative percentage transport for insulin across excised pig duodenum in the presence of *Aloe vera* whole leaf materials

Time (min)	%Transport chamber 1	%Transport chamber 2	%Transport chamber 3	Average % transport	SD
0	0	0	0	0	0
20	0.105	0.105	0.103	0.105	0.001
40	0.167	0.158	0.150	0.158	0.008
60	0.211	0.228	0.207	0.215	0.011
80	0.264	0.274	0.240	0.259	0.018
100	0.306	0.317	0.283	0.302	0.018
120	0.346	0.370	0.302	0.338	0.033

B.2 Cumulative percentage insulin transport across excised pig duodenum in the everted sac technique

Table B.4: Average cumulative percentage transport for insulin across excised pig duodenum in the absence of *Aloe vera* leaf materials

Time (min)	%Transport sac 1	%Transport sac 2	%Transport sac 3	Average % transport	SD
0	0	0	0	0	0
20	0	0	0	0	0
40	0	0	0	0	0
60	0.214	0.142	0.151	0.169	0.039
80	0.366	0.236	0.210	0.271	0.083
100	0.551	0.412	0.397	0.453	0.085
120	0.735	0.558	0.548	0.614	0.105

Table B.5: Average cumulative percentage transport for insulin across excised pig duodenum in the presence of *Aloe vera* gel

Time (min)	%Transport sac 1	%Transport sac 2	%Transport sac 3	Average % transport	SD
0	0	0	0	0	0
20	0.213	0.215	0	0.143	0.124
40	0.666	0.357	0.358	0.460	0.178
60	1.097	0.870	0.640	0.869	0.228
80	1.207	1.284	0.916	1.136	0.194
100	1.455	1.483	1.159	1.366	0.179
120	1.554	2.131	1.621	1.769	0.316

Table B.6: Average cumulative percentage transport for insulin across excised pig duodenum in the presence of *Aloe vera* whole leaf materials

Time (min)	%Transport sac 1	%Transport sac 2	%Transport sac 3	Average % transport	SD
0	0	0	0	0	0
20	0	0	0	0	0
40	0	0	0	0	0
60	0.144	0.241	0.161	0.180	0.052
80	0.530	0.545	0.001	0.359	0.310
100	0.628	0.822	0.354	0.602	0.235
120	0.913	1.170	0.602	0.895	0.284

B.3 Cumulative percentage insulin transport across excised pig jejunum (proximal) in the Sweetana-Grass diffusion chamber technique

Table B.7: Average cumulative percentage transport for insulin across excised pig jejunum (proximal) in the absence of *Aloe vera* leaf materials

Time (min)	%Transport chamber 1	%Transport chamber 2	%Transport chamber 3	Average % transport	SD
0	0	0	0	0	0
20	0.072	0.047	0.030	0.050	0.012
40	0.075	0.062	0.043	0.060	0.005
60	0.088	0.086	0.052	0.075	0.003
80	0.104	0.089	0.064	0.086	0.007
100	0.111	0.093	0.063	0.090	0.009
120	0.105	0.087	0.071	0.090	0.009

Table B.8: Average cumulative percentage transport for insulin across excised pig jejunum (proximal) in the presence of *Aloe vera* gel

Time (min)	%Transport chamber 1	%Transport chamber 2	%Transport chamber 3	Average % transport	SD
0	0	0	0	0	0
20	0.036	0.037	0.033	0.036	0.002
40	0.048	0.049	0.047	0.048	0.001
60	0.053	0.060	0.061	0.058	0.004
80	0.063	0.068	0.071	0.067	0.004
100	0.070	0.083	0.074	0.075	0.007
120	0.074	0.088	0.083	0.082	0.007

Table B.9: Average cumulative percentage transport for insulin across excised pig jejunum (proximal) in the presence of *Aloe vera* whole leaf materials

Time (min)	%Transport chamber 1	%Transport chamber 2	%Transport chamber 3	Average % transport	SD
0	0	0	0	0	0
20	0.137	0.128	0.143	0.136	0.008
40	0.208	0.173	0.201	0.194	0.019
60	0.246	0.227	0.238	0.237	0.010
80	0.278	0.270	0.278	0.275	0.005
100	0.308	0.326	0.299	0.311	0.014
120	0.325	0.361	0.330	0.339	0.020

B.4 Cumulative percentage insulin transport across excised pig jejunum (proximal) in the everted sac technique

Table B.10: Average cumulative percentage transport for insulin across excised pig jejunum (proximal) in the absence of *Aloe vera* leaf materials

Time (min)	%Transport sac 1	%Transport sac 2	%Transport sac 3	Average % transport	SD
0	0	0	0	0	0
20	0	0	0	0	0
40	0.169	0	0.188	0.119	0.103
60	0.386	0.197	0.251	0.278	0.098
80	0.106	0.305	0.351	0.254	0.130
100	0.623	0.467	0.454	0.515	0.094
120	1.044	0.708	0.789	0.847	0.175

Table B.11: Average cumulative percentage transport for insulin across excised pig jejunum (proximal) in the presence of *Aloe vera* gel

Time (min)	%Transport sac 1	%Transport sac 2	%Transport sac 3	Average % transport	SD
0	0	0	0	0	0
20	0.365	0.458	0.502	0.441	0.070
40	0.498	0.665	0.739	0.634	0.123
60	0.729	0.909	0.970	0.869	0.125
80	0.911	1.191	1.124	1.075	0.147
100	1.200	1.216	1.485	1.300	0.160
120	1.653	1.414	1.719	1.596	0.160

Table B.12: Average cumulative percentage transport for insulin across excised pig jejunum (proximal) in the presence of *Aloe vera* whole leaf materials

Time (min)	%Transport sac 1	%Transport sac 2	%Transport sac 3	Average % transport	SD
0	0	0	0	0	0
20	0.469	1.078	1.084	0.877	0.353
40	1.461	1.115	2.237	1.604	0.575
60	1.542	1.441	1.688	1.557	0.125
80	1.571	1.635	1.825	1.677	0.132
100	2.043	2.136	2.426	2.202	0.200
120	3.085	3.116	3.429	3.210	0.190

B.5 Cumulative percentage insulin transport across excised pig jejunum (medial) in the Sweetana-Grass diffusion chamber technique

Table B.13: Average cumulative percentage transport for insulin across excised pig jejunum (medial) in the absence of *Aloe vera* leaf materials

Time (min)	%Transport chamber 1	%Transport chamber 2	%Transport chamber 3	Average % transport	SD
0	0	0	0	0	0
20	0.128	0.122	0.106	0.119	0.012
40	0.148	0.152	0.142	0.147	0.005
60	0.200	0.194	0.199	0.198	0.003
80	0.231	0.241	0.227	0.233	0.007
100	0.270	0.266	0.283	0.273	0.009
120	0.302	0.303	0.317	0.308	0.009

Table B.14: Average cumulative percentage transport for insulin across excised pig jejunum (medial) in the presence of *Aloe vera* gel

Time (min)	%Transport chamber 1	%Transport chamber 2	%Transport chamber 3	Average % transport	SD
0	0	0	0	0	0
20	0.142	0.101	0.130	0.124	0.021
40	0.197	0.168	0.178	0.181	0.015
60	0.243	0.211	0.223	0.226	0.016
80	0.280	0.259	0.271	0.270	0.010
100	0.310	0.299	0.312	0.307	0.007
120	0.345	0.346	0.346	0.346	0.001

Table B.15: Average cumulative percentage transport for insulin across excised pig jejunum (medial) in the presence of *Aloe vera* whole leaf materials

Time (min)	%Transport chamber 1	%Transport chamber 2	%Transport chamber 3	Average % transport	SD
0	0	0	0	0	0
20	0.064	0.064	0.015	0.047	0.029
40	0.081	0.085	0.032	0.066	0.029
60	0.099	0.105	0.066	0.090	0.021
80	0.141	0.126	0.827	0.117	0.030
100	0.168	0.137	0.188	0.164	0.026
120	0.176	0.145	0.193	0.171	0.024

B.6 Cumulative percentage insulin transport across excised pig jejunum (medial) in the everted sac technique

Table B.16: Average cumulative percentage transport for insulin across excised pig jejunum (medial) in the absence of *Aloe vera* leaf materials

Time (min)	%Transport sac 1	%Transport sac 2	%Transport sac 3	Average % transport	SD
0	0	0	0	0	0
20	0	0	0	0	0
40	0	0	0	0	0
60	0.695	1.215	0.925	0.945	0.261
80	0.659	1.486	1.214	1.120	0.422
100	1.186	0.209	1.310	0.902	0.603
120	1.181	0.279	1.938	1.132	0.831

Table B.17: Average cumulative percentage transport for insulin across excised pig jejunum (medial) in the presence of *Aloe vera* gel

Time (min)	%Transport sac 1	%Transport sac 2	%Transport sac 3	Average % transport	SD
0	0	0	0	0	0
20	0.257	0.447	0.300	0.334	0.100
40	0.343	0.639	0.443	0.475	0.151
60	0.516	0.981	0.647	0.715	0.240
80	0.820	1.312	1.021	1.051	0.248
100	1.075	1.524	1.378	1.326	0.229
120	1.402	1.724	1.699	1.608	0.179

Table B.18: Average cumulative percentage transport for insulin across excised pig jejunum (medial) in the presence of *Aloe vera* whole leaf materials

Time (min)	%Transport sac 1	%Transport sac 2	%Transport sac 3	Average % transport	SD
0	0	0	0	0	0
20	0.471	0.325	0.523	0.439	0.103
40	0.650	0.435	0.485	0.523	0.112
60	0.616	0.492	0.708	0.605	0.109
80	0.822	0.672	1.039	0.844	0.184
100	1.098	0.912	1.448	1.153	0.272
120	1.177	1.343	1.759	1.426	0.300

B.7 Cumulative percentage insulin transport across excised pig jejunum (distal) in the Sweetana-Grass diffusion chamber technique

Table B.19: Average cumulative percentage transport for insulin across excised pig jejunum (distal) in the absence of *Aloe vera* leaf materials

Time (min)	%Transport chamber 1	%Transport chamber 2	%Transport chamber 3	Average % transport	SD
0	0	0	0	0	0
20	0	0	0.044	0.015	0.025
40	0.056	0.068	0.081	0.068	0.013
60	0.091	0.118	0.126	0.112	0.018
80	0.138	0.152	0.139	0.143	0.008
100	0.158	0.175	0.150	0.161	0.013
120	0.172	0.188	0.164	0.175	0.012

Table B.20: Average cumulative percentage transport for insulin across excised pig jejunum (distal) in the presence of *Aloe vera* gel

Time (min)	%Transport chamber 1	%Transport chamber 2	%Transport chamber 3	Average % transport	SD
0	0	0	0	0	0
20	0.151	0.112	0.1421	0.135	0.020
40	0.162	0.185	0.263	0.203	0.053
60	0.210	0.272	0.342	0.275	0.066
80	0.375	0.325	0.395	0.365	0.036
100	0.418	0.336	0.424	0.393	0.049
120	0.460	0.344	0.452	0.419	0.064

Table B.21: Average cumulative percentage transport for insulin across excised pig jejunum (distal) in the presence of *Aloe vera* whole leaf materials

Time (min)	%Transport chamber 1	%Transport chamber 2	%Transport chamber 3	Average % transport	SD
0	0	0	0	0	0
20	0.082	0.086	0.084	0.084	0.002
40	0.097	0.106	0.110	0.104	0.007
60	0.105	0.124	0.120	0.116	0.010
80	0.116	0.127	0.120	0.121	0.005
100	0.129	0.139	0.136	0.135	0.005
120	0.144	0.146	0.145	0.145	0.001

B.8 Cumulative percentage insulin transport across excised pig jejunum (distal) in the everted sac technique

Table B.22: Average cumulative percentage transport for insulin across excised pig jejunum (distal) in the absence of *Aloe vera* leaf materials

Time (min)	%Transport sac 1	%Transport sac 2	%Transport sac 3	Average % transport	SD
0	0	0	0	0	0
20	0	0	0	0	0
40	0	0	0.532	0.177	0.307
60	1.162	0.751	0.848	0.920	0.215
80	1.133	1.300	1.112	1.183	0.105
100	1.882	2.183	2.335	2.133	0.231
120	2.315	2.474	2.968	2.586	0.340

Table B.23: Average cumulative percentage transport for insulin across excised pig jejunum (distal) in the presence of *Aloe vera* gel

Time (min)	%Transport sac 1	%Transport sac 2	%Transport sac 3	Average % transport	SD
0	0	0	0	0	0
20	0	0	0	0	0
40	0.275	0.391	0.243	0.303	0.078
60	0.498	0.530	0.588	0.538	0.046
80	0.795	0.861	0.998	0.885	0.104
100	1.652	1.701	2.413	1.922	0.426
120	2.691	3.609	4.806	3.702	1.061

Table B.24: Average cumulative percentage transport for insulin across excised pig jejunum (distal) in the presence of *Aloe vera* whole leaf materials

Time (min)	%Transport sac 1	%Transport sac 2	%Transport sac 3	Average % transport	SD
0	0	0	0	0	0
20	0	0.158	0.332	0.163	0.166
40	0.299	0.389	0.399	0.362	0.055
60	0.501	0.350	0.467	0.440	0.079
80	0.559	0.505	0.609	0.558	0.052
100	0.815	0.860	0.100	0.892	0.096
120	1.132	1.176	1.189	1.166	0.030

B.9 Cumulative percentage insulin transport across excised pig ileum in the Sweetana-Grass diffusion chamber technique

Table B.25: Average cumulative percentage transport for insulin across excised pig ileum in the absence of *Aloe vera* leaf materials

Time (min)	%Transport chamber 1	%Transport chamber 2	%Transport chamber 3	Average % transport	SD
0	0	0	0	0	0
20	0	0	0.237	0.079	0.137
40	0.268	0.292	0.308	0.289	0.020
60	0.318	0.292	0.336	0.315	0.022
80	0.347	0.292	0.360	0.333	0.036
100	0.368	0.292	0.377	0.345	0.047
120	0.378	0.292	0.378	0.349	0.050

Table B.26: Average cumulative percentage transport for insulin across excised pig ileum in the presence of *Aloe vera* gel

Time (min)	%Transport chamber 1	%Transport chamber 2	%Transport chamber 3	Average % transport	SD
0	0	0	0	0	0
20	0.250	0.311	0.250	0.302	0.090
40	0.330	0.406	0.335	0.357	0.042
60	0.393	0.468	0.394	0.397	0.007
80	0.440	0.503	0.431	0.426	0.018
100	0.471	0.530	0.458	0.445	0.035
120	0.500	0.548	0.481	0.462	0.050

Table B.27: Average cumulative percentage transport for insulin in across excised pig ileum in the presence of *Aloe vera* whole leaf materials

Time (min)	%Transport chamber 1	%Transport chamber 2	%Transport chamber 3	Average % transport	SD
0	0	0	0	0	0
20	0.036	0.037	0.033	0.036	0.002
40	0.048	0.049	0.047	0.048	0.001
60	0.053	0.060	0.061	0.058	0.004
80	0.063	0.068	0.071	0.067	0.004
100	0.070	0.083	0.074	0.075	0.007
120	0.074	0.088	0.083	0.082	0.007

B.10 Cumulative percentage insulin transport across excised pig ileum in the everted sac technique

Table B.28: Average cumulative percentage transport for insulin in across excised pig ileum in the absence of *Aloe vera* leaf materials

Time (min)	%Transport sac 1	%Transport sac 2	%Transport sac 3	Average % transport	SD
0	0	0	0	0	0
20	0	0.781	0.798	0.526	0.456
40	0.639	0.791	0.970	0.800	0.166
60	1.742	1.321	1.459	1.507	0.215
80	1.936	1.717	1.816	1.823	0.110
100	2.124	2.198	2.056	2.126	0.071
120	2.244	2.843	2.448	2.512	0.304

Table B.29: Average cumulative percentage transport for insulin across excised pig ileum in the presence of *Aloe vera* gel

Time (min)	%Transport sac 1	%Transport sac 2	%Transport sac 3	Average % transport	SD
0	0	0	0	0	0
20	4.758	2.410	3.309	3.492	1.185
40	7.946	3.410	7.086	6.147	2.409
60	11.217	4.717	8.657	8.197	3.275
80	13.645	6.153	9.913	9.904	3.746
100	16.296	7.773	12.335	12.135	4.265
120	18.033	12.248	12.820	14.367	3.188

Table B.30: Average cumulative percentage transport for insulin across excised pig ileum in the presence of *Aloe vera* whole leaf materials

Time (min)	%Transport sac 1	%Transport sac 2	%Transport sac 3	Average % transport	SD
0	0	0	0	0	0
20	0	0	0	0	0
40	0.150	0.177	0	0.109	0.095
60	0.325	0.312	0.141	0.259	0.103
80	0.501	0.483	0.257	0.414	0.136
100	0.706	0.670	0.372	0.583	0.184
120	0.805	0.882	0.493	0.727	0.206

B.11 Cumulative percentage insulin transport across excised pig colon in the Sweetana-Grass diffusion chamber technique

Table B.31: Average cumulative percentage transport for insulin across excised pig colon in the absence of *Aloe vera* leaf materials

Time (min)	%Transport chamber 1	%Transport chamber 2	%Transport chamber 3	Average % transport	SD
0	0	0	0	0	0
20	0	0	0	0	0
40	0.029	0.026	0.023	0.026	0.003
60	0.035	0.033	0.028	0.032	0.003
80	0.041	0.037	0.033	0.037	0.004
100	0.048	0.041	0.036	0.042	0.006
120	0.054	0.043	0.040	0.046	0.008

Table B.32: Average cumulative percentage transport for insulin across excised pig colon in the presence of *Aloe vera* gel

Time (min)	%Transport chamber 1	%Transport chamber 2	%Transport chamber 3	Average % transport	SD
0	0	0	0	0	0
20	0.476	0.496	0.553	0.508	0.040
40	0.639	0.672	0.703	0.672	0.032
60	0.709	0.734	0.832	0.758	0.065
80	0.775	0.772	0.853	0.800	0.046
100	0.809	0.793	0.865	0.822	0.038
120	0.844	0.813	0.874	0.844	0.031

Table B.33: Average cumulative percentage transport for insulin across excised pig colon in the presence of *Aloe vera* whole leaf materials

Time (min)	%Transport chamber 1	%Transport chamber 2	%Transport chamber 3	Average % transport	SD
0	0	0	0	0	0
20	0.021	0.022	0.017	0.020	0.003
40	0.026	0.029	0.025	0.027	0.002
60	0.033	0.038	0.030	0.034	0.004
80	0.037	0.047	0.035	0.040	0.007
100	0.040	0.052	0.038	0.043	0.007
120	0.044	0.058	0.042	0.048	0.008

B.12 Cumulative percentage insulin transport across excised pig colon in the everted sac technique

Table B.34: Average cumulative percentage transport for insulin across excised pig colon in the absence of *Aloe vera* leaf materials

Time (min)	%Transport sac 1	%Transport sac 2	%Transport sac 3	Average % transport	SD
0	0	0	0	0	0
20	1.001	1.116	1.033	1.050	0.059
40	1.816	1.687	2.072	1.858	0.196
60	3.405	1.888	3.059	2.784	0.795
80	3.341	1.996	3.605	2.981	0.863
100	3.899	2.295	3.719	3.304	0.879
120	4.243	2.466	4.376	3.695	1.067

Table B.35: Average cumulative percentage transport for insulin across excised pig colon in the presence of *Aloe vera* gel

Time (min)	%Transport sac 1	%Transport sac 2	%Transport sac 3	Average % transport	SD
0	0	0	0	0	0
20	0	0.528	2.427	0.985	1.277
40	3.348	2.318	2.775	2.814	0.516
60	4.638	3.016	3.812	3.822	0.811
80	5.763	4.172	4.936	4.957	0.796
100	7.230	5.644	5.931	6.268	0.845
120	7.779	6.808	7.226	7.271	0.487

Table B.36: Average cumulative percentage transport for insulin across excised pig colon in the presence of *Aloe vera* whole leaf materials

Time (min)	%Transport sac 1	%Transport sac 2	%Transport sac 3	Average % transport	SD
0	0	0	0	0	0
20	0	0	0	0	0
40	0	0	0	0	0
60	0	0	0	0	0
80	0	0	0.181	0.060	0.104
100	0.219	0.252	0.374	0.282	0.082
120	0.338	0.377	0.556	0.424	0.116

ADDENDUM C

P_{APP} VALUES FOR *IN VITRO* TRANSPORT OF INSULIN ACROSS EXCISED PIG INTESTINAL TISSUE

C.1 P_{app} values for the cumulative percentage insulin transport across excised pig duodenum in the Sweetana-Grass diffusion chamber technique

Table C.1: P_{app} values for the cumulative percentage insulin transport across excised pig duodenum in the absence of *Aloe vera* leaf materials

P_{app} Chamber 1 (10^{-07})	P_{app} Chamber 2 (10^{-07})	P_{app} Chamber 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
2.584	2.219	2.665	2.489	0.194

Table C.2: P_{app} values for the cumulative percentage insulin transport across excised pig duodenum in the presence of *Aloe vera* gel

P_{app} Chamber 1 (10^{-07})	P_{app} Chamber 2 (10^{-07})	P_{app} Chamber 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
4.724	3.531	3.958	4.071	0.493

Table C.3: P_{app} values for the cumulative percentage insulin transport across excised pig duodenum in the presence of *Aloe vera* whole leaf materials

P_{app} Chamber 1 (10^{-07})	P_{app} Chamber 2 (10^{-07})	P_{app} Chamber 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
2.743	2.891	2.514	2.716	0.155

C.2 P_{app} values for the cumulative percentage insulin transport across excised pig duodenum in the everted sac technique

Table C.4: P_{app} values for the cumulative percentage insulin transport across across excised pig duodenum in the absence of *Aloe vera* leaf materials

P_{app} Sac 1 (10^{-07})	P_{app} Sac 2 (10^{-07})	P_{app} Sac 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
0.312	0.232	0.225	0.257	0.039

Table C.5: **P_{app} values for the cumulative percentage insulin transport across excised pig duodenum in the presence of *Aloe vera* gel**

P_{app} Sac 1 (10^{-07})	P_{app} Sac 2 (10^{-07})	P_{app} Sac 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
0.654	0.838	0.658	0.717	0.086

Table C.6: **P_{app} values for the cumulative percentage insulin transport across excised pig duodenum in the presence of *Aloe vera* whole leaf materials**

P_{app} Sac 1 (10^{-07})	P_{app} Sac 2 (10^{-07})	P_{app} Sac 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
0.385	0.485	0.214	0.454	0.112

C.3 P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (proximal) in the Sweetana-Grass diffusion chamber technique

Table C.7: **P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (proximal) in the absence of *Aloe vera* leaf materials**

P_{app} Chamber 1 (10^{-07})	P_{app} Chamber 2 (10^{-07})	P_{app} Chamber 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
0.706	0.635	0.501	0.614	0.085

Table C.8: **P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (proximal) in the presence of *Aloe vera* gel**

P_{app} Chamber 1 (10^{-07})	P_{app} Chamber 2 (10^{-07})	P_{app} Chamber 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
0.508	0.625	0.590	0.574	0.049

Table C.9: **P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (proximal) in the presence of *Aloe vera* whole leaf materials**

P_{app} Chamber 1 (10^{-07})	P_{app} Chamber 2 (10^{-07})	P_{app} Chamber 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
2.314	2.636	2.306	2.419	0.153

C.4 P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (proximal) in the everted sac technique

Table C.10: **P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (proximal) in the absence of *Aloe vera* leaf materials**

P_{app} Sac 1 (10^{-07})	P_{app} Sac 2 (10^{-07})	P_{app} Sac 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
0.367	0.286	0.292	0.315	0.037

Table C.11: **P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (proximal) in the presence of *Aloe vera* gel**

P_{app} Sac 1 (10^{-07})	P_{app} Sac 2 (10^{-07})	P_{app} Sac 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
0.599	0.535	0.639	0.591	0.043

Table C.12: **P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (proximal) in the presence of *Aloe vera* whole leaf materials**

P_{app} Sac 1 (10^{-07})	P_{app} Sac 2 (10^{-07})	P_{app} Sac 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
1.064	1.019	1.068	1.050	0.022

C.5 P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (medial) in the Sweetana-Grass diffusion chamber technique

Table C.13: P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (medial) in the absence of *Aloe vera* leaf materials

P_{app} Chamber 1 (10^{-07})	P_{app} Chamber 2 (10^{-07})	P_{app} Chamber 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
2.125	2.151	2.324	2.200	0.088

Table C.14: P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (medial) in the presence of *Aloe vera* gel

P_{app} Chamber 1 (10^{-07})	P_{app} Chamber 2 (10^{-07})	P_{app} Chamber 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
2.432	2.550	2.496	2.492	0.048

Table C.15: P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (medial) in the presence of *Aloe vera* whole leaf materials

P_{app} Chamber 1 (10^{-07})	P_{app} Chamber 2 (10^{-07})	P_{app} Chamber 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
1.328	1.039	1.634	1.334	0.243

C.6 P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (medial) in the everted sac technique

Table C.16: P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (medial) in the absence of *Aloe vera* leaf materials

P_{app} Sac 1 (10^{-07})	P_{app} Sac 2 (10^{-07})	P_{app} Sac 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
0.559	0.233	0.820	0.537	0.240

Table C.17: P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (medial) in the presence of *Aloe vera* gel

P_{app} Sac 1 (10^{-07})	P_{app} Sac 2 (10^{-07})	P_{app} Sac 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
0.538	0.680	0.666	0.628	0.064

Table C.18: P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (medial) in the presence of *Aloe vera* whole leaf materials

P_{app} Sac 1 (10^{-07})	P_{app} Sac 2 (10^{-07})	P_{app} Sac 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
0.422	0.46	0.65	0.512	0.110

C.7 P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (distal) in the Sweetana-Grass diffusion chamber technique

Table C.19: P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (distal) in the absence of *Aloe vera* leaf materials

P_{app} Chamber 1 (10^{-07})	P_{app} Chamber 2 (10^{-07})	P_{app} Chamber 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
1.528	1.668	1.273	1.490	0.164

Table C.20: P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (distal) in the presence of *Aloe vera* gel

P_{app} Chamber 1 (10^{-07})	P_{app} Chamber 2 (10^{-07})	P_{app} Chamber 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
3.555	2.709	3.431	3.232	0.373

Table C.21: P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (distal) in the presence of *Aloe vera* whole leaf materials

P_{app} Chamber 1 (10^{-07})	P_{app} Chamber 2 (10^{-07})	P_{app} Chamber 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
0.909	0.945	0.918	0.924	0.015

C.8 P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (distal) in the everted sac technique

Table C.22: P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (distal) in the absence of *Aloe vera* leaf materials

P_{app} Sac 1 (10^{-07})	P_{app} Sac 2 (10^{-07})	P_{app} Sac 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
1.007	1.113	1.200	1.108	0.080

Table C.23: P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (distal) in the presence of *Aloe vera* gel

P_{app} Sac 1 (10^{-07})	P_{app} Sac 2 (10^{-07})	P_{app} Sac 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
1.011	1.245	1.701	1.321	0.029

Table C.24: P_{app} values for the cumulative percentage insulin transport across excised pig jejunum (distal) in the presence of *Aloe vera* whole leaf materials

P_{app} Sac 1 (10^{-07})	P_{app} Sac 2 (10^{-07})	P_{app} Sac 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
0.450	0.429	0.435	0.438	0.009

C.9 P_{app} values for the cumulative percentage insulin transport across excised pig ileum in the Sweetana-Grass diffusion chamber technique

Table C.25: P_{app} values for the cumulative percentage insulin transport across excised pig ileum in the absence of *Aloe vera* leaf materials

P_{app} Chamber 1 (10^{-07})	P_{app} Chamber 2 (10^{-07})	P_{app} Chamber 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
3.258	3.335	2.450	3.014	0.400

Table C.26: P_{app} values for the cumulative percentage insulin transport across excised pig ileum in the presence of *Aloe vera* gel

P_{app} Chamber 1 (10^{-07})	P_{app} Chamber 2 (10^{-07})	P_{app} Chamber 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
3.432	3.643	3.269	3.448	0.153

Table C.27: P_{app} values for the cumulative percentage insulin transport across excised pig ileum in the presence of *Aloe vera* whole leaf materials

P_{app} Chamber 1 (10^{-07})	P_{app} Chamber 2 (10^{-07})	P_{app} Chamber 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
0.508	0.625	0.590	0.574	0.049

C.10 P_{app} values for the cumulative percentage insulin transport across excised pig ileum in the everted sac technique

Table C.28: P_{app} values for the cumulative percentage insulin transport across excised pig ileum in the absence of *Aloe vera* leaf materials

P_{app} Sac 1 (10^{-07})	P_{app} Sac 2 (10^{-07})	P_{app} Sac 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
1.044	1.040	0.911	0.100	0.063

Table C.29: P_{app} values for the cumulative percentage insulin transport across excised pig ileum in the presence of *Aloe vera* gel

P_{app} Sac 1 (10^{-07})	P_{app} Sac 2 (10^{-07})	P_{app} Sac 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
7.047	4.270	5.046	5.454	1.170

Table C.30: P_{app} values for the cumulative percentage insulin transport across excised pig ileum in the presence of *Aloe vera* whole leaf materials

P_{app} Sac 1 (10^{-07})	P_{app} Sac 2 (10^{-07})	P_{app} Sac 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
0.355	0.365	0.211	0.310	0.071

C.11 P_{app} values for the cumulative percentage insulin transport across excised pig colon in the Sweetana-Grass diffusion chamber technique

Table C.31: P_{app} values for the cumulative percentage insulin transport across excised pig colon in the absence of *Aloe vera* leaf materials

P_{app} Chamber 1 (10^{-07})	P_{app} Chamber 2 (10^{-07})	P_{app} Chamber 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
0.452	0.371	0.335	0.386	0.049

Table C.32: P_{app} values for the cumulative percentage insulin transport across excised pig colon in the presence of *Aloe vera* gel

P_{app} Chamber 1 (10^{-07})	P_{app} Chamber 2 (10^{-07})	P_{app} Chamber 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
5.572	5.239	5.679	5.497	0.188

Table C.33: P_{app} values for the cumulative percentage insulin transport across excised pig colon in the presence of *Aloe vera* whole leaf materials

P_{app} Chamber 1 (10^{-07})	P_{app} Chamber 2 (10^{-07})	P_{app} Chamber 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
0.302	0.417	0.299	0.340	0.055

C.12 P_{app} values for the cumulative percentage insulin transport across excised pig colon in the everted sac technique

Table C.34: P_{app} values for the cumulative percentage insulin transport across excised pig colon in the absence of *Aloe vera* leaf materials

P_{app} Sac 1 (10^{-07})	P_{app} Sac 2 (10^{-07})	P_{app} Sac 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
1.705	0.856	1.700	1.421	0.400

Table C.35: P_{app} values for the cumulative percentage insulin transport across excised pig colon in the presence of *Aloe vera* gel

P_{app} Sac 1 (10^{-07})	P_{app} Sac 2 (10^{-07})	P_{app} Sac 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
3.419	2.764	2.623	2.936	0.347

Table C.36: P_{app} values for the cumulative percentage insulin transport across excised pig colon in the presence of *Aloe vera* whole leaf materials

P_{app} Sac 1 (10^{-07})	P_{app} Sac 2 (10^{-07})	P_{app} Sac 3 (10^{-07})	Average P_{app} (10^{-07})	SD (10^{-07})
0.124	0.139	0.221	0.161	0.043

ADDENDUM D

TEER MEASUREMENTS FOR *IN VITRO* TRANSPORT OF INSULIN ACROSS EXCISED PIG INTESTINAL TISSUE IN THE SWEETANA-GRASS DIFFUSION CHAMBER TECHNIQUE

D.1 TEER measurements across regional excised pig intestinal tissue in the absence of *Aloe vera* leaf materials

Table D.1: TEER values measured across the regional intestinal membranes before the commencement of the transport studies and thereafter in the absence of *Aloe vera* leaf materials

Diffusion chamber Control												
	Duodenum		Proximal jejunum		Medial jejunum		Distal jejunum		Ileum		Colon	
	Start	End	Start	End	Start	End	Start	End	Start	End	Start	End
C 1	87	63	53	35	153	122	42	28	40	32	62	51
C 2	82	74	50	33	112	102	43	32	44	35	57	32
C 3	77	52	55	48	126	115	42	38	66	48	70	37
Av	82.0	63.0	52.7	38.7	130.3	113	42.3	32.7	50	38.3	63	40

D.2 TEER measurements across regional excised pig intestinal tissue in the presence of *Aloe vera* gel

Table D.2: TEER values measured across the regional intestinal membranes before the commencement of the transport studies and thereafter in the presence of *Aloe vera* gel

Diffusion chamber *Aloe vera* gel												
	Duodenum		Proximal jejunum		Medial jejunum		Distal jejunum		Ileum		Colon	
	Start	End	Start	End	Start	End	Start	End	Start	End	Start	End
C 1	51	30	45	43	72	70	42	31	33	23	36	24
C 2	58	21	40	27	77	68	63	32	36	32	36	18
C 3	70	44	58	29	68	67	50	39	43	25	34	24
Av	59.7	31.7	47.7	33	72.3	68.3	51.7	34	37.3	26.7	35.3	22

D.3 TEER measurements across regional excised pig intestinal tissue in the presence of *Aloe vera* whole leaf materials

Table D.3: TEER values measured across the regional intestinal membranes before the commencement of the transport studies and thereafter in the presence of *Aloe vera* whole leaf

Diffusion chamber *Aloe vera* whole leaf												
	Duodenum		Proximal jejunum		Medial jejunum		Distal jejunum		Ileum		Colon	
	Start	End	Start	End	Start	End	Start	End	Start	End	Start	End
C 1	54	27	155	132	79	74	40	38	56	48	53	44
C 2	49	39	178	112	84	78	59	51	58	47	50	45
C 3	55	37	179	95	116	92	72	58	73	49	42	36
Av	52.7	34.3	56.9	37.7	93	81.3	57	49	62.3	48	48.3	41.7

ADDENDUM E

STATISTICAL ANALYSIS OF DATA FROM *IN VITRO* TRANSPORT STUDIES OF INSULIN ACROSS EXCISED PIG INTESTINAL TISSUE

E.1 Statistical analysis of the *in vitro* transport of insulin across excised pig duodenum in the Sweetana–Grass diffusion chamber technique

Table E.1: Descriptive statistics for the cumulative percentage insulin transport across excised pig duodenum

Descriptive statistics						
Type = Control						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	24.894	25.843	22.189	26.651	2.3778
Type = *Aloe vera* gel						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	40.710	39.579	35.313	47.238	6.0424
Type = *Aloe vera* whole leaf						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	27.158	27.434	25.140	28.900	1.895

Table E.2: Levene's test for homogeneity of variances across excised pig duodenum

Levene's test for homogeneity of variances Effect: Type Degrees of freedom for all F's: 2, 6				
	MS Effect	**MS Error**	**F**	**P**
p_{app}verm	7.872	3.250	2.422	0.169

Table E.3: Dunnett's post hoc test for cumulative percentage insulin transport across excised pig duodenum

Dunnett's test; variable p_{app}verm Probabilities for post hoc tests (2-sided) Error: between MS = 15.252, df = 6.0000		
Cell no.	**Type**	**{1} 24.894**
1	Control	
2	*Aloe vera* gel	0.004591
3	*Aloe vera* whole leaf	0.714492

Table E.4: Analysis test of variance for insulin across excised pig duodenum

Analysis of variance Marked effects are significant at p < 0.05000								
Variable	**SS effect**	**df effect**	**MS effect**	**SS error**	**df error**	**MS error**	**F**	**P**
p_{app} verm	438.931	2	219.466	91.512	6	15.252	14.389	0.005135

E.2 Statistical analysis of the *in vitro* transport of insulin across excised pig jejunum (proximal) in the Sweetana–Grass diffusion chamber technique

Table E.5: Descriptive statistics for the cumulative percentage insulin transport across excised pig jejunum (proximal)

Descriptive Statistics						
Type = Control						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	6.139	6.350	5.010	7.057	1.040
Type = *Aloe vera* gel						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	5.739	5.890	5.077	6.250	0.600
Type = *Aloe vera* whole leaf						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	24.214	23.143	23.100	26.400	1.893

Table E.6 Levene's test for homogeneity of variances across excised pig jejunum (proximal)

Levene's test for homogeneity of variances Effect: Type Degrees of freedom for all F's: 2, 6				
	MS Effect	**MS Error**	**F**	**P**
p_{app}verm	0.813	0.233	3.491	0.099

Table E.7: Dunnett's post hoc test for cumulative percentage insulin transport across excised pig jejunum (proximal)

Dunnett's test; variable p_{app}verm Probabilities for post hoc tests (2-sided) Error: between MS = 15.252, df = 6.0000		
Cell no.	**Type**	**{1} 6.1388**
1	Control	
2	*Aloe vera* gel	0.903023
3	*Aloe vera* whole leaf	0.000014

Table E.8: Analysis test of variance for insulin across excised pig jejunum (proximal)

Analysis of variance Marked effects are significant at p < 0.05000								
Variable	**SS effect**	**df effect**	**MS effect**	**SS error**	**df error**	**MS error**	**F**	**P**
p_{app} verm	668.216	2	334.108	10.049	6	1.675	199.480	0.000003

E.3 Statistical analysis of the *in vitro* transport of insulin across excised pig jejunum (medial) in the Sweetana–Grass diffusion chamber technique

Table E.9: Descriptive statistics for the cumulative percentage insulin transport across excised pig jejunum (medial)

Descriptive Statistics						
Type = Control						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	21.997	21.500	21.250	23.240	1.084
Type = *Aloe vera* gel						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	24.932	25.000	24.300	25.496	0.601
Type = *Aloe vera* whole leaf						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	13.327	13.282	10.400	16.300	2.950

Table E.10: Levene's test for homogeneity of variances across excised pig jejunum (medial)

Levene's test for homogeneity of variances Effect: Type Degrees of freedom for all F's: 2, 6				
	MS Effect	**MS Error**	**F**	**P**
p_{app}verm	1.965	1.017	1.931	0.225

Table E.11: Dunnett's post hoc test for cumulative percentage insulin transport across excised pig jejunum (medial)

Dunnett test; variable p_{app}verm Probabilities for post hoc tests (2-sided) Error: between MS = 3.4135, df = 6.0000		
Cell no.	**Type**	**{1} 21.997**
1	Control	
2	*Aloe vera* gel	0.167114
3	*Aloe vera* whole leaf	0.002187

Table E.12: Analysis test of variance for insulin across excised pig jejunum (medial)

Analysis of variance Marked effects are significant at p < 0.05000								
Variable	**SS effect**	**df effect**	**MS effect**	**SS error**	**df error**	**MS error**	**F**	**P**
p_{app} verm	218.436	2	109.218	20.481	6	3.413	31.996	0.00063

E.4 Statistical analysis of the *in vitro* transport of insulin across excised pig jejunum (distal) in the Sweetana–Grass diffusion chamber technique

Table E.13: Descriptive statistics for the cumulative percentage insulin transport across excised pig jejunum (distal)

Descriptive Statistics						
Type = Control						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	14.900	15.300	12.700	16.700	2.030
Type = *Aloe vera* gel						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	32.318	34.312	27.090	35.551	4.569
Type = *Aloe vera* whole leaf						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	9.240	9.180	9.089	9.452	0.189

Table E.14: Levene's test for homogeneity of variances across excised pig jejunum (distal)

Levene's test for homogeneity of variances Effect: Type Degrees of freedom for all F's: 2, 6				
	MS Effect	**MS Error**	**F**	**P**
p_{app}verm	8.507	1.187	7.168	0.026

Table E.15: Dunnett's post hoc test for cumulative percentage insulin transport across excised pig jejunum (distal)

Dunnett's test; variable p_{app}verm Probabilities for post hoc tests (2-sided) Error: between MS = 8.3451, df = 6.0000		
Cell no.	**Type**	**{1} 14.900**
1	Control	
2	*Aloe vera* gel	0.000583
3	*Aloe vera* whole leaf	0.091499

Table E.16: Analysis test of variance for insulin across excised pig jejunum (distal)

Analysis of variance Marked effects are significant at p < 0.05000								
Variable	**SS effect**	**df effect**	**MS effect**	**SS error**	**df error**	**MS error**	**F**	**P**
p_{app} verm	867.966	2	433.983	50.071	6	8.345	52.004	0.000162

E.5 Statistical analysis of the *in vitro* transport of insulin across excised pig ileum in the Sweetana–Grass diffusion chamber technique

Table E.17: Descriptive statistics for the cumulative percentage insulin transport across excised pig ileum

Descriptive Statistics						
Type = Control						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	30.150	32.600	24.500	33.350	4.908
Type = *Aloe vera* gel						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	34.467	34.300	32.700	36.400	1.856
Type = *Aloe vera* whole leaf						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	5.739	5.890	5.077	6.250	0.601

Table E.18: Levene's test for homogeneity of variances across excised pig ileum

Levene's test for homogeneity of variances Effect: Type Degrees of freedom for all F's: 2, 6				
	MS Effect	**MS Error**	**F**	**P**
p_{app}verm	8.958	1.274	7.033	0.0673

Table E.19: Dunnett's post hoc test for cumulative percentage insulin transport across excised pig ileum

Dunnett's test; variable p_{app}verm Probabilities for post hoc tests (2-sided) Error: between MS = 9.2956, df = 6.0000		
Cell no.	**Type**	**{1} 30.150**
1	Control	
2	*Aloe vera* gel	0.2200776
3	*Aloe vera* whole leaf	0.000127

Table E.20: Analysis test of variance for insulin across excised pig ileum

Analysis of variance Marked effects are significant at p < 0.05000								
Variable	**SS effect**	**df effect**	**MS effect**	**SS error**	**df error**	**MS error**	**F**	**P**
p_{app} verm	1439.790	2	719.898	55.773	6	9.296	77.445	0.000052

E.6 Statistical analysis of the *in vitro* transport of insulin across excised pig colon in the Sweetana–Grass diffusion chamber technique

Table E.21: Descriptive statistics for the cumulative percentage insulin transport across excised pig colon

Descriptive Statistics						
Type = Control						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	3.860	3.710	3.350	4.520	0.599
Type = *Aloe vera* gel						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	54.973	55.718	52.400	56.800	2.293
Type = *Aloe vera* whole leaf						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	3.393	3.020	2.990	4.170	0.673

Table E.22: Levene's test for homogeneity of variances across excised pic colon

Levene's test for homogeneity of variances Effect: Type Degrees of freedom for all F's: 2, 6				
	MS Effect	**MS Error**	**F**	**P**
p_{app}verm	1.533	0.321	4.774	0.057

Table E.23: Dunnett's post hoc test for cumulative percentage insulin transport across excised pig colon

Dunnett's test; variable p_{app}verm Probabilities for post hoc tests (2-sided) Error: between MS = 2.0229, df = 6.0000		
Cell no.	**Type**	**{1} 3.8600**
1	Control	
2	*Aloe vera* gel	0.000010
3	*Aloe vera* whole leaf	0.891643

Table E.24: Analysis test of variance for insulin across excised pig colon

Analysis of variance Marked effects are significant at p < 0.05000								
Variable	**SS effect**	**df effect**	**MS effect**	**SS error**	**df error**	**MS error**	**F**	**P**
p_{app} verm	5273.170	2	2636.590	12.137	6	2.023	1303.391	0.000316

E.7 Statistical analysis of the *in vitro* transport of insulin across excised pig duodenum in the everted sac technique

Table E.25: Descriptive statistics for the cumulative percentage insulin transport across excised pig duodenum

Descriptive statistics						
Type = Control						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	2.566	2.323	2.253	3.125	0.483
Type = *Aloe vera* gel						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	7.166	6.580	6.536	8.381	1.053
Type = *Aloe vera* whole leaf						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	3.610	3.847	2.138	4.846	1.369

Table E.26: Levene's test for homogeneity of variances across excised pig duodenum

Levene's test for homogeneity of variances Effect: Type Degrees of freedom for all F's: 2, 6								
Variable	**SS effect**	**df effect**	**MS Effect**	**SS error**	**df error**	**MS Error**	**F**	**P**
p_{app} verm	0.596	2	0.298	1.161	6	0.193	1.540	0.288

Table E.27: Dunnett's post hoc test for cumulative percentage insulin transport across excised pig duodenum

Dunnett test; variable p_{app}verm Probabilities for post hoc tests (2-sided) Error: between MS = 1..0720, df = 6.0000		
Cell no.	**Type**	**{1} 2.5658**
1	Control	
2	*Aloe vera* gel	0.002890
3	*Aloe vera* whole leaf	0.412542

Table E.28: Analysis test of variance for insulin across excised pig duodenum

Analysis of variance Marked effects are significant at p < 0.05000								
Variable	**SS effect**	**df effect**	**MS effect**	**SS error**	**df error**	**MS error**	**F**	**P**
p_{app} verm	34.892	2	17.446	6.432	6	1.072	16.274	0.00377

E.8 Statistical analysis of the *in vitro* transport of insulin across excised pig jejunum (proximal) in the everted sac technique

Table E.29: Descriptive statistics for the cumulative percentage insulin transport across excised pig jejunum (proximal)

Descriptive Statistics						
Type = Control						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	3.151	2.941	2.859	3.669	0.450
Type = *Aloe vera* gel						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	5.906	5.987	5.346	6.386	0.525
Type = *Aloe vera* whole leaf						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	10.502	10.639	10.189	10.678	0.272

Table E.30: Levene's test for homogeneity of variances across excised pig jejunum (proximal)

Levene's test for homogeneity of variances Effect: Type Degrees of freedom for all F's: 2, 6								
Variable	**SS effect**	**df effect**	**MS Effect**	**SS error**	**df error**	**MS Error**	**F**	**P**
p_{app} verm	0.0466	2	0.023	0.196	6	0.033	0.713	0.527

Table E.31: Dunnett's post hoc test for cumulative percentage insulin transport across excised pig jejunum (proximal)

Dunnett's test; variable p_{app}verm Probabilities for post hoc tests (2-sided) Error: between MS = 18.379, df = 6.0000		
Cell no.	**Type**	**{1} 3.1507**
1	Control	
2	*Aloe vera* gel	0.000412
3	*Aloe vera* whole leaf	0.000011

Table E.32: Analysis test of variance for insulin across excised pig jejunum (proximal)

Analysis of variance Marked effects are significant at p < 0.05000								
Variable	**SS effect**	**df effect**	**MS effect**	**SS error**	**df error**	**MS error**	**F**	**P**
p_{app} verm	82.755	2	41.378	1.010	6	0.184	225.139	0.000002

E.9 Statistical analysis of the *in vitro* transport of insulin across excised pig jejunum (medial) in the everted sac technique

Table E.33: Descriptive statistics for the cumulative percentage insulin transport across excised pig jejunum (medial)

Descriptive Statistics						
Type = Control						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	5.374	5.588	2.330	8.204	2.942
Type = *Aloe vera* gel						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	6.278	6.658	5.376	6.801	0.785
Type = *Aloe vera* whole leaf						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	5.124	4.627	4.215	6.532	1.236

Table E.34: Levene's test for homogeneity of variances across excised pig jejunum (medial)

Levene's test for homogeneity of variances Effect: Type Degrees of freedom for all F's: 2, 6								
Variable	**SS effect**	**df effect**	**MS Effect**	**SS error**	**df error**	**MS Error**	**F**	**P**
p_{app} verm	3.340	2	1.670	5.526	6	0.921	1.813	0.242

Table E.35: Dunnett's post hoc test for cumulative percentage insulin transport across excised pig jejunum (medial)

Dunnett test; variable p_{app}verm Probabilities for post hoc tests (2-sided) Error: between MS = 3.6009, df = 6.0000		
Cell no.	**Type**	**{1} 5.3742**
1	Control	
2	*Aloe vera* gel	0.791591
3	*Aloe vera* whole leaf	0.981189

Table E.36: Analysis test of variance for insulin across excised pig jejunum (medial)

Analysis of variance Marked effects are significant at p < 0.05000								
Variable	**SS effect**	**df effect**	**MS effect**	**SS error**	**df error**	**MS error**	**F**	**P**
p_{app} verm	2.211	2	1.106	21.605	6	3.601	0.307	0.747

E.10 Statistical analysis of the *in vitro* transport of insulin across excised pig jejunum (distal) in the everted sac technique

Table E.37: Descriptive statistics for the cumulative percentage insulin transport across excised pig jejunum (distal)

Descriptive Statistics						
Type = Control						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	11.079	11.132	10.070	12.036	0.984
Type = *Aloe vera* gel						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	13.207	12.500	10.114	17.006	3.500
Type = *Aloe vera* whole leaf						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	4.378	4.348	4.292	4.495	0.105

Table E.38: Levene's test for homogeneity of variances across excised pig jejunum (distal)

Levene's test for homogeneity of variances Effect: Type Degrees of freedom for all F's: 2, 6								
Variable	**SS effect**	**df effect**	**MS Effect**	**SS error**	**df error**	**MS Error**	**F**	**P**
p_{app} verm	9.843	2	4.921	5.833	6	0.972	5.062	0.052

Table E.39: Dunnett's post hoc test for cumulative percentage insulin transport across excised pig jejunum (distal)

Dunnett's test; variable p_{app}verm Probabilities for post hoc tests (2-sided) Error: between MS = 4.4098, df = 6.0000		
Cell no.	**Type**	**{1} 11.079**
1	Control	
2	*Aloe vera* gel	0.409976
3	*Aloe vera* whole leaf	0.014069

Table E.40: Analysis test of variance for insulin across excised pig jejunum (distal)

Analysis of variance Marked effects are significant at p < 0.05000								
Variable	**SS effect**	**df effect**	**MS effect**	**SS error**	**df error**	**MS error**	**F**	**P**
p_{app} verm	127.365	2	63.683	26.459	6	4.410	14.441	0.00509

E.11 Statistical analysis of the *in vitro* transport of insulin across excised pig ileum in the everted sac technique

Table E.41: Descriptive statistics for the cumulative percentage insulin transport across excised pig ileum

Descriptive Statistics						
Type = Control						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	9.998	10.441	9.105	10.448	0.774
Type = *Aloe vera* gel						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	54.542	50.458	42.697	70.470	14.330
Type = *Aloe vera* whole leaf						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	3.104	3.554	2.109	3.651	0.863

Table E.42: Levene's test for homogeneity of variances across excised pig ileum

Levene's test for homogeneity of variances Effect: Type Degrees of freedom for all F's: 2, 6								
Variable	**SS effect**	**df effect**	**MS Effect**	**SS error**	**df error**	**MS Error**	**F**	**P**
p_{app} verm	199.593	2	99.797	72.713	6	12.119	8.235	0.01904

Table E.43: Dunnett's post hoc test for cumulative percentage insulin transport across excised pig ileum

Dunnett's test; variable p_{app}verm Probabilities for post hoc tests (2-sided) Error: between MS = 9.2956, df = 6.0000		
Cell no.	**Type**	**{1}**
1	Control	
2	*Aloe vera* gel	0.001085
3	*Aloe vera* whole leaf	0.528519

Table E.44: Analysis test of variance for insulin across excised pig ileum

Analysis of variance Marked effects are significant at p < 0.05000								
Variable	**SS effect**	**df effect**	**MS effect**	**SS error**	**df error**	**MS error**	**F**	**P**
p_{app} verm	4677.517	2	2338.759	413.107	6	68.901	33.943	0.000535

E.12 Statistical analysis of the *in vitro* transport of insulin across excised pig colon in the everted sac technique

Table E.45: Descriptive statistics for the cumulative percentage insulin transport across excised pig colon

Descriptive Statistics						
Type = Control						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	14.214	17.035	8.559	17.050	4.898
Type = *Aloe vera* gel						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	29.355	27.643	26.229	34.193	4.249
Type = *Aloe vera* whole leaf						
Variable	**Valid N**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
p_{app}verm	3	1.611	1.390	1.236	2.209	0.524

Table E.46: Levene's test for homogeneity of variances across excised pig colon

Levene's test for homogeneity of variances Effect: Type Degrees of freedom for all F's: 2, 6				
	MS Effect	**MS Error**	**F**	**P**
p_{app}verm	9.830	1.718	5.723	0.041

Table E.47: Dunnett's post hoc test for cumulative percentage insulin transport across excised pig colon

Dunnett's test; variable p_{app}verm Probabilities for post hoc tests (2-sided) Error: between MS = 14.108, df = 6.0000		
Cell no.	**Type**	**{1} 14.214**
1	Control	
2	*Aloe vera* gel	0.004697
3	*Aloe vera* whole leaf	0.011217

Table E.48: Analysis test of variance for insulin across excised pig colon

Analysis of variance Marked effects are significant at p < 0.05000								
Variable	**SS effect**	**df effect**	**MS effect**	**SS error**	**df error**	**MS error**	**F**	**P**
p_{app} verm	1157.78	2	578.891	84.652	6	14.109	41.031	0.00032

ADDENDUM F

LANGUAGE EDITING CERTIFICATE

Declaration

This is to declare that I, Annette L Combrink, accredited language editor and translator of the South African Translators' Institute, have language-edited the dissertation by

Elizca Pretorius (20260350)

With the title

Gastrointestinal region specific insulin absorption enhancement by *Aloe vera* leaf materials

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