Development of a double phase dosage form for enhanced peptide drug delivery

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It all starts here ™

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This dissertation is dedicated to my beloved parents, Piet and Karin de Bruyn, for always supporting me and for being the driving force in my life and career. Throughout my life, they have actively supported me in my determination to find and realise my potential. They have always believed in me and encouraged me to go on every adventure, especially this one.

ABSTRACT

Parenteral administration remains the most utilised route of administration for therapeutic peptides due to low intestinal epithelial permeability. However, the most convenient and popular route of drug administration remains the oral route. Oral delivery of protein and peptide drugs encounters challenges such as poor penetration of intestinal mucosa and pre-systemic enzymatic degradation. The former can be overcome with the inclusion of effective and safe absorption enhancing agents in dosage forms. In previous studies, both *Aloe vera* leaf materials and bile salts have shown the capability of increasing drug transport across *in vitro* intestinal epithelial models.

The purpose of this study was to develop and evaluate a double phase drug delivery system for effective oral insulin delivery. Spherical beads were prepared by means of extrusion spheronisation containing insulin as active ingredient and chitosan as muco-adhesive agent. Four other bead formulations were prepared by means of extrusion spheronisation, each containing a different drug absorption enhancing agent, which included *Aloe vera* whole leaf, *Aloe vera* gel, a bile salt mixture (50% sodium cholate acid and 50% sodium deoxycholate) and a single bile salt (sodium glycocholate). The physical and muco-adhesive properties of the different bead formulations were evaluated. Mixtures of the beads containing insulin with beads containing an absorption enhancer were loaded into hard gelatin capsules to prepare four different double phase drug delivery systems. The insulin delivery performance of the double phase drug delivery systems was evaluated across excised pig intestinal tissues in a Sweetana-Grass diffusion apparatus.

All the bead formulations complied with the specified requirements regarding physical properties and showed relatively narrow size distribution values. Inclusion of chitosan pronouncedly improved the muco-adhesive properties of the beads. All the double phase drug delivery systems showed enhanced transport of insulin across excised pig intestinal tissues, which was significantly higher than that of the control group (insulin alone) when pre-exposed to *A. vera* whole leaf containing beads.

Key words: absorption enhancer, *Aloe vera* gel/whole leaf, bile salt, chitosan, extrusion-spheronisation, insulin, muco-adhesion, oral route, sodium glycocholate

UITTREKSEL

Parenterale toediening is steeds die mees benutte roete van toediening vir terapeutiese peptiede as gevolg van hul swak deurlaatbaarheid deur intestinale epiteel. Die orale roete is egter die mees gerieflike en gewildste roete vir geneesmiddeltoediening. Orale toediening van proteïen en peptiedgeneesmiddels staar uitdagings soos swak penetrasie van intestinale mukosa en pre-sistemiese ensiematiese afbraak in die gesig. Eersgenoemde kan oorkom word met die insluiting van effektiewe en veilige absorpsiebevorderaars in doseervorme. In vorige studies het beide *Aloe vera* blaarmateriale en galsoute die vermoë getoon om die beweging van geneesmiddels oor *in vitro* intestinale epiteelmodelle te bevorder.

Die doel van hierdie studie was om 'n dubbelfase doseervorm vir effektiewe aflewering van orale insulin te ontwikkel en te evalueer. Sferiese krale is voorberei deur middel van uitpers-sferonisasie, met insulien as aktiewe middel en kitosaan as ‘n mukoadhesiewe middel. Vier ander formulerings van krale was berei met behulp van ekstrusie-sferonisasie, elk het 'n ander absorpsiebevorderaar bevat wat *Aloe vera* heelblaar, *Aloe vera* jel, ‘n galsoutmengsel (50% natriumkolaat en 50% natriumdeoksilaat) en die enkel galsout, natrium glikocholaat bevat. Die fisiese en mukoadhesiewe eienskappe van die verskillende kraalbereidings is geëvalueer. Mengsels van die krale wat insulien bevat asook elk van die kraalformulerings wat ‘n absorpsiebevorderaar bevat was in harde-gelatien kapsules gelaai om vier verskillende dubbelfase geneemiddelafleweringstelsels te berei. Die insulienafleweringsprestasie van die dubbelfase afleweringstelsels was geëvalueer oor uitgesnyde varkderm intestinale-weefsel in ‘n Sweetana-Grass diffusieapparaat.

Al die kraalformulerings het aan die vereistes ten opsigte van fisiese eienskappe voldoen en het relatief noue deeltjiegrootteverspreidingswaardes getoon. Insluiting van kitosaan het noemenswaardige verbetering in die mukoadhesiewe eienskappe van die kraalformulerings gemaak. Al die dubbelfase afleweringstelsels het verbeterde transport van insulien oor uitgesnyde varkderm intestinale weefsel getoon, wat statisties betekenisvol hoër as dié van die kontrole groep (insulien alleen) was vir die *A. vera* heelblaarbevattende krale.

Sleutelwoorde: Absorpsiebevorderaar, orale toedieningsroete, insulien, , mukoadhesie, *Aloe vera* jel/heelblaar*,* galsoute, natrium glikocholaat, chitosan, ekstrusie-sferonisasie

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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND AND JUSTIFICATION

1.1.1 Absorption enhancement of protein and peptide drugs

Therapeutic proteins and peptides such as insulin, calcitonin, vasopressin, growth factors, human growth hormone, somatostatin, leuprolide and cytokins usually require frequent administration over relatively long periods of time for treatment of chronic diseases (Lee, 2002). These protein and peptide drugs rely mostly on the parenteral route of administration such as injections due to poor oral bioavailability. The oral route of administration is, however, one of the most acceptable ways of administering therapeutics and presents many advantages over the parenteral route of administration. These advantages include no need to manufacture dosage forms under sterile conditions with reduced production costs and the avoidance of discomfort, pain and infections normally associated with injections (Fassano, 1998).

There are many barriers that need to be overcome for protein and peptide drugs to be successfully delivered via the oral route of administration. These barriers include enzymatic and chemical degradation, poor aqueous solubility, low intrinsic membrane permeability and pre-systemic metabolism (Chen *et al.*, 2009). Oral bioavailability of protein and peptide drugs may be improved by co-administration of chemical absorption enhancers amongst other techniques. There are three main categories of absorption enhancement, namely formulation techniques (e.g. chemical absorption enhancers, enzyme inhibitors, muco-adhesive systems, particulate carrier systems), chemical modifications (e.g. pro-drugs, structural modifications, peptidomimetics) and targeting of receptors and transporters (Hamman *et al.*, 2005).

1.1.2 *Aloe vera* leaf materials as drug absorption enhancers

Many compounds have shown the ability to enhance intestinal absorption of polypeptides, but they are not yet clinically used in commercial products due to insufficient activity and/or toxic effects (Chen *et al.*, 2009). Damage to the intestinal epithelium is, for example, a major problem associated with drug absorption enhancing agents. However, certain drug absorption enhancing agents have the ability to increase intestinal drug absorption in a reversible way without causing toxic effects. This has ignited renewed interest in safe and effective oral drug absorption enhancement (Whitehead, Karr and Mitragotri, 2008).

Aloes are perennial succulent xerophytes belonging to the Asphodelaceae family (Chen *et al.*, 2009). The leaves consist of three distinct parts that are known for their medicinal uses, namely the exudate, gel and whole leaf extract (Hamman, 2008). *In vivo* studies in humans showed that *A. vera* (L.) Burm.f. (*Aloe barbadensis* Miller) gel and whole leaf extract liquid preparations have the ability to enhance the bioavailability of vitamins C and E (Vinson, Kharrat and Andreoli, 2005). Furthermore, the whole leaf extract and gel of *A. vera* have been found to increase *in vitro* drug transport by opening the tight junctions between intestinal epithelial cells in a reversible manner. These *A. vera* leaf materials have previously been reported to significantly reduce transepithelial electrical resistance (TEER) of Caco-2 cell monolayers and to significantly enhance the transport of insulin across monolayers of this cell culture model (Chen *et al.,* 2009).

1.1.3 Bile salts as drug absorption enhancers

Bile acids are natural substrates that undergo enterohepatic circulation involving the small intestine and liver. Bile acids exist as bile salts at physiological conditions. Bile salts, such as sodium glycocholate, demonstrated the ability to increase insulin bioavailibility through various suggested mechanisms (Morimoto *et al.*, 1998). These mechanisms include inhibition of protease's activity, dissociation of molecular aggregates through micellar solubilisation and alteration of biological membrane integrity (Gordon *et al.*, 1985; Donovan & Carey, 1990). One factor that may be responsible for an increase in paracellular drug movement is the formation of calcium complexes by bile salts (Lillienau *et al.*, 1992). It was shown that lowering the concentration of free calcium in the extracellular environment may affect the integrity of intercellular tight junctions (Michael *et al.*, 2000).

1.1.4 Beads in multiple-unit dosage forms

Multiple-unit dosage forms contain a number of sub-units, each one containing a certain portion of the total drug dose. Multiple-unit dosage forms offer several advantages over conventional single-unit drug delivery systems, which include a higher degree of homogenous dispersion of the drug in the gastro-intestinal tract, a reduced risk of dose dumping and a reduced risk of tissue irritation (Ishida *et al.*, 2008). Beads are spherical pellets used in multiple-unit solid oral dosage forms such as filled hard-gelatine capsules. Beads can be manufactured through different techniques (e.g. hot melt extrusion, granulation, layer-by-layer techniques and extrusion spheronisation) of which extrusion spheronisation is one of the most popular methods (Mallipeddi *et al.*, 2010).

1.2 AIM AND OBJECTIVES

1.2.1 General aim

The aim of this study is to develop and evaluate a double phase multiple-unit dosage form for the delivery of an absorption enhancer and a peptide drug. The multiple-unit dosage form consisted of beads loaded in hard gelatine capsules where a part of the beads contained an absorption enhancing agent and the other part of the beads contained an active ingredient, namely insulin, together with a muco-adhesive agent (i.e. chitosan). The intention was to develop a dosage form that consists of beads that releases the drug absorption enhancer directly after administration (first phase) to interact with the intestinal epithelium in order to open tight junctions followed by delayed release of the insulin (second phase) from the beads that move relatively slowly along the gastrointestinal tract due to muco-adhesion.

1.2.2 Specific objectives

The following objectives were set for the study:

- To prepare and evaluate different bead formulations manufactured by means of extrusion spheronisation containing different absorption enhancing agents (i.e. *A. vera* gel, *A. vera* whole leaf material, bile salt mixture and sodium glycocholate).
- To prepare and evaluate beads manufactured by means of extrusion spheronisation containing insulin as active ingredient and chitosan as muco-adhesive agent.
- To prepare and evaluate a double phase multiple-unit drug delivery system by loading a mixture of the prepared beads in hard gelatine capsules.

- To evaluate the drug delivery performance of the different double phase multiple-unit dosage forms in terms of reduction of transepithelial electrical resistance as well as insulin transport across excised porcine intestinal tissues in Sweetana-Grass diffusion chambers.

1.3 DESIGN OF THE STUDY

In this study, the permeation of a model drug (i.e. insulin) was manipulated by formulation of chemical absorption enhancers in a double phase solid oral dosage form. Control groups were included to eliminate the effect of chance interferences. In order to manufacture double phase drug delivery systems, different types of beads were combined in hard gelatine capsules. The bead formulations containing different selected absorption enhancing agents were each combined with beads containing insulin and chitosan to prepare double phase drug delivery systems (Table 1.1).

Table 1.1: Composition of the double phase drug delivery systems prepared and investigated for their drug absorption enhancing effects in this study

Double phase drug delivery system	Composition
Formulation 1	Beads containing *A. vera* whole leaf and beads containing insulin and chitosan
Formulation 2	Beads containing *A. vera* gel and beads containing insulin and chitosan
Formulation 3	Beads containing sodium glycocholate and beads containing insulin and chitosan
Formulation 4	Beads containing a mixture of bile salts and beads containing insulin and chitosan
Formulation 5	**Control group:** Beads containing excipient only (without absorption enhancer) and beads containing insulin and chitosan

Each double phase formulation was evaluated not only in terms of physical properties, but also in terms of drug delivery performance in an *in vitro* diffusion model.

1.4 LAYOUT OF DISSERTATION

The first chapter gives a brief overview of background and the research problem as well as a summary of the motivation for the research undertaken in this study. This is followed in Chapter 2 by a recapitulation of related and applicable literature, placing the research project in the context of oral protein and peptide drug delivery. Chapter 3 details the experimental and statistical methods used, while the results and discussions are conveyed in Chapter 4. The final conclusion is discussed in Chapter 5 along with recommendations for future studies.

CHAPTER 2

ORAL DELIVERY OF THERAPEUTIC PROTEINS AND PEPTIDES

2.1 INTRODUCTION

Peptide and protein drugs have a compelling and expeditiously expanding role in medicinal therapy as a result of their superior compatibility and precision, reduced toxicity and capability to modify protein-protein interactions. Therapeutic peptide and protein drugs universally launched on the commercial market stand at about 220 at present (Buchanan & Revell, 2015:171). Metabolic diseases and cancer are the main conditions urging the therapeutic use of protein and peptide drugs. The progression of the pharmaceutical industry regarding rare conditions and orphan drugs has also expanded towards peptides such as teduglutide for the treatment of short bowel syndrome and pasireotide for Cushing's syndrome. Research for the treatment of infectious diseases and inflammation is also trending towards peptide drugs (Fosgerau & Hoffmann, 2015:123).

Peptide and protein drugs are typically classified by the Biopharmaceutical Classification System (BCS) as class III drugs. Reasons for their poor bioavailability include considerable molecular sizes, enzymatic degradation, hydrophilic characteristics and low solubility (Lee, 2002:572). These physico-chemical characteristics cause challenges with their systemic delivery. Unfortunately the rate of advancement for improved delivery systems has not matched the relatively rapid rise in the development of biotechnology based therapeutics (Van der Walle & Olejnick, 2011:1-23).

Most protein-based drugs are currently administered using the parenteral route. Around 75% of peptide drugs are formulated as injectables (Fosgerau & Hoffman, 2015:123). Therefore there is a demand for a less invasive route of administration, particularly with treatments that are administered chronically. This need exists due to a number of shortcomings associated with injections namely pain and discomfort and a risk of infection. But more importantly, the physiological release pattern is not mimicked. Insulin is a good example of this occurrence, with its post-prandial and basal release patterns being difficult to mimic (Roach, 2008:595-610). Furthermore, non-invasive oral peptide and protein drug delivery could potentially

improve efficacy by means of resembling the physiological release pattern more accurately because after oral administration, the insulin first moves to the liver before reaching the systemic circulation and peripheral sites (Brayden & Maher, 2010:5-9). Further clinical advantages include eliminating resistance to self-injection and the fear of needles, which limits patient compliance (Brandt & Boss, 2006:9-12).

2.2 DRUG ABSORPTION FROM THE GASTRO-INTESTINAL TRACT

2.2.1 Transport pathways

There are two principal pathways by which peptide and protein-based drug molecules can move through the intestinal epithelium (refer to Figure 2.1 for a schematic illustration). These include the paracellular transport pathway (through the intercellular spaces between adjacent epithelial cells) and the transcellular transport pathway (through the epithelial cells) (Liu *et al.*, 2009:267).

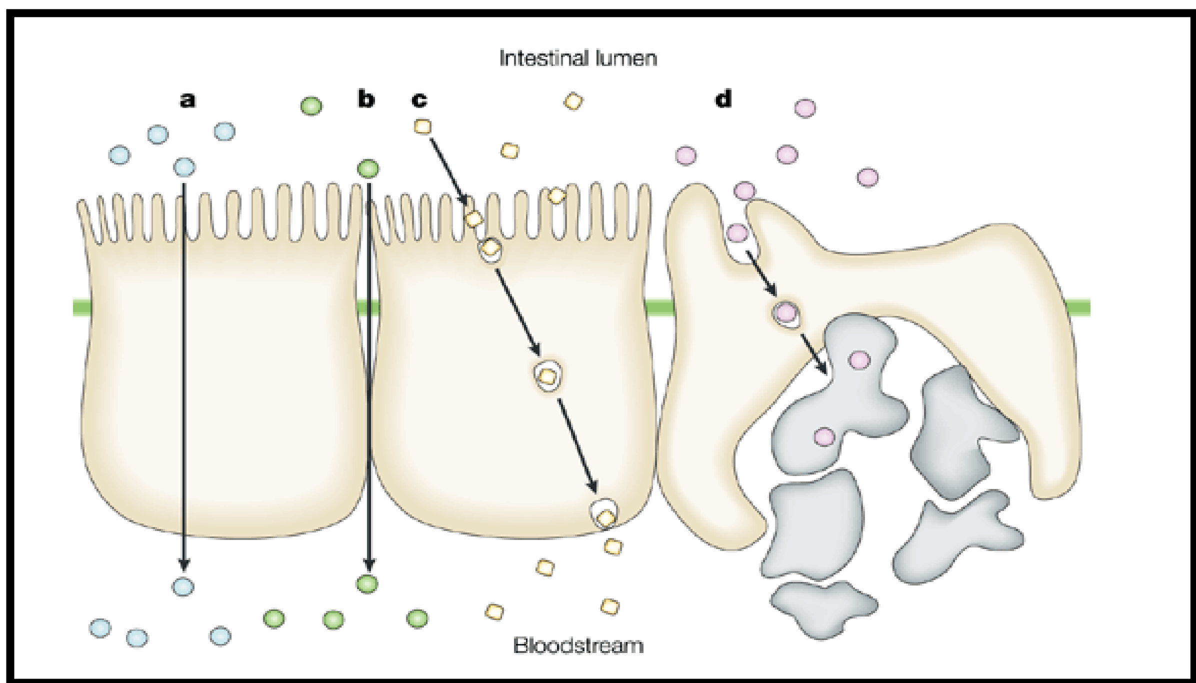

Figure 2.1: Schematic illustration of the mechanisms and pathways of molecule movement across the intestinal epithelium: a) Transcellular pathway (through the epithelial cells), b) Paracellular pathway (in between adjacent cells), c) Transcytosis and receptor-mediated endocytosis, d) Absorption into the lymphatic circulation via M-cells of Peyer's patches (Goldberg & Gomez-Orellana, 2003:90)

Drug molecules must be transported across the brush border and epithelial cell membrane during transcellular absorption. This form of uptake/absorption can take place through simple diffusion, pinocytosis or carrier mediation. The apical and basolateral membranes are identical to other plasma membranes in terms of their permeability characteristics (Lui *et al.*, 2009:267).

The paracellular pathway delineates an aqueous extracellular space separating adjacent cells. As such, the uptake through the paracellular pathway depends on the transfer of substances across a region of packed, hydrophobic intercellular proteins that circumscribe all intestinal epithelial cells under the brush border that forms a continuous barrier known as the 'tight junction' complex (Hamman *et al.*, 2005:167; Lapierre, 2000:255). As such, tight junctions form an intercellular border reducing paracellular movement of solutes through the epithelial layer (Van Itallie & Anderson, 2014:157). The tight junctions hinder the passage of molecules through the intercellular spaces (otherwise known as the fence mechanism) (Artursson *et al.*, 2012:282).

2.2.2 Mechanisms of drug absorption

2.2.2.1 Transcellular passive diffusion

The transcellular passive diffusion mechanism represents a situation where drug molecules transfer from areas of high concentration (e.g. the gastrointestinal tract lumen) via the cellular lipid membrane to an area of low concentration (e.g. the blood). The molecules therefore have to pass through the apical membrane of epithelial cells, then move through the cytoplasm before exiting the cell via the basolateral membrane (Lui *et al.*, 2009:268; Kerns & Di, 2008:87).

It is important to mention that this transport process only takes place after the drug is dissolved. After dissolution occurred in the aqueous liquids found in the gastrointestinal tract, the molecules have to partition into the apical membrane of the epithelial cells and then partition out of the membrane on the other side into the cytosolic fluid. Subsequently, the solute diffuses across the epithelial cell's cytoplasm and ends up in a network of blood capillaries. A much lower accumulation will be sustained in the blood in comparison to the concentration at the site of absorption due to the fast rate of blood flow and quick distribution into the tissues (Ashford, 2007b:279).

2.2.2.2 Carrier-mediated transport

A large number of uptake transporter proteins is present in the small intestinal mucosa and facilitates the transfer of specific drugs, vitamins, and nutrients (Hidalgo, 2001:388). Transporter proteins can be categorized functionally into pumps, channels, and carriers based on the differences in the mechanism through which they use to facilitate the transport of non-electrolytes and ions. Principally, two dedicated carrier-mediated transport frameworks are present in the human body, including facilitated diffusion and active transport (Grassl, 2012:153; Dobson & Kell, 2008:205).

2.2.2.2.1 Active transport

Active transport defines a form of transport that involves the active engagement of transporter proteins in the movement of molecules across the epithelium. The process takes place in the apical membrane of the absorptive epithelial cells located in the columnar lining. A carrier-drug complex is created when the transporter protein (carrier) binds to the molecules of the drug, after which the complex is transferred across the membrane. The molecules of the drug are released on the other side of the epithelial membrane. The carrier then goes back to the plane of the cell membrane where it awaits the arrival of other drug molecules (Ashford, 2007a:281). The transport mechanism may end up being saturated because of the limited availability of carrier molecules (Shargel *et al.*, 2005:380).

Active transport, otherwise termed as active transfer, is branded with the ability to transfer drug molecules against the concentration gradient. Resultantly, active transport is an energy-dependent mechanism that draws its energy from the electrical potential (the trans-membranous sodium gradient) or ATP hydrolysis (Grassl, 2012:154).

2.2.2.2.2 Facilitated diffusion or transport

Facilitated diffusion differs from active transport because it does not involve the transfer of drug molecules against the concentration gradient. This mechanism, therefore, does not depend on energy in order to take place. On the contrary, facilitated transport occurs in a reversible and passive manner, where the path of general transfer out of or into the cell depends on the orientation of electrochemical potential difference of the transported molecules (Grassl, 2012:154). The mechanism may also encounter saturation and presents competitive inhibition (Shargel *et al.*, 2005:380).

2.2.2.3 Endocytosis

Endocytosis defines a transport process where a cell's plasma membrane invaginates to form a small intercellular membrane-covered vesicle (that surrounds a volume of materials). Endocytosis depends on energy to facilitate the uptake process where the invaginated matter is transferred to lysosomes or vesicles. The contents of some vesicles evade the enzymatic digestion and transfer to the basolateral membrane of the cell after which it undergoes exocytosis. The uptake process (endocytosis) can further be categorized into receptor-mediated endocytosis, pinocytosis, transcytosis and phagocytosis (Silverstein *et al.*, 1977:673).

2.2.2.3.1 Pinocytosis

Pinocytosis refers to the vesicular uptake of small particles (such as colloids, lipoproteins and immune complexes), low molecular-weight solutes, fluids, and soluble macromolecules (such as hormones, enzymes, and antibodies). Small particles making up extracellular fluids and the materials named above are interiorized within the membrane vesicles that are then taken up into the epithelial cells (Silverstein *et al.*, 1977:673).

2.2.2.3.2 Receptor-mediated endocytosis

The binding of receptors and appropriate ligands facilitate the formation of ligand-receptor complexes (Ashford, 2007b:283). Binding occurs on the surface of the cell, and this explains why the receptor undergoes a conformational transformation. This change causes the ligand-receptor complexes to cling on the surface of the cell in clusters, after which they invaginate and detach from the membrane forming layered vesicles. The coating of the layered vesicle gets lost upon entering the cell's cytoplasm, allowing the exposed vesicle to release their content to the endosomes. The internalised receptors automatically return to the surface of the cell for additional binding, while the internalised ligand is arranged and transferred to the lysosomes where disintegration takes place (Sato *et al.*, 1996:446).

2.2.2.3.3 Phagocytosis

Phagocytosis represents the process that facilitates the absorption of particles that are comparatively large (typically larger than 500 nm), including viruses (Ball, 2004:76). The absorption mechanism takes place when a part of the plasma membrane resists the surface of the particles, rejecting most and sometimes all the nearby fluids. Phagocytosis explains the

mechanism that facilitates the absorption of some vaccines, including the polio vaccine, from the gastrointestinal tract (Ashford, 2007a:283; Silverstein *et al.*, 1977:673).

2.2.2.3.4 Transcytosis

Transcytosis is defined as an active process that allows for materials such as vitamins, macromolecules and ions to be transported in vesicles from one side of the cell to the other side. Transcytosis is likely to be discriminatively receptor-mediated, but may at times be non-discriminative in the fluid stage of the vesicle (Di Paquale & Chiorini, 2006:506).

2.2.2.4 Paracellular pathway

The paracellular pathway is best conceptualized as the transport route that allows the passage of drug molecules through extracellular, aqueous paths between epithelial cells rather than through cell membranes. This uptake process has three key driving forces causing the movement of molecules through the paracellular route, namely the electrochemical potential gradient, electrical potential and hydrostatic pressure between the two sides of the epithelium (Ashford, 2007a:283).

In general terms, the movement of drug molecules through the intestinal epithelium via the paracellular pathway is minimal because of the existence of the tight junctions between neighbouring cells. Only hydrophilic molecules that are very small are permitted to use the space between adjacent cells as their transport pathway (Liu *et al.*, 2009:267).

Investigations to discover new strategies to enhance the passage of protein and peptide drug molecules through the gastrointestinal tract epithelium are underway. A close evaluation of such strategies groups them into two categories namely controlling the tight junctions associated with the paracellular pathway and physico-chemical transformation of the drug molecule (Salamat-Miller & Johnston, 2005:203).

2.3 LIMITATIONS TO ORAL BIOAVAILABILITY OF PEPTIDE DRUGS

The principle function of the gastrointestinal tract is to ensure that the body gets a sufficient supply of nutrients that are necessary for biological processes. As such, it is more than justifiable to note that the very adaptation and design of the gastro-intestinal tract is to facilitate digestion and absorption of fluids, electrolytes and nutrients from the lumen of the gastro-intestinal tract into the systemic circulation of the body that allows their transportation to

various points of need. However, it should not be ignored that the same tract has multiple responsibilities. For example, efflux (which is the active transport of molecules from the epithelium back into the digestive tract lumen) takes place to prevent the effects that might amount from the uptake of harmful substances. The gastrointestinal tract is therefore well adapted for this function and is committed to protect the body from the systemic attack of dangerous agents such as antigens, toxins and pathogens (Lennernäs, 1998:406).

Factors that may prevent a drug from reaching the systemic circulation include the mucus layer, the varied pH at different points of the digestive tract, digestive enzymes in the gastrointestinal tract lumen, unstirred layer of water, and the tight junctions, while the metabolic and liver enzymes presents additional challenges. All these factors present practical barriers that diminish the bioavailability of drugs (Daugherty & Mrsny, 1999:144 Liu *et al.*, 2009:235). Figure 2.2 is a diagram illustrating the barriers that limit the uptake of particular drugs. Such barriers are categorized into two sets; biochemical barriers and physical barriers.

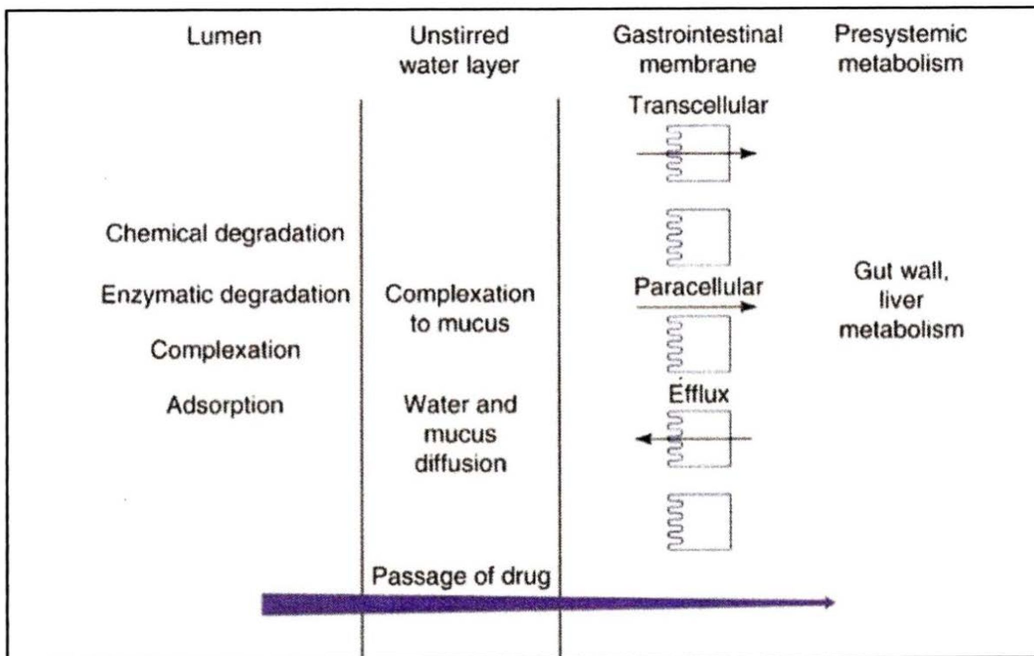

Figure 2.2: Schematic illustration of the factors limiting the uptake of certain drugs. These factors include physical barriers such as the unstirred water layer, the intestinal epithelial cell membrane and tight junctions as well as biochemical barriers such as efflux of drug molecules from the intestine, enzymatic degradation in the digestive tract lumen and pre-systemic metabolism by liver and digestive enzymes (Ashford, 2007: 276)

2.3.1 Physical limitations

The main physical barriers to the absorption/uptake and bioavailability of drug molecules include the unstirred water layer, epithelial cell membrane (for transcellular uptake) as well as the tight junctions (for paracellular uptake) that are positioned between epithelial cells (Hamman *et al.*, 2005:166).

2.3.1.1 Unstirred water layer

The unstirred water layer primarily consists of mucus, water and the glycocalyx, which limit drug molecules from reaching the membrane of the epithelial cells. The mechanical faction of muscles aligning the intestinal tract does not present sufficient mechanical force to facilitate complete mixing of the free contents, which leaves a layer of unstirred water (approximately 30 to 100 um thick) near the surface of the intestinal mucosal layer. Drug molecules must cross this layer to get close enough to the surface of intestinal cells where they can be absorbed (Ashford, 2007b:279; Hamman, 2007:102).

2.3.1.2 Epithelium and cell membranes

Regardless of the fact that the membranes of the epithelial cells are adapted to facilitate the uptake of nutrients, vitamins and other useful substances from the gastro-intestinal lumen, they also act as a barrier that prevents the absorption of certain drug molecules from the gastrointestinal tract. This barrier function is possible since the membrane acts as physical separation that divides the lumen from the systemic circulation (Van de Waterbeemd, Lennernäs, & Artursson, 2003:10).

As Figure 2.3 illustrates, the cell membrane of the epithelium presents a two-layer structure consisting of lipids, proteins, polysaccharides, lipoproteins and carrier molecules/transporter proteins. Like other cell membranes, the cell membrane of epithelial cells is selectively permeable; a feature that allows the selective transportation of molecules that dissolve in lipids. As such, hydrophilic molecules must make use of aqueous pores to be transported across the membrane (Daugherty & Mrsny, 1999:144; Choonara *et al.*, 2014:1269).

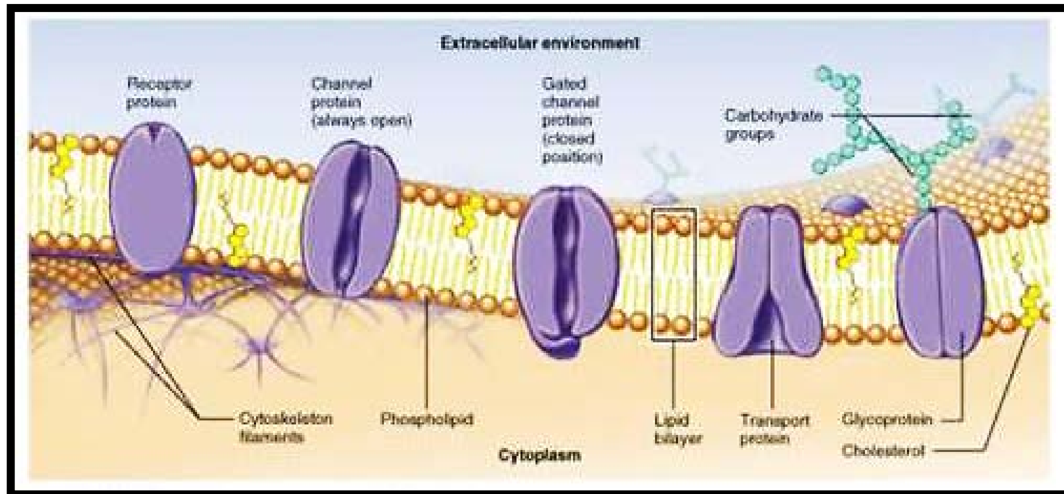

Figure 2.3: Schematic illustration of the structure of a biological cell membrane (Unklab Nursing Portal, 2013)

2.3.1.3 Tight junctions

The lining of the gastro-intestinal tract is made up of a single layer of epithelial cells. Nearby epithelial cells of this monolayer are clustered together by intercellular junction complexes that are classified as follows: the tight junctions (zonula occludens) located closest to the apical side, the adherence junctions (zonula adherens) underlying the tight junctions and the desmosomes (macula adherens) that are located closest to the basolateral side (Van Itallie & Anderson, 2014:157). The tight junction defines the only junctional complex that forms an occluding barrier, which limits the transfer of molecules across the epithelium through the intercellular spaces. Tight junctions are multi-faceted structures consisting of transmembrane proteins that are connected to a cytoplasmic plaque that consists of a complex connection of adaptor and scaffolding proteins, actin-binding cytoskeleton linkers and signalling components (Kosinska & Andlauer, 2013:951; Hamman *et al.*, 2005:169).

The change of movement of ions across the epithelium through intercellular spaces can be determined through a measurement known as the transepithelial electrical resistance (TEER). TEER expresses the level of permeability of the space between adjacent epithelial cells and may be utilized in *in vitro* models in measuring the resistance offered by the tight junction (Salama *et al.*, 2006:15).

2.3.2 Biochemical limitations

In addition to the physical barriers described above, biochemical barriers exist that may diminish the bioavailability of drugs. A tertiary function of the human gastrointestinal tract is to prevent systemic intoxication of the body by harmful agents such as antigens, toxins and pathogens (Aguiar *et al.*, 2005:60). Drug molecules are exposed to different pH environments at diverse points of the gastrointestinal tract and they have to be resilient to these variations to retain their effectiveness and purpose. As drug molecules transit the gastro-intestinal tract, these molecules are at a risk of enzymatic degradation, a condition that persist even after they enter the systemic circulation (Hamman *et al.*, 2005:168; Choonara *et al.*, 2014:1271). The transfer of drug molecules through the gastro-intestinal epithelium into the circulatory system therefore does not mark an end to the factors challenging the bioavailability of these drug molecules. Drug molecules that manage to enter the systemic circulation are exposed to a range of challenges linked to biochemical processes (Krishna & Yu, 2007:256).

2.3.2.1 Efflux pumps

Epithelial cells contain numerous transporter proteins. While some transporter molecules are inherently beneficial in aiding the absorption of drug molecules, it is well founded that specific transporter proteins diminish the bioavailability of specific drug molecules by hindering their uptake. Transporter proteins that obscure the uptake of drug molecules, otherwise termed as P-glycoproteins or counter-transporter efflux proteins, define a set of proteins that function by facilitating the movement of drug molecules from the inner side of the epithelial cells back to the gastrointestinal lumen (Avdeef, 2003:83). P-glycoprotein is an energy-enabled, membrane-sealed protein represented at the top levels of the apical layer that exist at the membrane of the brush border. The same also exists in other tissues and may be found in the liver, blood-brain barrier and kidneys (Anderle, 2009:23).

2.3.2.2 Enzymatic degradation

Enzymatic degradation is one of the most profound barriers to the bioavailability of many drug molecules and is one of the most notable downsides of the oral administration of peptides. Furthermore, enzymatic degradation is very challenging to overcome. This is because of the fact that enzymes are ubiquitous and their degradation action takes place at numerous sites (Krishna & Yu, 2007:256).

The acidic gastric fluid causes the degradation by denaturation of protein molecules (Anderle, 2009:23; Cantor, 1994:95). Additionally, enzymatic actions facilitate the irreversible hydrolytic cleavage of peptide and protein molecules into relatively soluble oligo-peptides and amino acids (Krishna & Yu, 2007:256; Fei *et al.*, 1994:563; Zhou, 1994:239). The chemical breakdown of proteins in the gastrointestinal tract is also stimulated in the presence of pepsin (Lee *et al.*, 2001:573). Enzymes originating from the brush border and those secreted by the pancreas have a major hand in the digestion of peptide and protein molecules into supplementary amino acids (Krishna & Yu, 2007:256; Lee, 2002:572).

2.4 STRATEGIES TO IMPROVE ORAL DRUG DELIVERY

By understanding the factors that may limit or present challenges to the bioavailability of peptide and protein drug molecules, strategies may be deployed in devising enhanced delivery of peptide and protein drugs (Grassi, 2007:576). From a general perspective, such strategies can be divided into two groups including formulation methods and chemical modification methods. Protein therapeutics can be subjected to chemical changes that may be achieved through the synthesis of pro-drugs; structural transformations that target particular receptors or transporters or the preparation of peptidomimetics (Brady, 2005:314). The issue of low bioavailability can also be met through the formulation of novel dosage forms that include the incorporation of absorption enhancers and/or enzyme inhibitors into the drug delivery systems (Liu *et al.*, 2009:267).

2.4.1 Formulation approaches

2.4.1.1 Drug absorption enhancers

The intestinal absorption of peptide and protein molecules can be improved by absorption facilitating agents that operate as functional adjuvants in dosage forms. Permeation enhancers/absorption enhancers can be conceptualized as molecules that circumvent the challenges of the exterior layer of the body tissue in a reversible way and with low tissue injury, thus allowing the drug molecules to pass through the epithelial cells to enter the systemic and lymphatic circulation (Muranishi, 1990:3). Absorption enhancers initiate their drug absorption enhancing function by implementing one or a series of mechanisms including tight junction opening, decreasing the viscosity of the mucus layer and improving membrane fluidity (Choonara *et al.*, 2014:1269; Hamman *et al.*, 2005:168).

Numerous compounds with different chemical characteristics have demonstrated the capacity to improve the intestinal uptake of polypeptide drugs as summarized in Table 2.1.

Table 2.1: A list of chemical compounds with potential to act as intestinal drug absorption enhancers (Hamman, 2007:187)

Absorption enhancer	**Examples**	**Mechanism of action**
Bile salts	Sodium glycocholate, taurocholate, deoxycholate, taurodihydrofusidate	Reduction in mucus viscosity and membrane integrity disruption through phospholipid solubilisation
Fatty acids	Long chain fatty acid esthers (palmitoylcarnitine) and medium chain glycerides	Dilates tight junctions (paracellular) and causes disruption of cell membranes (transcellular)
Surfactants	*Nonionic:* Polysorbate (Tween 80) *Ionic:* Sodium dioctyl sulfosuccinate	Extraction of membrane proteins or lipids causes membrane damage, phospholipid acyl chain perturbation
Chelating agents	Ethylene glycol tetraacetic acid (EGTA), salicylates, Ethylene diamine tetraacetic acid (EDTA), citric acid	Magnesium and calcium complexation which opens tight junctions
Salicylates	Salicylate ion and sodium salicylate	Increases fluidity of cell membranes, prevents protein aggregation or self-association
Complexation agents	Cyclodextrins	Increases the dissolution rate and solubility
Ion pairing	Counterion	Form an ion pair that is more lipophilic which can partition the membrane
Efflux pump inhibitors	First, second and third generation	Blocks the drug binding site on P-gp, interfere with ATP hydrolysis
Anionic polymers	Poly(acrylic acid) derivatives	Enzyme inhibition as well as extracellular calcium depletion
Cationic polymers	Chitosan salts, N-trimethyl chitosan chloride	Ionic interactions with the cell membrane to open tight junctions

The principal consideration for effective drug uptake facilitation by chemical permeation enhancers is ensuring that the drug permeability is predictable, reversible and reproducible. The absorption enhancer should further promote intestinal permeability without risking toxic outcomes (Legen *et al.*, 2005: 184).

2.4.1.1.1 Aloe leaf materials

Aloe vera (L.) Burm.f. (*Aloe barbadensis* Miller) is a plant characterised by leaves containing water storage tissue in order to survive in dry areas. The translucent inner pulp of the leaves consists of a soft tissue with large thin-walled parenchyma cells that contains a viscous mucilage or gel (Hamman, 2008:1600). The gel consists of water (98%) and polysaccharides such as pectin, mannose derivatives, hemicelluloses, cellulose, acemannan and glucomannan. The structure of acemannan can be described as an extended chain of acetylated polymannose and is regarded as the principal functional molecule of the aloe leaf gel (Chen *et al.*, 2009:588).

The outcomes of *A. vera* juice on oral bioavailability of vitamins C and E in humans was studied by Vinston, Kharrat and Andreoli, (2005:760) by means of a double-blind, randomised clinical trial. The bioavailability of vitamin C was 3 times higher when administered with *A. vera* juice compared to the control, and the level of the vitamin continued to be higher than the baseline even after 24 hours ($p < 0.05$). The bioavailability of vitamin E was approximately 3.7 times higher when the vitamin was co-administered with the *A. vera* juice than when it was taken alone (control). An *in vitro* study demonstrated that *A. vera* gel notably lowered the transepithelial electrical resistance (TEER) of the epithelial cell monolayer (Caco-cell line). The ability of *A. vera* to reduce TEER in the intestinal epithelial cells indicated the loosening of the tight junction between nearby epithelial cells. Furthermore, *A. vera* gel notably improved the transfer of insulin across Caco-2 cell monolayers (Chen *et al.*, 2009:587).

Efforts to delineate the absorption enhancement properties of aloe leaf materials included experiments where the *in vitro* transport enhancement capacity of the gel components of three aloe species namely *Aloe ferox*, *Aloe speciosa*, and *Aloe morlothii* were evaluated using excised rat tissues as well as Caco-2 cell monolayers. The gel components demonstrated the potential to improve the uptake of several model compounds as well as lowering the TEER (Beneke *et al.,* 2012:475; Lebitsa *et al.*, 2012:297).

2.4.1.1.2 Chitosan and derivatives

Chitosan is a ß-(1,4) connected carbohydrate polymer of 2-amino-2-deoxy-D-glucose and is developed through the deacetylation of chitin, the most copious natural polymer after cellulose. Chitosan is inherently a biocompatible and non-toxic polymer that can improve the paracellular permeability of peptide drug molecules across the mucosal epithelium, thereby functioning like an absorption facilitator of hydrophilic macromolecular model complexes such as buserelin and insulin (Thanou *et al.*, 2001:117; Thanou *et al.*, 1999:74). Chitosan's low effectiveness as small intestinal drug uptake enhancer and low solubility at neutral and alkaline pH environments have triggered the synthesis of chitosan derivatives, including N-trimethyl chitosan chloride (TMC) (Thanou *et al.*, 2001:117).

2.4.1.2 Polymeric hydrogels

Natural polymers have been studied for use in potential carrier delivery systems for the delivery of a variety of drugs including protein and peptide therapeutics, as they are inherently non-toxic and biocompatible (Peppas *et al.*, 2000:27). For example, microparticles that consist of poly(methacrylic acid) grafted with poly(ethylene glycol) proved to improve bioavailability and protect insulin in the gastrointestinal tract (Yamagata *et al.*, 2006, 343; Ichikawa & Peppas, 2003:609).

2.4.1.3 Muco-adhesive systems

Bio-adhesion explains the extended connection between drug delivery systems and the gastrointestinal mucosa. Two terms that are commonly used to describe bio-adhesion include "cyto-adhesion" (connection between an adhesive agent and the surface of the cell) and "muco-adhesion" (the connection between the drug delivery system and the mucus layer) (Rekha & Sharma, 2013:54). The creation of bio-adhesive drug delivery systems aims at prolonging the intestinal transit time by slowing down the movement of the delivery system through the gastro-intestinal tract by adhering to the mucosa. This enhanced contact with the mucosa causes a high drug concentration gradient and localizing the delivery of the drug at a specific site (Peppas, 2004:11).

In a previous study, a muco-adhesive hydrogel shuttle drug delivery system was developed for effective gastrointestinal delivery of peptide and protein drugs. This system included drug absorption enhancers as well as enzyme inhibitors, which were intended to be released first

(first phase) followed by the protein drug (second phase) as illustrated in Figure 2.4. The system was designed to swell and adhere to the wall of the small intestine after which the double phase release was supposed to occur. This drug delivery system managed to protect the drug from proteolytic enzymes, while being dependent on environmental pH for drug release (Dorkoosh *et al.,* 2001:11). The drug delivery system developed in this study was based on this principle, however, instead of a swellable shuttle system a multiple-unit drug delivery system was designed.

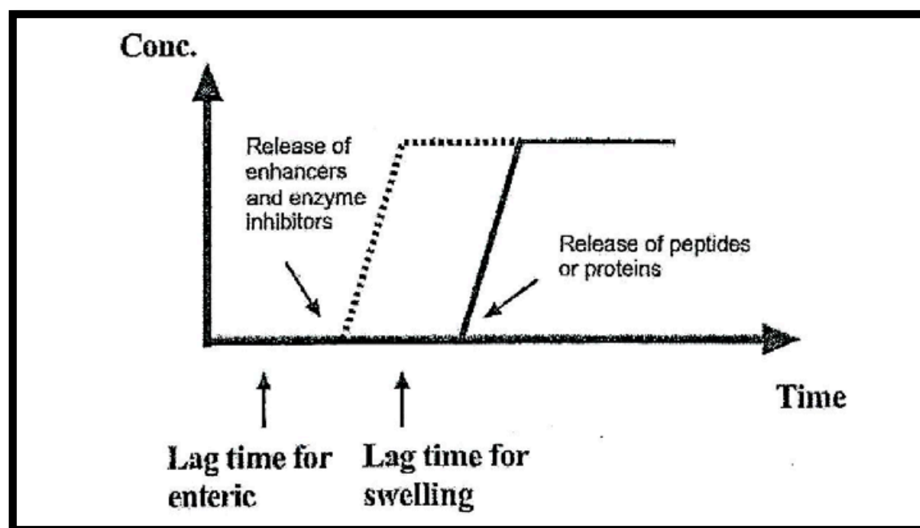

Figure 2.4: Graph illustrating a double phase time controlled release profile as theoretically expected from a polymeric hydrogel shuttle system (Dorkoosh *et al*., 2001:11)

2.4.1.4 Particulate and nanoscale technologies

The application of colloidal polymeric particulate drug delivery systems has demonstrated the potential to reduce the challenges associated with the oral delivery of these drugs. The majority of particle carrier systems for peptide and protein drug uptake such as nanoparticles, emulsions, liposomes, and microspheres have been applied to defend proteins against the enzymes and acidic medium found in the gastrointestinal tract lumen and controlling the rate of drug release (Hildalgo, 2001, p. 388). Numerous illustrations of polymeric systems for enhancing drug absorption exist in literature, one demonstrating that a liposomal system consisting of sodium taurocholate and insulin has a significant potential to reduce the level of blood sugar upon oral administration. The same formula demonstrated a significant *in vivo*/*in vitro* correlation (Petrus, Fairchild & Doyle, 2009:37).

Nanoparticle-based oral drug delivery systems for peptide and protein drugs have proven to be useful in efforts to enhance bioavailability because they can provide a shielding effect against biochemical degradation. An oral peptide and protein drug formulation was designed by structuring caseins surrounding PEG-insulin nanoparticles. Casein provides muco-adhesive characteristics as well as a defence mechanism against the acidic environment in the stomach. For example, administering this dosage form directly into the gastrointestinal tract (stomach) of fasted diabetic mice reduced the glucose level by 80% in the first hour, and enhanced the half-life of insulin, which enhanced therapeutic action (Barrett & Donowitz, 2001:11; Chitchumroonchokchai, 2004:23).

2.4.1.5 Enzyme Inhibitors

Enzyme inhibitors (such as aprotinin (inhibiting chymotrypsin and trypsin), FK448 (inhibiting chy-381 motrypsin), soybean trypsin inhibitor (inhibiting pancreatic endopeptidases), and chicken ovomucoid (inhibiting trypsin) can influence protein and peptide drug bioavailability by decreasing the activities of the protein degradation enzymes. Unfortunately, the deployment of enzyme inhibitors remains questionable bearing in mind the possible feedback-controlled protease secretion, adverse outcomes, the breakdown of dietary proteins, and intestinal mucosal damage. A possibility to overcome these undesirable outcomes include the application of delivery systems that offer simultaneous discharge of the inhibitor and the drug while limiting their concentration in a localized area, controlling the movement of the inhibitor out of the delivery system or ensuring that a close contact exists between the mucosa and the delivery system (Park & Mrsny, 2000:32; Tillement, 2006:695; Kerns & Di, 2008:86; Náray-Szabó, 2014:254).

2.4.2 Chemical modifications

Chemical methods to improve the bioavailability of peptide and protein drugs include pro-drug strategies, structural transformations, peptidomimetics, lipidisation, PEGylation, amino acid substitution and targeting membrane receptors and transporters (Anderle, 2009:23).

2.4.2.1 Pro-drugs

A pro-drug entails a pharmacological inert compound that needs biotransformation to turn into the pharmacologically active entity (Krishna & Yu, 2007:256). The majority of pro-drug methods used for drugs focus on changing one functional group (Anderle, 2009:23). Pro-drugs

focusing on membrane transporters are chemically designed to become substrates for membrane transporters; a feature that facilitates their uptake (Krishna & Yu, L 2007:256). The pro-drug gets transferred across the epithelial membrane and may reach the systemic circulation in its original state (after which it undergoes biotransformation to the active drug) or can immediately be subjected to enzymatic hydrolysis in the intracellular surrounding (after which it is released as active drug in the systemic circulation) as illustrated in Figure 2.5 (Brady, 2005:314; Herkenne, 2005:268).

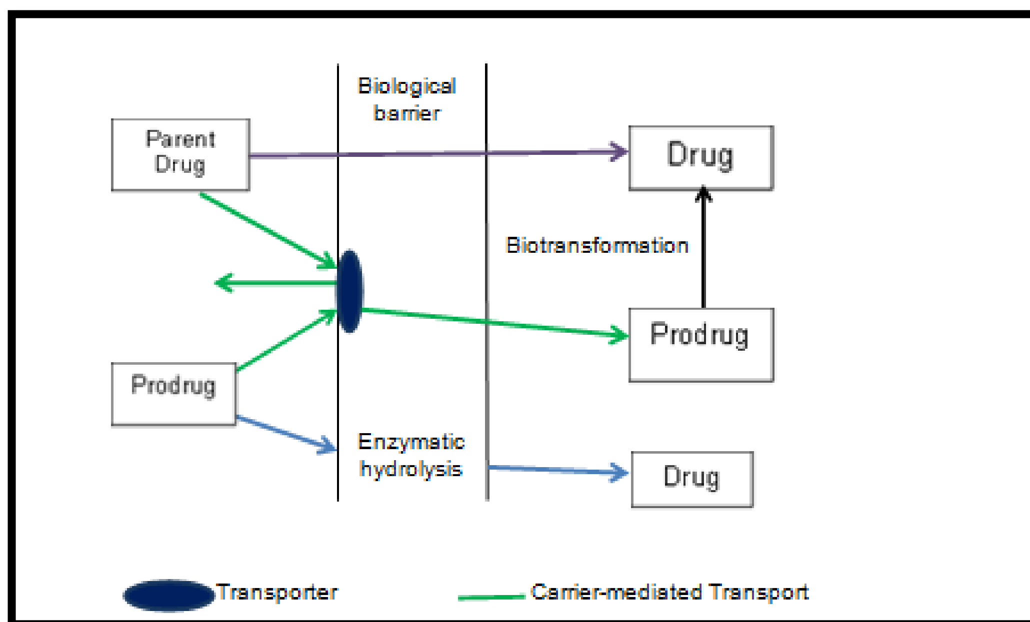

Figure 2.5: Schematic illustration of the pro-drug approach (Majumdar *et al.*, 2004:1439)

2.4.2.2 Amino acid substitution

Chemical transformation of protein and peptide drugs that depends on amino acid substitution can be attained by using an alternative amino acid or replacing the D-amino acid with the L-amino acid or by changing the sequence of amino acids (Krishna & Yu, 2007:256).

2.4.2.3 Lipidisation

Lipidisation occurs by conjugating a fatty acid onto the peptide or protein molecule, which improves the bioavailability of the macromolecule by enhancing its lipophilicity and therefore also its diffusion across biological membranes (Anderle, 2009:23).

2.4.2.4 Polyethylene glycolation (PEGylation)

Polyethylene glycol (PEG) refers to a biocompatible and non-toxic polymer that can dissolve in aqueous and organic solvents. The pharmacokinetic characteristics of peptide and protein drugs can be enhanced through covalent linking to PEG, which is known as PEGylation (Chitchumroonchokchai, 2004:23; Mathiowitz, Chickering & Lehr, 2009:53). PEGylation is renowned for its benefits in boosting the *in vivo* circulation half-life of peptides and proteins by preventing them from breakdown, reducing their renal disposal and enhancing their physico-chemical characteristics (Chitchumroonchokchai, 2004:23). PEGylation has become an advanced field of chemical modifications of peptide molecules and multifaceted strategies exist to link PEG to the macromolecule as illustrated in Figure 2.6 below.

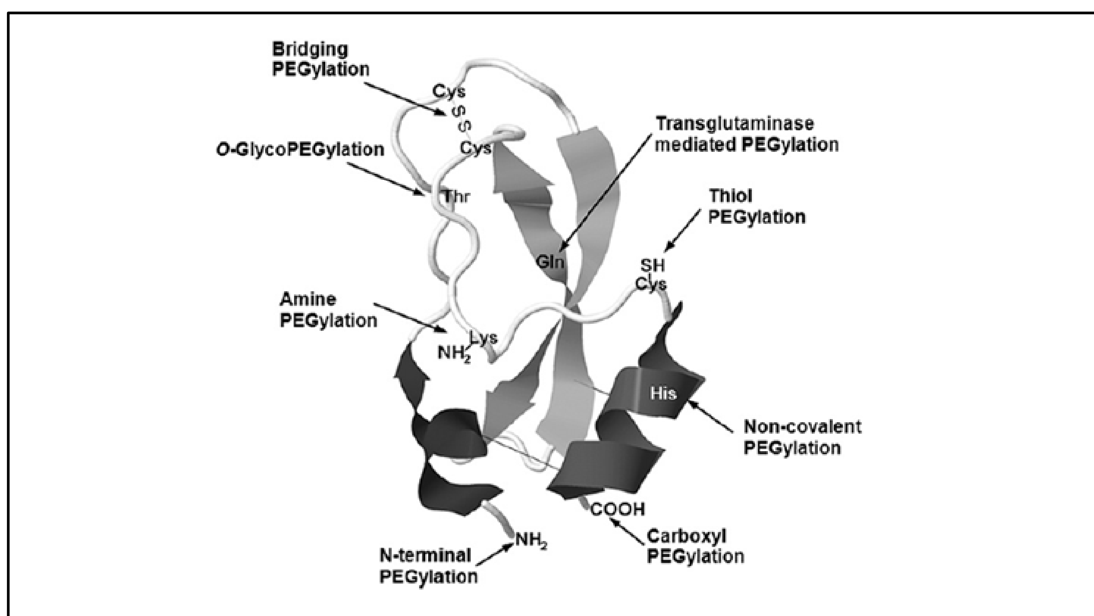

Figure 2.6: The illustration of diverse PEGylation strategies (Pfister & Morbidelli, 2014:137)

2.5 SUMMARY

The oral route would be a more preferable option for the delivery of protein and peptide drugs, but poor bioavailability and pre-systemic degradation are important challenges that need to be addressed when formulating protein and peptides in an oral dosage form. Protein and peptide drugs encounter physical barriers after oral administration, including the unstirred water layer, the intestinal epithelium itself, tight junctions and efflux systems. The oral delivery of protein and peptide drugs is also pH dependent and susceptible to biochemical degradation due to enzymes and micro-organisms present in the gastro-intestinal tract.

Approaches that have been studied to overcome the above mentioned challenges include chemical modifications and formulation technologies. The use of absorption enhancers is an important formulation strategy to enhance protein and peptide transport across the gastro-intestinal tract epithelium. A double phase drug delivery system consisting of a drug absorption enhancer and a peptide drug combined into one swellable solid oral dosage form exhibited successful protein delivery in a previous study. Such a system aims to release the drug absorption enhancer first to overcome the absorption barrier and thereafter the peptide drug is released and subsequently absorbed.

CHAPTER 3
MATERIALS AND METHODS

3.1 INTRODUCTION

To achieve the aim of this study, two types of beads were formulated that could be mixed and loaded into hard gelatine capsules. This hard gelatine capsule filled with spherical beads was developed in such a way as to fulfil the requirements of a double phase drug delivery system for effective oral delivery of insulin. The idea was that the beads containing the drug absorption enhancing agent will move immediately after administration to the small intestine in order to open the tight junctions between epithelial cells. The beads containing insulin will follow at a slower rate due to the presence of a muco-adhesive agent. Once the insulin containing beads reach the site of absorption, delivery of insulin into the blood stream will be facilitated via the paracellular route due to the already opened tight junctions.

In order to test the drug delivery potential of this double phase dosage form, an *in vitro* permeation technique was used where excised pig intestinal tissue was mounted in a diffusion apparatus. The two types of beads were applied consecutively after each other to the excised pig intestinal tissues (therefore separated from each other and not as a single unit loaded into hard gelatine capsules) to mimic the *in vivo* situation more realistically. The *in vitro* technique also faced limitations in terms of the use of uncoated beads only, since coated beads would release the insulin at a slower rate or after a delayed period of time, while the excised tissue has a limited viability period after removal from the animal. This problem would not exist *in vivo* as the beads will first move through the stomach (which takes approximately 2 h).

Different types of bead formulations were designed; the one type contained a drug absorption enhancing agent (each formulation contained one of four selected drug absorption enhancing agents), whereas another type of bead formulation contained insulin as active ingredient and chitosan as muco-adhesive agent. The bead formulations were evaluated in terms of physical properties including friability, mass variation, particle size distribution, muco-adhesion and dissolution. Double phase dosage forms were designed by loading a mixture of beads (half contained a drug absorption enhancing agent and the other half contained insulin together with chitosan) into hard gelatine capsules.

3.2 MATERIALS

3.2.1 Materials used for formulating the beads

- *Aloe vera* gel powder and *Aloe vera* whole leaf was sourced from Warren Chem Pharmaceuticals (Pty) (LTD) (Johannesburg, South Africa).
- Insulin, bile salt mixture (50% sodium salt of cholic acid and 50% sodium salt of deoxycholic acid) and sodium glycocholate were purchased from Sigma Aldrich (Johannesburg, South Africa).
- Chitosan was purchased from Warren Chem Pharmaceuticals (Pty) (LTD) (Johannesburg, South Africa).
- MicroceLac®100 (co-processed excipient consisting of lactose and microcrystalline cellulose) was obtained from Meggle (Wasserburg, Germany).
- Ethanol was purchased from Rochelle Chemicals (Johannesburg, South Africa).
- Glacial acetic acid, acetone and isopropanol were purchased from Associated Chemical Enterprises (Johannesburg, South Africa).
- Ac-di-sol® (Crosscarmellose Sodium) was obtained from BASF (Midrand, South Africa).
- Eudragit® S100 and Eudragit® L100 were obtained from Evonik Industries (Midrand, South Africa).
- Triethyl citrate was purchased from Sigma Aldrich (Johannesburg, South Africa).

3.2.2 Materials used in the transepithelial electrical resistance and transport studies

- Krebs-Ringer bicarbonate (KRB) buffer was purchased from Sigma-Aldrich (Johannesburg, South Africa).
- Sodium bicarbonate was purchased from Sigma Aldrich (Johannesburg, South Africa).
- Porcine proximal jejunum tissue was collected from the local abattoir (Potchefstroom, South Africa).

3.2.3 Materials used in dissolution studies

- Hydrochloric acid was purchased from Associated Chemical Enterprises (Johannesburg, South Africa).

3.3 FORMULATION AND PREPERATION OF BEADS

3.3.1 Preparation of beads containing absorption enhancing materials

An extrusion-spheronisation technique was used for the preparation of the different bead formulations, each containing a different absorption enhancing agent as shown in Table 3.1. Weighed amounts of all the dry powder ingredients namely 10% w/w of each absorption enhancer, which included aloe materials (i.e. *A. vera* gel and *A. vera* whole leaf); sodium glycocholate and bile salt mixture (50% cholic acid sodium salt and 50% deoxycholic acid sodium salt) together with 2% w/w Ac-di-sol® and MicroceLac® 100 were mixed in a Turbula® mixer (Willy. A. Bachofen, Switzerland) for 5 min at 69 rpm for each bead formulation.

Table 3.1: Composition of bead formulations containing different drug absorption enhancing agents

Absorption enhancer (10% w/w)	Disintegrant (2% w/w)	Filler material (qs)
A. vera gel	Ac-di-sol®	MicroceLac® 100
A. vera whole leaf	Ac-di-sol®	MicroceLac® 100
Sodium glycocholate	Ac-di-sol®	MicroceLac® 100
Bile salt mixture (50% sodium cholic acid salt and 50% sodium deoxycholic acid salt)	Ac-di-sol®	MicroceLac® 100

The total weight of the powder mixture for each bead formulation was 100 g. A volume of 50 ml of 20% v/v ethanol was slowly added to the powder mixture of each bead formulation while blending the powder mass in a mortar with a pestle. The wetted powder mass of each bead formulation was passed through a 1 mm extrusion screen (Type 20 Caleva® extruder, Caleva Process Solutions, England) at a speed of 25 rpm to form spaghetti-like extrudates. This was followed by spheronisation of the extrudate using a Caleva® spheroniser apparatus (Caleva Process Solutions, England) at 1200 rpm for 6 min to form spherical beads. The beads were lyophilised by first freezing the beads in a -80°C freezer and then drying them under vacuum (Virtis, Gardiner N.Y. USA) for up to 48 h.

3.3.2 Preparation of beads containing insulin and chitosan

Beads containing insulin as the active ingredient and chitosan as muco-adhesive agent was prepared in a similar manner as described for the beads containing the absorption enhancers. In this case, the bead formulation consisted of 0.1% w/w insulin, 15% w/w chitosan , 2% w/w Ac-di-sol® (disintegrant) and MicroceLac®100 (filler). The dry powders were weighed (to make a total of 100 g) and mixed in a Turbula® mixer for 5 min at 69 rpm.

A mixture of de-ionised water, 20% v/v ethanol and 2% v/v glacial acetic acid (50 ml) was added to this powder mixture, while blending the powder mass in a mortar with a pestle. The wetted powder mass was passed through the 1 mm extrusion screen (Type 20 Caleva® extruder, Caleva Process Solutions, England) at a speed of 25 rpm followed by spheronisation of the extrudate (Caleva® spheroniser, Caleva Process Solutions, England) at 1800 rpm for 12 min to form spherical beads. The beads were lyophilised by first freezing the beads (- 80°C) and then drying them under vacuum (Virtis, Gardiner N.Y. USA) for up to 48 h.

3.3.3 Film coating of beads containing insulin

3.3.3.1 Coating formulation

A suspension for film coating of the insulin containing beads was prepared using the formulation as shown in Table 3.2.

The Eudragit® S100 and Eudragit® L100 powders were added slowly into half of the diluent mixture (i.e. acetone, isopropanol and water) and this mixture was stirred with a high shear mixer until the polymers were completely dissolved. The talc and triethyl citrate was added to the remaining half of the diluent mixture, which was stirred for 10 min with a high shear mixer. This mixture was slowly poured into the Eudragit® solution while stirring with a conventional stirrer. The coating suspension was passed through a 0.5 mm sieve before it was sprayed onto the beads in a coating pan.

Table 3.2: Ingredients used to prepare the suspension for film coating of the beads

Ingredient	**Function**	**Quantity**	**% w/w**
Eudragit® S100	Polymer	15.65 g	3.13
Eudragit® L100	Polymer	15.65 g	3.13
Triethyl citrate	Plasticizer	3.15 g	0.63
Talc	Anti-tacking	15.65 g	3.13
Acetone	Diluent	171.5 g	34.3
Isopropanol	Diluent	257 g	51.4
Water	Diluent	21.4 g	4.28
Total		500 g	100

3.3.3.2 Spray coating process

The insulin containing beads (30 g) were coated with the Eudragit® suspension using a pan-coater (Associated Electrical Industries Pty Ltd, SA). The beads were added to the drum of the pan-coater which was rotated at a speed of 8 rpm and then sprayed with the coating suspension using a spray gun with a 1.2 mm nozzle at a distance of 15 cm from the beads. The beads were coated at a spray rate of approximately 3 ml/min for 80 min, while simultaneously drying the beads by means of an inlet air temperature of approximately 40°C.

3.4 EVALUATION OF THE BEAD FORMULATIONS

3.4.1 Assay

The insulin content of a sample (1 g) of each insulin bead formulation was determined by means of high performance liquid chromatography (HPLC). The bead sample was crushed using a mortar and pestle and transferred to a volumetric flask, which was made up to volume (100 ml) with distilled water. The insulin quantity that was present in the solution was used to obtain the experimental value of insulin in the beads. This experimental value was compared to that of the theoretical value in order to express the insulin content as a percentage of the

dose that was intended to be contained in the dosage form. The percentage insulin content was calculated with the following equation:

$$\% \text{ Content} = \frac{\text{(experimental value of insulin content)}}{\text{(theoretical value of insulin content)}} \times 100 \qquad \text{(Equation 3.1)}$$

3.4.2 Mass variation

Ten hard gelatine capsules (size 0) were individually filled (by hand) with beads taken randomly from each bead formulation and the contents of each capsule was weighed. The mass of the beads in each capsule was compared to that of the average mass. The mass variation of capsules containing beads (uncoated, single–dose) weighing more than 300 mg should not have a percentage deviation of more than 7.5% from the average (USP, 2014:492).

Since an assay of drug content was done on the beads, mass variation would indicate the distribution of insulin in the different capsules. An acceptable mass variation will therefore indicate an acceptable dose variation in the final product.

3.4.3 Friability

Friability is the reduction in mass of solid dosage forms (e.g. beads) when they are subjected to mechanical strains during handling and packaging, which can be mimicked by tumbling produced by a friability tester. Tumbling of the beads may cause abrasion, deformation or breakage and indicate their ability to withstand physical strain (BP, 2013: XVII).

A sample of beads (3 g) from each formulation was placed in a friability tester (NWU, Potchefstroom, South Africa) along with 25 glass beads (diameter of 5 mm). The friability tester was operated at 25 rpm for 4 min to apply 100 revolutions to each test sample. The content was emptied onto a 425 µm sieve, the glass beads were removed and smaller powder particles were allowed to pass through the sieve prior to weighing the beads. Friability (F) was determined by calculating the percentage loss in mass according to the following equation:

$$F = \frac{W_1 - W_2}{W_1} \times 100 \qquad \text{(Equation 3.2)}$$

Where W_1 is the initial mass of the beads and W_2 is the mass of the same beads after they were exposed to the friability test. The friability of each formulation of beads was assessed in triplicate (BP, 2013: XVII).

3.4.4 Particle size analysis

Laser light diffraction is one technique that can be used to determine the particle size distribution of solid particles. A representative sample is dispersed at an adequate concentration in a suitable liquid, which is passed through a beam of monochromatic light. A multi-element detector then measures the light scattering caused by the particles (e.g. beads) at various angles. Further analysis is done on the numerical values collected by the detectors through the use of mathematical algorithms and an appropriate optical model. These calculations then provide the proportion of total volume to a number of size ranges, which forms a volumetric particle-size distribution (BP, 2013: XVII).

The size and size distribution of the beads from each formulation was determined by a Malvern® Mastersizer 2000 (Malvern Instruments Ltd. Worcestershire, UK) fitted with a Hydro 2000MU sample dispersion unit. A volume of 600 ml ethanol was used to flush the system and to align the optics within the apparatus and it was also used as the liquid dispersant for each bead sample. The Mastersizer software was used to capture and process the data to obtain the mean particle size and particle size distribution.

3.4.5 Drug release from the bead formulation

Dissolution of insulin from the different bead formulations was conducted in 0.1 M HCl at pH 1.3 with the paddle method in a six vessel dissolution apparatus (Distek 2500 dissolution apparatus, North Brunswick, NJ, USA). The stirring rate was set at 150 rpm in 200 ml dissolution medium and the temperature was maintained at 37 ± 0.5°C. Dissolution of insulin from the coated bead formulation as well as the uncoated bead formulation was evaluated in triplicate by means of placing one capsule of each formulation in three individual dissolution vessels, respectively.

Samples (200 µl) were withdrawn from each dissolution vessel using a pipette at time intervals of 5, 12, 21, 30, 60, 90 and 120 min, which were each immediately replaced with 200 µl of fresh dissolution medium. The samples obtained from the dissolution study were analysed with an HPLC method to determine the amount of insulin in the dissolution medium at each time point. This was done to evaluate the efficacy of the film coating in delaying insulin release in an acidic environment that represents the stomach environment that the formulation will encounter in the *in vivo* situation.

3.4.6 Muco-adhesion

The muco-adhesive properties of the bead formulations were evaluated by a method adapted from the "falling liquid film method" and the "adhesion number" measurement (Vasir *et al.*, 2003:28). This is done by placing 1 g of each bead formulation onto a piece of excised pig proximal jejunum. The jejenum tissue was cut open and mounted onto a channel which was set at a 15° slope as illustrated in Figure 3.1. Distilled water (1000 ml) was allowed to flow from a separating funnel over the beads placed on the tissue, which caused some beads to wash off and accumulate in a beaker. The beads remaining on the intestine was scraped off and placed onto a 425 µm sieve and rinsed with distilled water to remove any mucus from the beads. The beads were then dried in a conventional oven at 40°C for 60 min to remove any moisture which would affect accurate weight measurements.

The percentage of the beads retained on the intestine was then calculated using the following equation, which was used as an indication of muco-adhesion:

$$\%\ \text{mucoadhesion} = \frac{\text{Mass beads retained}}{\text{Total bead mass}} \times 100 \qquad \text{(Equation 3.3)}$$

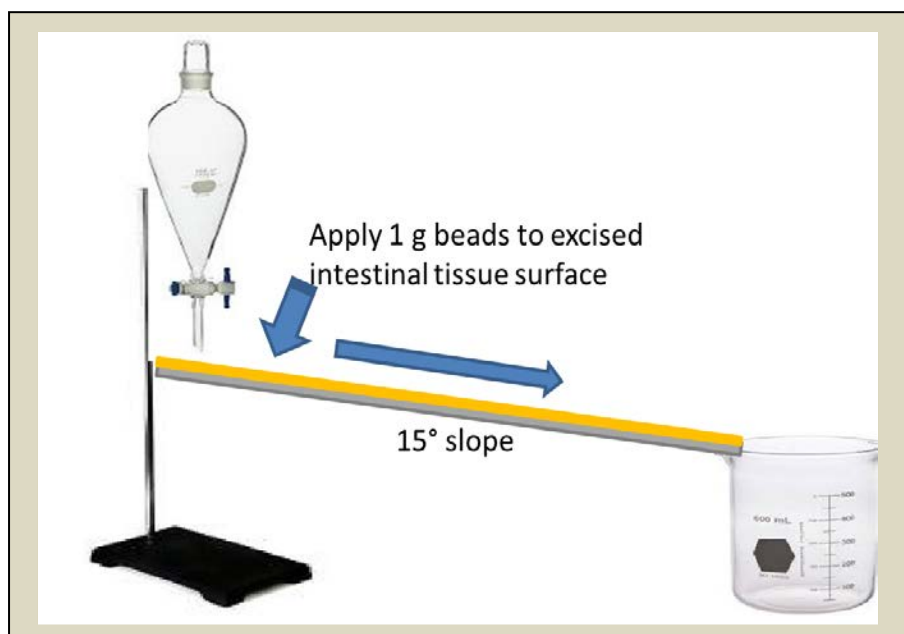

Figure 3.1: Schematic illustration of the apparatus used to measure the muco-adhesive properties of the beads

3.5 TRANS-EPITHELIAL ELECTRICAL RESISTANCE AND TRANSPORT STUDIES

3.5.1 Preparation and mounting of excised porcine intestinal tissue on half-cells of the Sweetana-Grass diffusion apparatus

Approval from the Animal Ethics Committee at North-West University was obtained for the use of pig intestinal tissue (ethics approval number: NWU-00025-15-A5). Directly after pigs were slaughtered (Potch Abattoir, Potchefstroom, South Africa), a piece of approximately 30 cm of proximal jejunum tissue was collected from the gastro-intestinal tract. The tissue was rinsed with ice cold Krebs-Ringer bicarbonate buffer (KRB) and transported in cold KRB buffer in a cooler box to the laboratory.

In the laboratory, the jejunum was pulled onto a glass rod (Figure 3.2, A) where it was kept moist by applying KRB buffer. The serosa was removed by blunt dissection (Figure 3.2, B) and the tissue was cut along the mesenteric border with a scalpel blade. A piece of heavy duty filter paper was placed on a Perspex® plate positioned on ice. The tissue was washed from the glass rod onto the filter paper using cold KRB buffer (Figure 3.2, C & D). The tissue and filter paper were cut into approximately 2 cm pieces (Figure 3.2, E), while keeping the tissue moist with KRB buffer. These segments of tissue were mounted onto the half-cells of the Sweetana-Grass diffusion apparatus and the filter paper was removed (Figure 3.2, F). The two half-cells were clamped together with metal rings (Figure 3.2, G) and these combined chambers were placed into a heating block and filled with 7 ml pre-heated (37°C) KRB buffer. The half-cells of the chambers were connected to parallel gas flow (5% CO_2; 95%O_2) with a flow rate of 15-20 ml/min (Figure 3.2, H). The assembled cells were left for 20 min to reach an equilibrated state before transport studies commenced.

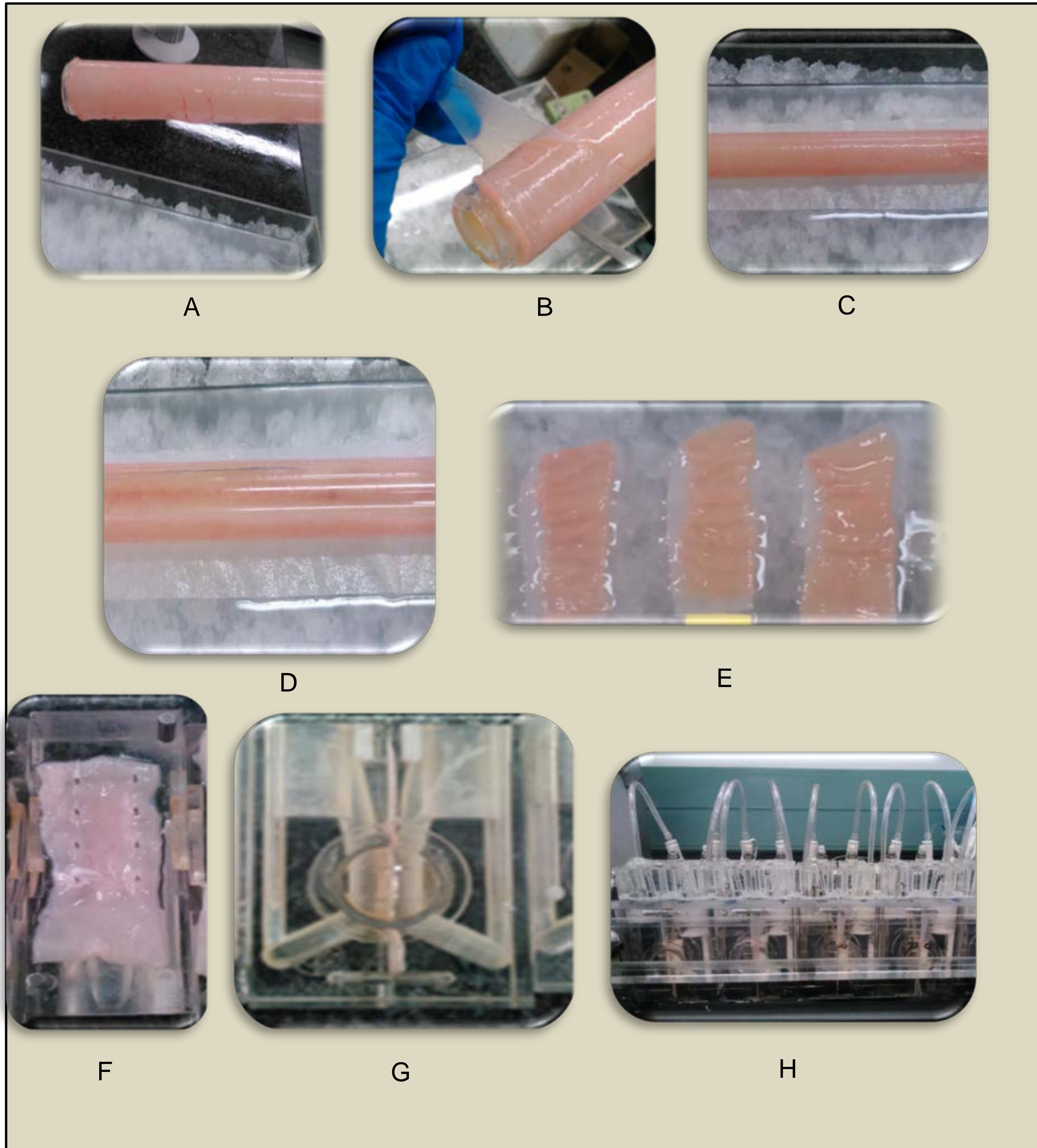

Figure 3.2: Images (A-H) illustrating the preparation and mounting of the pig jejunum on the Sweetana-Grass diffusion chamber. A: excised pig jejunum on glass rod, B: removal of serosa, C: rod with tissue placed on Perspex® plate, D: jejunum cut open, E: jejunum together with filter paper cut into rectangular pieces, F: jejunum mounted on half-cell, G: half-cells clamped together, H: chambers in heat block

3.5.2 Trans-epithelial electrical resistance (TEER) study

3.5.2.1 Measurement of TEER

The TEER experiments were performed on excised pig intestinal tissue when exposed to the four different bead formulations which each contained a different absorption enhancing agent (i.e. *Aloe vera* gel, *Aloe vera* whole leaf, sodium glycocholate and a bile salt mixture). A control group containing no absorption enhancing agent (i.e. beads consisting of MicroceLac®100 only) was also included.

The TEER of the excised porcine intestinal tissues mounted in the Sweetana-Grass diffusion chamber was measured using a Dual Channel Epithelial Voltage Clamp (Warner Instruments, Hamden, Connecticut, USA). An initial TEER measurement was taken directly before adding the beads (0.78 g) to the apical chamber, followed by a measurement every 20 min over a period of 2 h.

The percentage reduction in TEER was calculated by the following equation:

$$\%\ \text{TEER reduction} = \left(\frac{\text{TEER value at specific time interval}}{\text{Initial TEER value}}\right) \times 100 \qquad \text{(Equation 3.4)}$$

This percentage TEER reduction expresses the extent to which each bead formulation opened the tight junctions between the epithelial cells of the intestinal tissue.

3.5.3 *In vitro* transport studies

3.5.3.1 Insulin transport across excised pig intestinal tissue

The transport of insulin was determined across excised pig intestinal tissue after exposure of the tissues to beads in a specific order. Firstly, the intestinal tissue was exposed to beads containing an absorption enhancer suspended in the transport medium on the apical side for 60 min. After this initial 60 min period, the intestinal tissue was exposed to beads containing insulin and chitosan suspended in the transport medium on the apical side for 120 min. The absorption enhancer containing beads were completely removed from the chamber before adding the beads containing insulin and chitosan. Another transport study was conducted where the tissue was exposed to beads containing insulin and chitosan without initial exposure to an absorption enhancer containing bead formulation. This was done to determine if the inclusion of chitosan as muco-adhesive agent contributed to insulin transport enhancement

across the intestinal tissue. Insulin dissolved in KRB buffer served as a control group to determine if the insulin transport in the experimental groups were enhanced after pre-exposure to beads containing absorption enhancing agents.

A sufficient quantity of the insulin containing bead formulation was added to the apical chamber to provide an approximate concentration of 0.1 mg/ml insulin. Samples (200 µl) were withdrawn from the basolateral chamber every 20 min over a 2 h period and replaced immediately after every withdrawal with 200 µl pre-heated (37°C) KRB buffer. The insulin concentration in the transport samples was determined by means of a validated high performance liquid chromatography (HPLC) method.

The percentage insulin transported across the excised intestinal tissue was plotted as a function of time. Apparent permeability coefficient (P_{app}) values were calculated from these graphs using the following equation (Hellum & Nilsen, 2008:468; Hansen & Nilsen, 2009:88):

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A.C_0.60} \qquad \text{(Equation 3.4)}$$

Where P_{app} represents the apparent permeability coefficient (cm/s), dQ/dt ($\mu g.s^{-1}$) represents the increase in the amount of drug in the receiver chamber within a given time period, which is equivalent to the slope of the plot of drug concentration transported versus time. A (cm^2) represents the effective surface area of the excised pig intestinal tissue between the apical and basolateral chambers and C_o is the initial insulin concentration in the apical chamber ($\mu g.cm^{-3}$).

3.5.4 Statistical analysis

Data analyses on the transport results were performed with STATISTICA Ver 12. ANOVA's with Tukey's Honest significant post-hoc tests were performed and statistically significant differences were accepted when $p < 0.05$. All results were verified with non-parametric Kruskall-Wallis and Dunn's post-hoc tests.

3.5.5 High-performance liquid chromatography analysis of insulin

An HPLC analysis method (previously developed in the Analytical Technology Laboratory of Pharmacen, North-West University, Potchefstroom, South Africa) was validated as described below and used to analyse the dissolution and transport samples for insulin concentration.

3.5.5.1 Chromatographic conditions

HPLC is a multi-stage separation method where molecules of the analyte in solution are distributed between a stationary and a mobile phase and the analyte molecules that are eluded can be detected by a variety of techniques such as Ultra violet light absorbance. The stationary phase is packed in a column and the mobile phase is a liquid forced through the column under high pressure (USP, 2014:6378-6379). The chromatographic conditions used to analyse the insulin in this study are summarised in Table 3.3.

Table 3.3: Summary of the chromatographic conditions used to analyse the insulin

Parameters	Description
Analytical instrument	HP1100 series HPLC equipped with a pump, auto-sampler, UV detector and Chemstation Rev. A.10.01 data acquisition and analysis software.
Column	Vydac C18 Protein and peptide column, 218TP54, 300 Å, 250 x 4.6 mm (Grace Vydac, Hesperia, CA).
Mobile phase	Phase A: Degassed mixture of HPLC grade water and 0.1% orthophosphoric acid. Phase B: Acetonitrile
Flow rate	1.0 ml/min
Injection volume	50 µl
Detection	UV absorbance at 210 nm
Retention time	5.87 min
Stop time	12 min
Solvent	HPLC grade water

The mobile phase consisted of two components and was applied by means of a gradient, as shown in Table 3.4.

Table 3.4: Gradient conditions for the mobile phase used in the analytical method

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

3.5.5.2 Standard solution preparation

Approximately 5 mg of human recombinant insulin was accurately weighed and dissolved in 50 ml HPLC grade water. This standard insulin solution was transferred into an HPLC vial and volumes of 10, 20, 30, 40 and 50 µl were injected into the HPLC. This was repeated each time an HPLC analysis of dissolution and transport samples was conducted to create a standard curve (straight line), which was used to calculate the insulin concentration from the peak areas of the insulin peaks on the chromatograms by using linear regression as follows:

$$\text{Concentration in sample } (\mu g/ml) = \frac{(\text{peak area of sample} - \text{y-intercept})}{\text{slope}} \qquad \text{(Equation 3.5)}$$

3.6 VALIDATION OF THE CHROMATOGRAPHIC ANALYTICAL METHOD

3.6.1 Introduction

The validation of an analytical method can be defined as the procedure followed to establish whether the performance characteristics of an analytical method meet the requirements for the intended application of that analytical method (USP, 2014:1157). In essence, analytical test method validation provides evidence of reliability and accuracy of the analytical method in order to generate meaningful data (Shabir, 2003:57).

Since the HPLC method used in this study was previously developed and validated (Kleynhans, 2015), it was only necessary to determine the limit of quantification, limit of detection, specificity and linearity.

3.6.2 Limit of quantification and limit of detection

The limit of quantification (LOQ) is the lowest quantity of an analyte in a sample that can be determined with acceptable precision and accuracy under standard experimental conditions. It is a characteristic of importance of quantitative assays for low levels of compounds in samples (USP, 2014:1160). The LOQ was taken as the lowest concentration of insulin that could be analysed with a relative standard deviation (RSD) of ≤ 15% for six replicates.

The limit of detection (LOD) is the lowest concentration peak which is discernible from baseline noise of a chromatogram (USP, 2014:1160). The LOD was taken as an insulin peak on the chromatogram equal to three times the average baseline noise.

3.6.3 Specificity

Specificity is the ability of an analytical method to detect a substance (i.e. the analyte) in the presence of other substances, which may interfere with the detection of the analyte (USP, 2014:1159).

Samples of 0.1% w/w insulin solutions in the presence of 15% w/w chitosan, 10% w/w *A. vera* gel, 10% w/w *A. vera* whole leaf, 10% w/w bile salt and 10% w/w sodium glycocholate were analysed with the HPLC method, which represented the solutions used during the *in vitro* transport studies. The chromatograms were inspected to ensure the insulin peak was

completely separated from those of the other components for the analytical method to be acceptable.

3.6.4 Linearity

Linearity of an analytical method refers to the relationship between the analyte concentration and the analysis measurement (e.g. peak area) (USP, 2014:1160).

A standard insulin solution of 0.1 mg/ml was prepared in KRB buffer of which 2.5, 5, 10, 20, 30, 40, and 50 µl were injected in duplicate into the chromatograph. The peak areas on the chromatograms were plotted as a function of insulin concentration and a linear regression of the curve was done by using Microsoft Excel® software from which the correlation coefficient (R^2) was obtained. An R^2 value ≥ 0.99 was required for the analytical method to be acceptable.

3.7 SUMMARY

In order to design a double phase dosage form, two types of beads were prepared that could be combined as a mixture in hard gelatine capsules. The one type of bead formulation contained a drug absorption enhancer (i.e. one of the following: *A. vera* gel or *A. vera* whole leaf or sodium glycocholate or bile salt mixture), whereas the other type contained insulin as active ingredient and chitosan as muco-adhesive agent. The idea was to develop a dosage form that after administered will allow the one type of beads (i.e. those containing the drug absorption enhancer) to move relatively quickly into the small intestine to open the tight junctions. The other type of coated beads will follow due to the presence of a muco-adhesive component and once they arrive at the small intestine the insulin will be delivered across the modulated intestinal tissue.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 INTRODUCTION

A double phase drug delivery system consisting of different bead formulations loaded into hard gelatine capsules for oral peptide delivery was developed and evaluated. The physical characteristics of the bead formulations were tested with regards to insulin content (i.e. assay), mass variation, friability, particle size distribution and drug release. A portion of the beads containing insulin was film coated in an attempt to delay insulin release. A previously developed HPLC method for insulin analysis was validated in terms of linearity, specificity, limit of quantification and limit of detection.

The potential paracellular transport enhancing effect of the beads containing absorption enhancing agents was investigated by studying their ability to reduce transepithelial electrical resistance (TEER) as an indication of their effects on the tight junctions. This was followed by an *in vitro* transport study on the beads containing insulin across excised pig intestinal tissues, which were pre-exposed to the absorption enhancer containing beads.

4.2 EVALUATION OF THE BEAD FORMULATIONS

4.2.1 Assay

The following regression values were obtained from the insulin calibration curve:

c (y-intercept) = 0 and m (slope) = 161522.175

By substituting the HPLC insulin peak area value (1449.5 mAU; obtained from a sample of the 100 ml solution where 1 g of ground beads was dispersed) into the equation of a straight line (y = mx + c), the experimental value of insulin content (x) in the beads was obtained as follows:

$$\frac{(1449.5 - 0)}{161522.175} = 0.008974 \text{ mg/ml} = 0.8974 \text{ mg/100 ml}$$

It was determined that a quantity of 0.8974 mg insulin was present in the 1 g beads (or 0.08974 % w/w of the beads) that was dispersed in 100 ml of the solvent.

The % insulin content in the beads in terms of the target dose was calculated as follows:

$$\% \text{ insulin content} = \frac{0.08974}{0.1} \times 100 = 89.74\%$$

The percentage content of insulin in the bead formulation is 89.74% of the total amount of insulin that was intended to be contained in the bead formulation. This was calculated by dividing the true insulin content as analysed by the assay with the theoretical insulin content that was weighed and included into the mixture during formulation. Loss of the active ingredient (i.e. the 10.26% of the total dose of insulin) can probably be explained by chemical degradation and/or physical loss during the production (i.e. extrusion and spheronisation), drying, handling or storage of the beads. It should be investigated in future studies and loss of the active ingredient should be prevented by implementing changes to the production process in order to obtain a higher percentage insulin content (i.e. limits of 95% – 105%) in the final bead formulation.

4.2.2 Mass variation

A constant dose of active ingredient between individual dosing units within a batch is an important attribute of a pharmaceutical product. By measuring the uniformity of mass (or mass variation), an indirect indication of the uniformity of content or variation in dose can be obtained (Aulton, 2002:417-418).

The results obtained for mass variation of capsules filled with the different bead formulations (i.e. beads containing *A. vera* whole leaf, *A. vera* gel, sodium glycocholate, bile salt mixture, insulin and chitosan and MicroceLac®100 only) are shown in Table 4.1.

The requirement for the mass variation test is that the weight of individual bead filled capsules for each type of bead formulation must not deviate from the average mass of the bead filled capsules by more than ± 7.5% (USP, 2014:492). From Table 4.1, it is clear that all the bead formulations prepared in this study complied with the requirement prescribed by the USP.

Table 4.1: Mass variation results for hard gelatine capsules filled with different beads

Bead composition	Average mass (g)	% deviation (lower than average)	% deviation (higher than average)
A. vera gel (10% w/w)	0.3091	-7.0%	4.9%
A. vera whole leaf (10% w/w)	0.2824	-6.2%	4.4%
Sodium glycocholate (10% w/w)	0.3128	-5.5%	4.4%
Bile salt mixture (10% w/w)	0.2701	-6.4%	4.1%
Insulin (0.1% w/w) and Chitosan (15% w/w)	0.3209	-5.3%	4.7%
MicroceLac®100 (100% w/w)	0.2752	-7.2%	3.3%

4.2.3 Friability

According to the USP (2014:1146), the limit for acceptable friability of solid oral dosage forms is 1%. The percentage friability values for all the bead formulations are depicted in Table 4.2. From the results obtained for the friability of the bead formulations, it is clear that all the bead formulations investigated in this study complied with the requirement of less than 1% friability.

Table 4.2: Average percentage friability and standard deviations for all the bead formulations

Formulation composition	Percentage friability	Standard deviation
A. vera gel (10% w/w)	0.56%	0.28%
A. vera whole leaf (10% w/w)	0.98%	0.14%
Sodium glycocholate (10% w/w)	0.78%	0.31%
Bile salt mixture (10% w/w)	0.87%	0.67%
Insulin (0.1% w/w) and chitosan (15% w/w)	0.94%	0.28%
MicroceLac®100 (100% w/w)	0.85%	0.70%

The friability of solid oral dosage forms provides an indication of their ability to handle mechanical stresses. The higher the friability, the more easily abrasion of the dosage form (e.g. beads) can take place. The dosage form must be tough enough to prevent crumbling and abrasion during handling, but should be soft enough to disintegrate and release the drug upon contact with gastrointestinal fluids. By complying with the criterion for acceptable friability (i.e. ≤ 1%), the results in Table 4.2 indicate that the beads prepared in this study will most probably be able to handle mechanical stresses caused by packaging, handling and storage.

4.2.4 Particle size analysis

4.2.4.1 Bead formulation containing *Aloe vera* gel

The particle size distribution plot for the bead formulation containing *A. vera* gel is shown in Figure 4.1. The majority of the beads containing *A. vera* gel (i.e. 70.75%) ranged between 954.9 µm and 1258.9 µm based on volume distribution measurements. The average median of the distribution value (d(0.5)) for this bead formulation was 1056.1 ± 5.9 µm, wheras the average volume weighed size distribution value (D[4,3]) was 1084.9 ± 6.9 µm. The particle size distribution as expressed by span was 0.67. This span value indicates a relatively narrow particle size distribution, which is expected of beads prepared by means of extrusion spheronisation.

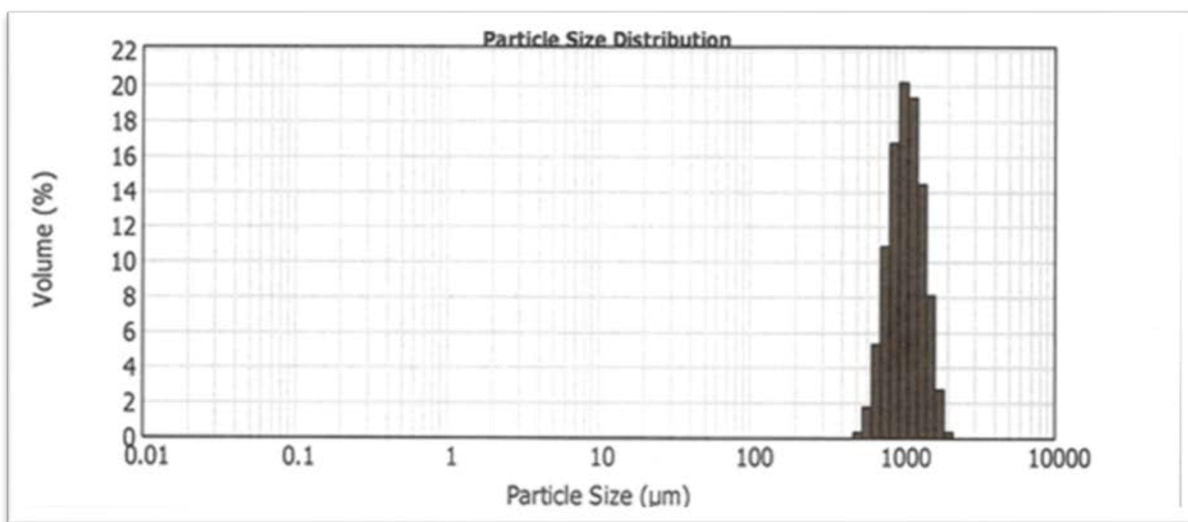

Figure 4.1: Particle size distribution plot for the bead formulation containing *Aloe vera* gel

4.2.4.2 Bead formulation containing *Aloe vera* whole leaf

The particle size distribution plot for the bead formulation containing *A. vera* whole leaf is presented in Figure 4.2. The majority of the beads (i.e. 73.59%) containing *A. vera* whole leaf ranged between 831.764 μm and 1258.925 μm based on volume distribution measurements. The average median of the distribution value (d(0.5)) for this bead formulation was 965.3 ± 28.4 μm, while the average volume weighed size distribution value (D[4,3]) was 1005.9 ± 25.02 μm. The particle size distribution as expressed by span was 0.64. This span value indicates a relatively narrow particle size distribution, which is expected of beads prepared by means of extrusion spheronisation.

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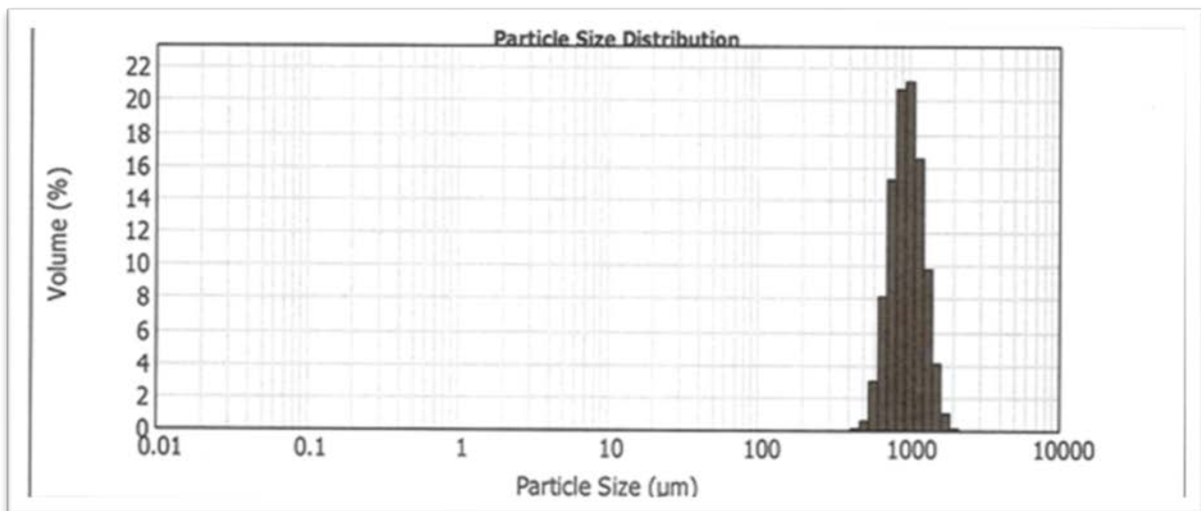

Figure 4.2: Particle size distribution plot for the bead formulation containing *Aloe vera* whole leaf

4.2.4.3 Bead formulation containing Sodium glycocholate

The particle size distribution plot for the bead formulation containing sodium glycocholate is depicted in Figure 4.3.

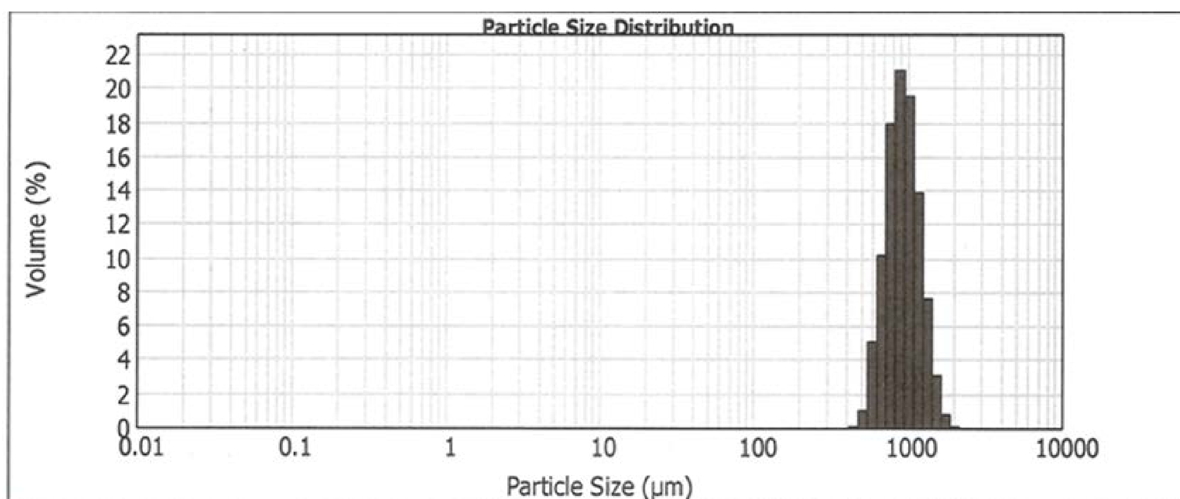

Figure 4.3: Particle size distribution plot for the optimised bead formulation containing sodium glycocholate

The majority of the beads (i.e. 72.23%) of this bead formulation containing sodium glycocholate varied between 831.764 μm and 1258.925μm based on volume distribution measurements. The average median of the distribution value (d(0.5)) for this bead formulation was 921.4 ± 10.8 μm, while the average volume weighed size distribution value (D[4,3]) was 951.3 ± 7.5 μm. The particle size distribution as expressed by span is 0.67. This span value indicates a relatively narrow particle size distribution, which is expected of beads prepared by means of extrusion spheronisation.

4.2.4.4 Bead formulation containing Bile salt mixture

The particle size distribution plot for the bead formulation containing bile salt mixture is shown in Figure 4.4.

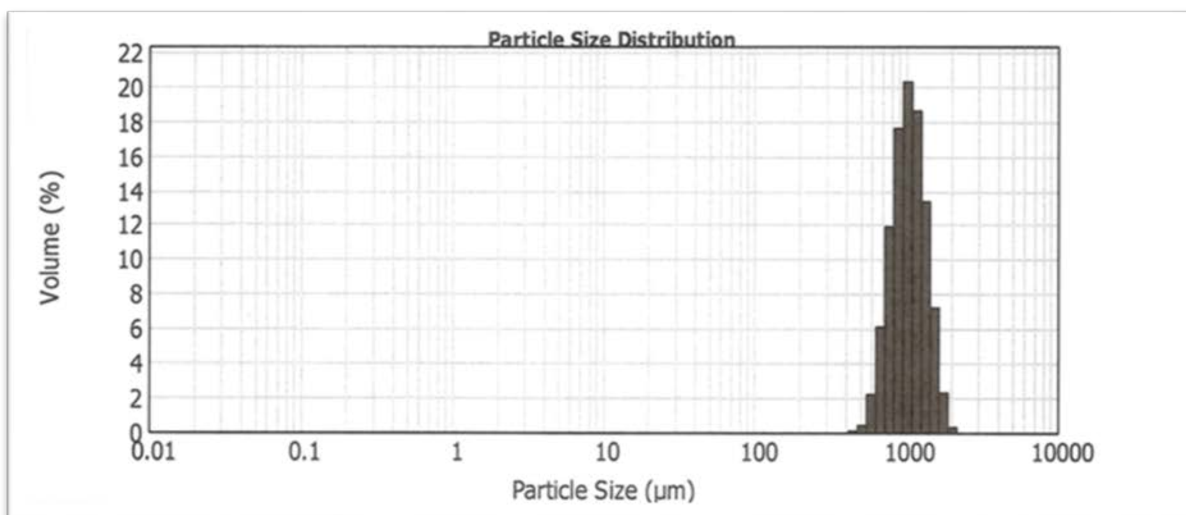

Figure 4.4: Particle size distribution plot for the bead formulation containing bile salt mixture

Most of the beads (i.e. 69.75%) containing a bile salt mixture (Figure 4.4) varied between 831.764 µm and 1258.925 µm based on volume distribution measurements. The average median of the distribution value (d(0.5)) for this bead formulation was 1035.1 ± 8.8 µm, while the average volume weighed size distribution value (D[4,3]) was 1066.23 ± 8.3 µm. The particle size distribution as expressed by span was 0.68. This span value indicates a relatively narrow particle size distribution, which is expected of beads prepared by extrusion spheronisation.

4.2.4.5 Bead formulation containing insulin and chitosan

The particle size distribution plot for the bead formulation containing insulin and chitosan is shown in Figure 4.5. The size of the bulk of the insulin containing beads (i.e. 71.9%) varied between 831.764 µm and 1096.478 µm based on volume distribution measurements. The average median of the distribution value (d(0.5)) for this bead formulation was 841 ± 7.9 µm, while the average volume weighed size distribution value (D[4,3]) was 872.4 ± 8.3 µm. The particle size distribution as expressed by span was 0.67. This span value indicates a relatively

narrow particle size distribution, which is expected of beads prepared by means of extrusion spheronisation.

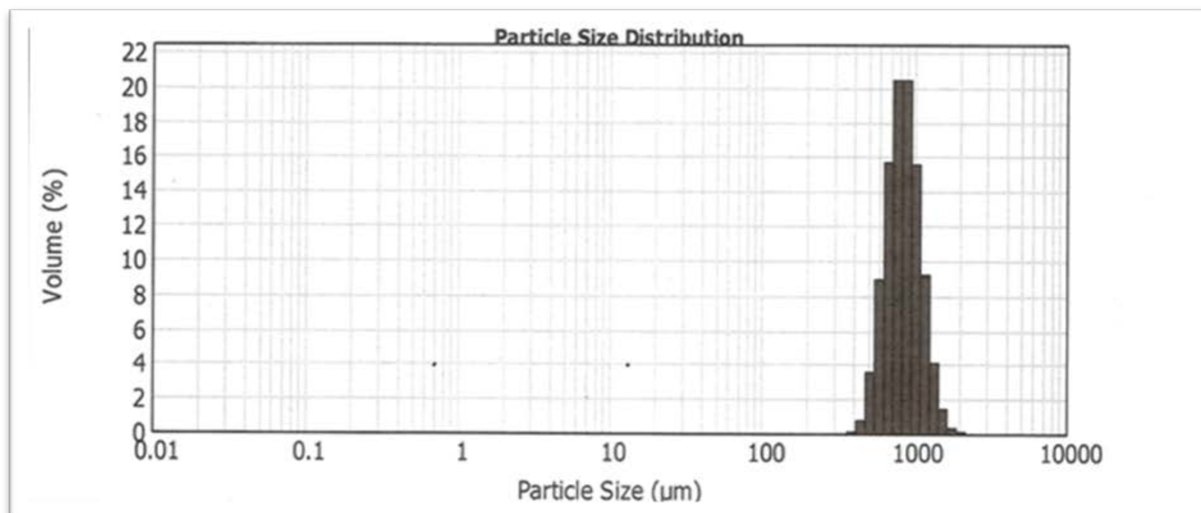

Figure 4.5: Particle size distribution plot for the beads containing insulin and chitosan

4.2.4.6 Bead formulation containing MicroceLac®100

The particle size distribution plot for the bead formulation containing only MicroceLac®100 is shown in Figure 4.6. The bulk of the beads (i.e. 73.36%) in this bead formulation containing MicroceLac®100 (Figure 4.6) varied between 831.764µm and 1096.478µm based on volume distribution measurements. The average median of the distribution value (d(0.5)) for this bead formulation was 966.7 ± 5.4 µm, while the average volume weighed size distribution value (D[4,3]) was 998.3 ± 5.0 µm. The particle size distribution as expressed by span was 0.65. This span value indicates a relatively narrow particle size distribution, which is expected of beads prepared by means of extrusion spheronisation.

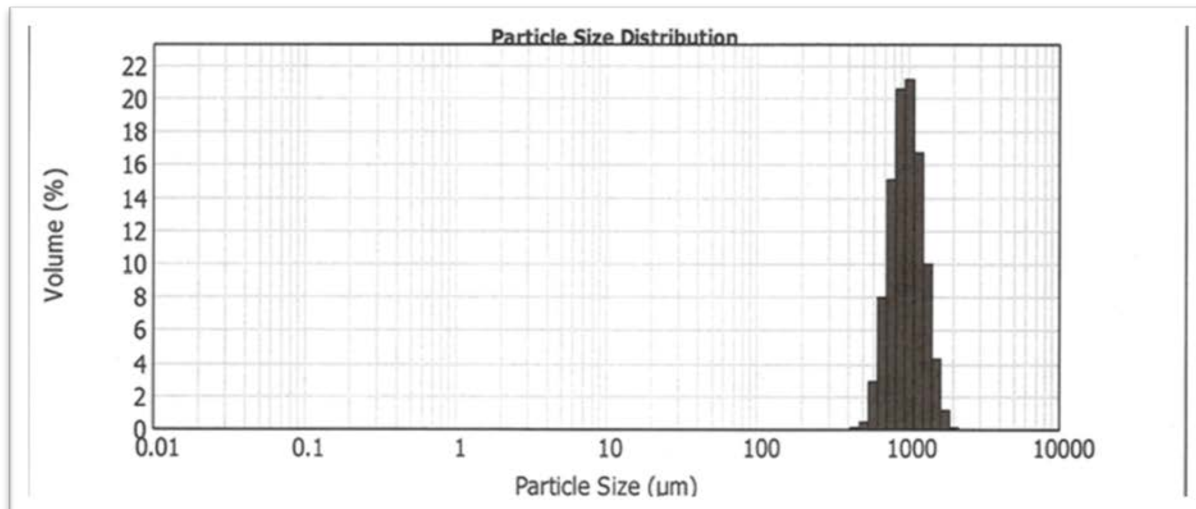

Figure 4.6: Particle size distribution plot for the beads consisting of MicroceLac®100 only

4.2.5 Dissolution

For the double phase dosage form developed in this study, the insulin beads should ideally have a delayed release. The reason for this is because time should be allowed for the absorption enhancing containing beads to first move to the site of absorption and open the tight junctions before the insulin becomes available for absorption. Since insulin is also sensitive to enzymatic degradation (Hamman *et al.*, 2005), especially in the stomach, an enteric coating may contribute to protect the insulin from degradation and in addition provide the delay in insulin release as explained.

The percentage dissolution for both coated and uncoated insulin bead formulations was plotted as a function of time and is shown in Figure 4.7. From Figure 4.7 it is clear that insulin was released at a faster rate and to a larger extent from the uncoated beads when compared to that of the coated beads in an acidic medium (0.1 M HCl). Furthermore, a plateau in insulin release was already reached after 30 min for the uncoated beads, whereas the coated bead formulations reached a plateau in insulin release only after 60 min. The plateau was probably caused by the fact that the beads did not disintegrate and stayed intact during the 120 min dissolution test. Dissolution studies over longer periods and in different media should be conducted in future studies. Although the coating of the beads resulted in a slower insulin release, it did not provide a lag phase to completely delay insulin release. It is therefore suggested that a thicker film coating on the surface of the beads be investigated in future.

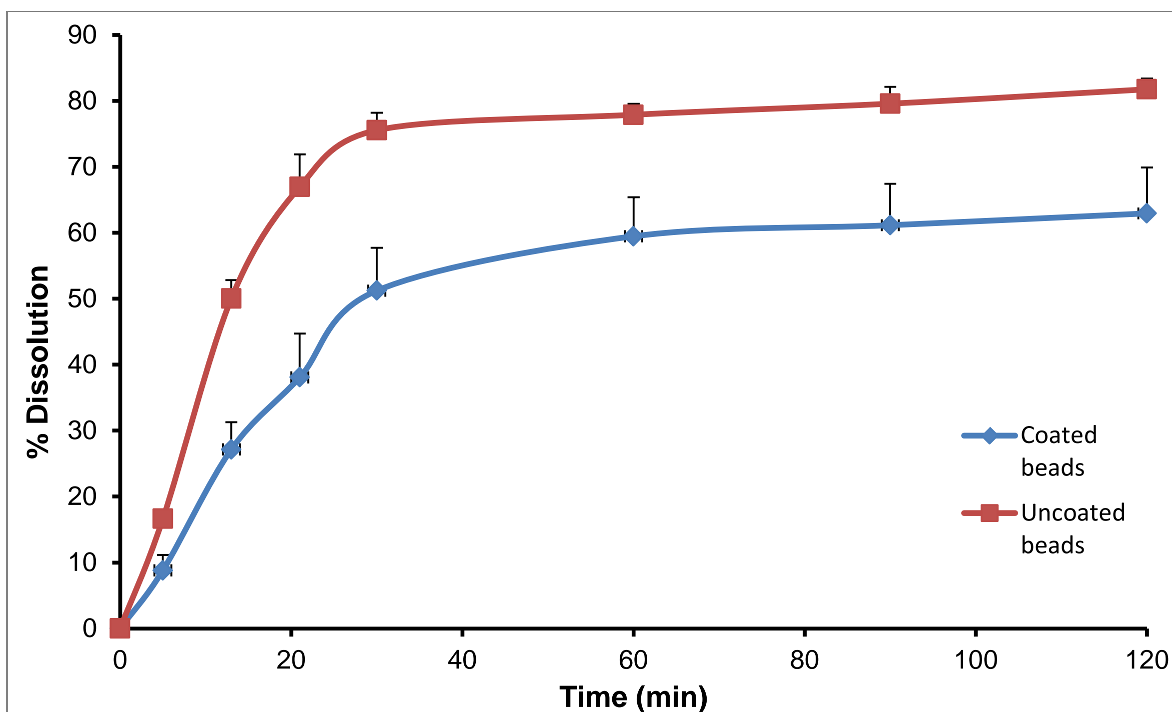

Figure 4.7: Percentage dissolution of insulin plotted as a function of time for coated and uncoated bead formulations

4.2.6 Muco-adhesion

A muco-adhesive agent (chitosan) was specifically added to the bead formulation containing the insulin to slow down the movement of these beads along the gastrointestinal tract. This will allow the beads containing the absorption enhancer to reach the target site (i.e. small intestine) first in order to open the tight junctions before the beads with the active ingredient (insulin) reach this particular absorption site. The muco-adhesive properties of all the bead formulations were tested, which would give an indication whether the movement of the different beads could potentially be controlled in the gastrointestinal tract.

The percentage muco-adhesion obtained for the bead formulations is shown in Figure 4.8. From this figure it is clear that the beads consisting of the filler material only (i.e. MicroceLac®100 without chitosan, which served as the control group) exhibited a relatively low percentage muco-adhesion of 26.1%. Addition of drug absorption enhancing agents such as the bile salt mixture and sodium glycocholate had a negligible change in the muco-adhesive properties of the beads compared to that of the control group with percentage muco-adhesion values of 28.6% and 26.5%, respectively.

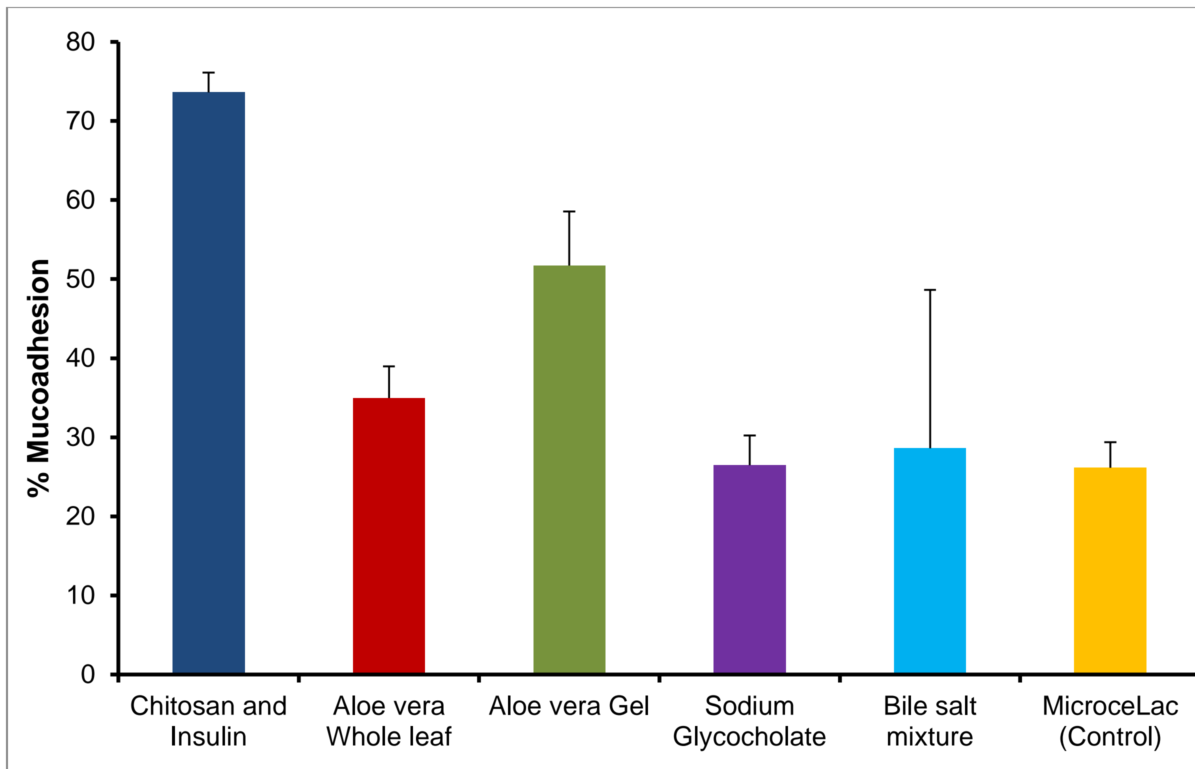

Figure 4.8: Percentage muco-adhesion of the bead formulations

However, addition of *A. vera* gel to the beads caused an increase in the percentage muco-adhesion compared to that of the control group (51.7% vs 26.1%), while the presence of *A. vera* whole leaf material in the beads increased the percentage muco-adhesion to a lesser extent (i.e. to 34.9% vs 26.1%). This increase in muco-adhesion for aloe leaf materials is to be expected since the gel part consists of large polysaccharide molecules (Hamman, 2008) that may interact with the mucus layer on the pig intestinal tissue and thereby cause adhesion.

On the other hand, addition of chitosan to the beads showed a pronounced increase in muco-adhesion when compared to the control group and exhibited the highest percentage muco-adhesion of all the bead formulations namely 73.6%. This indicates that the chitosan fulfilled the function of increasing the muco-adhesion of the beads, which may aid the double phase dosage form to achieve the delivery of insulin at the required time. The beads containing the drug absorption enhancer will probably reach the absorption site first due to relatively poor muco-adhesion and will then open the tight junctions. The beads containing the insulin will probably follow at a slower rate due to higher muco-adhesion.

4.3 TRANS-EPITHELIAL ELECTRICAL RESISTANCE (TEER) STUDY

The results obtained from the TEER experiment for the different bead formulations containing absorption enhancing agents are shown in Figure 4.9.

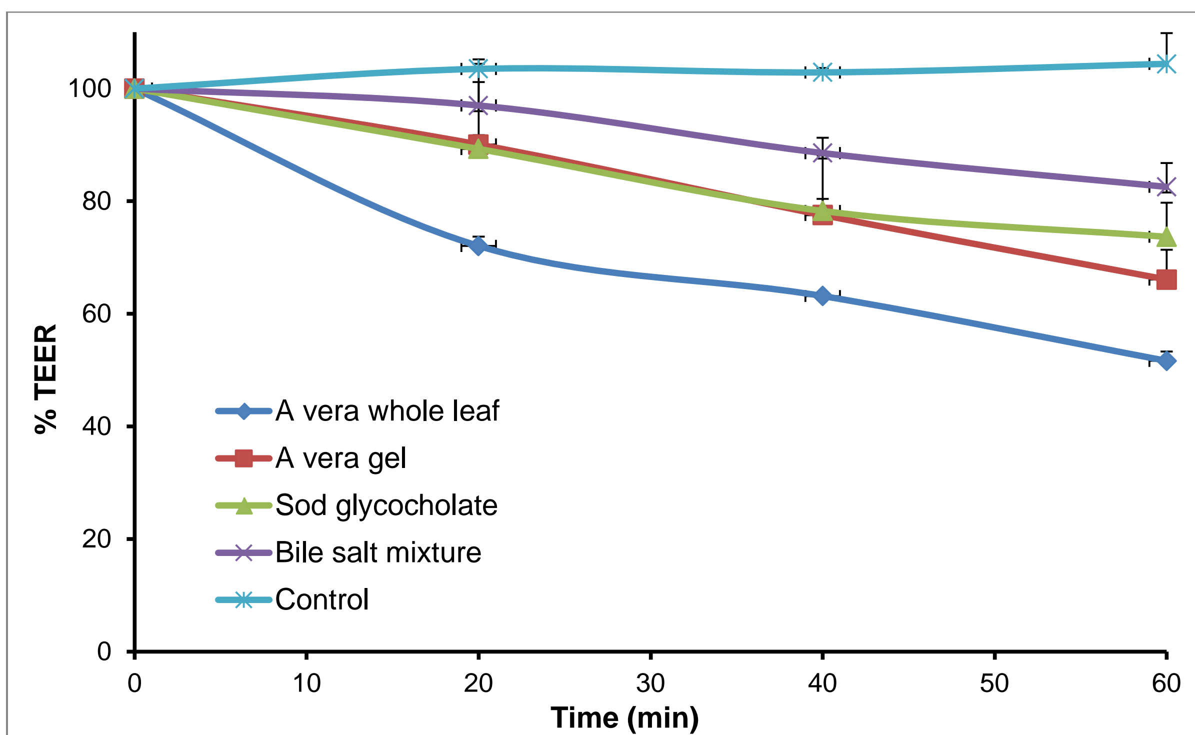

Figure 4.9: Percentage transepithelial electrical resistance (TEER) of excised porcine intestinal tissues exposed to different beads plotted as a function of time

4.3.1 Beads containing *Aloe vera* gel

The beads containing *A. vera* gel showed a relatively large reduction in TEER (up to 60.0 % of initial value) of the excised pig intestinal tissues. This corresponds with findings of a previous study where *A. vera* gel in solution reduced the TEER of Caco-2 cell monolayers (Chen *et al.*, 2009:297). This study therefore confirms that *A. vera* gel formulated in a solid oral dosage form such as beads is also capable of reducing the TEER of the intestinal epithelium. This reduction in TEER indicates opening of the tight junctions between adjacent epithelial cells, which is associated with enhanced paracellular absorption of poorly absorbable drugs (Lemmer & Hamman, 2013:104). Even though the TEER of the excised pig intestinal tissue was immediately reduced by exposure to the beads containing *A. vera* gel (90% of initial TEER value after 20 min), the TEER was continuously further reduced over the entire period.

4.3.2 Beads containing *Aloe vera* whole leaf

Beads containing *A. vera* whole leaf material displayed the largest reduction in the TEER of excised pig intestinal tissue when compared to the other bead formulations. This bead formulation had an immediate effect on TEER (the TEER was reduced to 72.1% of the initial value after 20 min), but it continued to reduce the TEER further over time (the TEER was reduced to 51.6% of the initial value after 60 min).

4.3.3 Beads containing Sodium glycocholate

Beads containing the bile salt, sodium glycocholate, showed a reduction in TEER of the excised pig intestinal tissue (it reduced the TEER to 74% of the initial value). This reduction in TEER of the intestinal epithelium corresponds with findings of a previous study indicating that sodium glycocholate was capable of decreasing TEER (Lindhardt & Bechgaard, 2003:187). Bile salts have shown the ability to enhance drug absorption by a combination of mechanisms including modulating tight junctions and changing membrane fluidity through phospholipid solubilisation (Hamman, 2005:172).

4.3.4 Beads containing Bile salt mixture

The beads containing the bile salt mixture (50% sodium cholic acid salt and 50% sodium deoxycholic acid salt) showed a less pronounced reduction in TEER (up to 83% of the initial TEER value), which is still a relatively large effect when compared to the control group but the lowest effect when compared to the other bead formulations. Although this was the lowest reduction in TEER as compared to the other bead formulations, it still indicates the potential to modulate tight junctions and thereby allow for enhanced paracellular transport of poorly absorbable drugs, albeit to a lower extent.

4.3.5 Beads consisting of MicroceLac®100 only (control group)

The bead formulation consisting of MicroceLac®100 showed no reduction in TEER of the excised pig intestinal tissue because the TEER values remained almost constant over the total period of exposure between 100% and 104%. This indicates that the filler material used in the bead formulations, namely MicroceLac®100, did not contribute to any TEER reduction of the intestinal epithelium and therefore did not cause interferences with the effects caused by the drug absorption enhancing agents. It is also an indication that the excised intestinal tissues remained intact over the entire period of the experiment.

4.4 INSULIN TRANSPORT ACROSS EXCISED PIG INTESTINAL TISSUE

4.4.1 Insulin solution (control group)

Figure 4.10 illustrates the percentage insulin transport obtained as a function of time when an insulin solution was applied to excised pig intestinal tissue in the Sweetana-Grass diffusion apparatus for a period of 120 min. This experimental group served as a control where the intestine was not exposed to any bead formulations containing absorption enhancing agents.

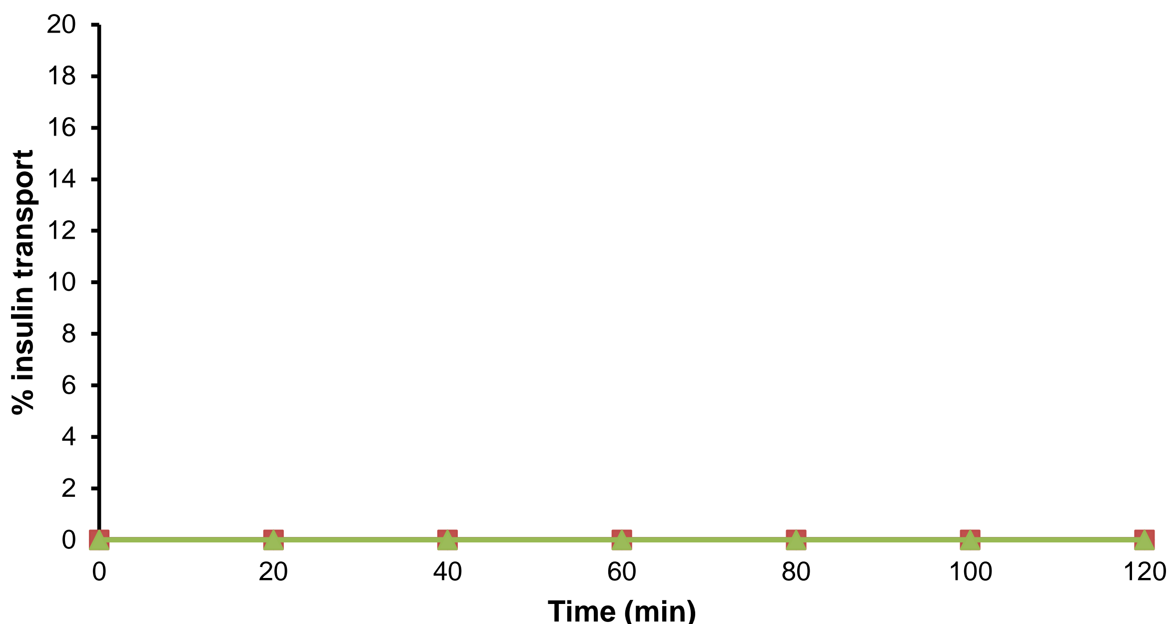

Figure 4.10: Graph of the percentage insulin transport across excised pig intestinal tissue plotted as a function of time for an insulin solution without exposure of the excised pig intestinal tissue to any bead formulation containing drug absorption enhancing agents

It is clear from Figure 4.10 that no insulin could be detected on the basolateral side of the excised pig intestinal tissues over the entire period of 120 min after application of an insulin solution. This is in line with the fact that peptide and protein drugs are usually very poorly bioavailable after oral administration (typically < 1% *in vivo*) (Renukuntla *et al*., 2013:75). Furthermore, it has been shown that *in vitro* models such as Caco-2 cell monolayers are more "leakier" than excised animal intestinal tissues (Westerhout *et al*., 2014:172). Therefore, although insulin has previously been shown to be transported across Caco-2 cell monolayers (Chen *et al*., 2009:594), it is not permeable across the robust, intact excised pig intestinal tissue.

4.4.2 Beads containing *Aloe vera* gel

Figure 4.11 illustrates the percentage insulin transport obtained when applying the bead formulation containing insulin for a period of 120 min after the excised pig intestinal tissue was pre-exposed to the *A. vera* gel bead formulation for 60 min.

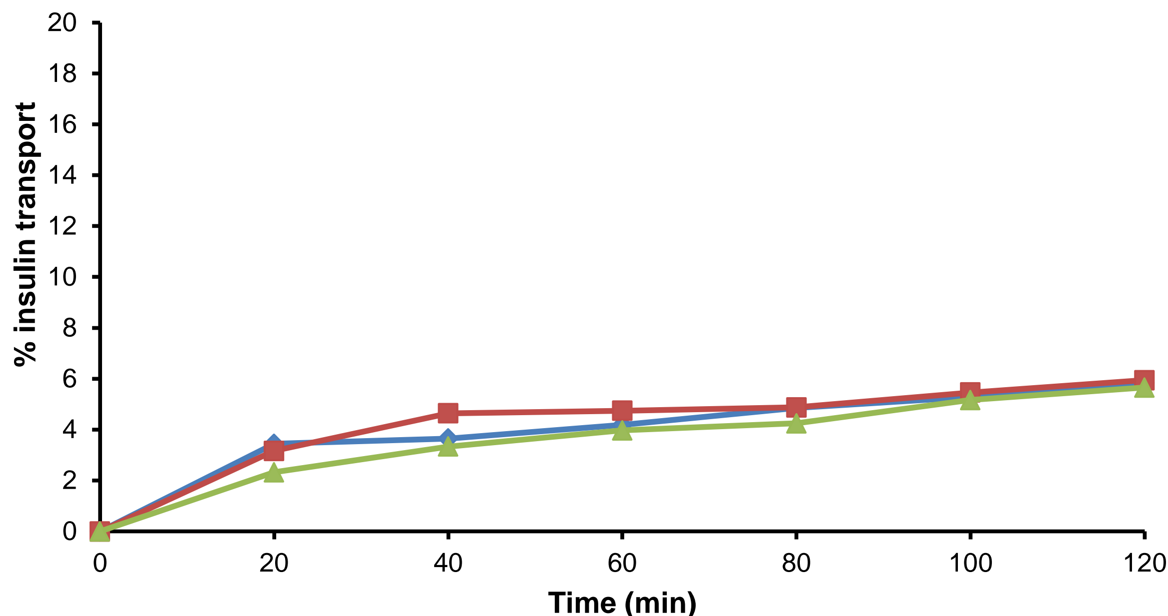

Figure 4.11: Graph of the percentage insulin transport across excised pig intestinal tissue plotted as a function of time for an insulin containing bead formulation after pre-exposure to beads containing *Aloe vera* gel

From Figure 4.11, it is clear that pre-exposure of excised pig intestinal tissue to beads containing *A. vera* gel exhibited enhanced transport of insulin (5.75% ± 0.14%) when compared to the control group (0% transport for the insulin solution, Figure 4.10). This enhanced transport of insulin is in accordance with the TEER reduction obtained when beads containing *A. vera* gel was applied to excised pig intestinal tissue. The opening of the tight junctions as indicated by a reduction in TEER when beads containing *A. vera* gel were applied resulted in an increased transport of insulin probably due to improved paracellular movement of the insulin molecules across the intestinal tissue.

A bioavailability of 5% has been considered as acceptable for a potent drug such as insulin (Hamman *et al.*, 2005). Although it has been shown previously that *A. vera* gel can enhance *in vitro* insulin transport across the intestinal epithelium when applied in solution (Chen *et al.*,

2009:593), this study confirmed that inclusion of the *A. vera* gel material in a solid oral dosage form such as beads can also produce enhanced delivery of insulin across the intestinal epithelium.

4.4.3 Beads containing *Aloe vera* whole leaf

Figure 4.12 illustrates the percentage insulin transport obtained when applying the bead formulation containing insulin for a period of 120 min after the excised pig intestinal tissue was pre-exposed to the *A. vera* whole leaf material bead formulation for 60 min.

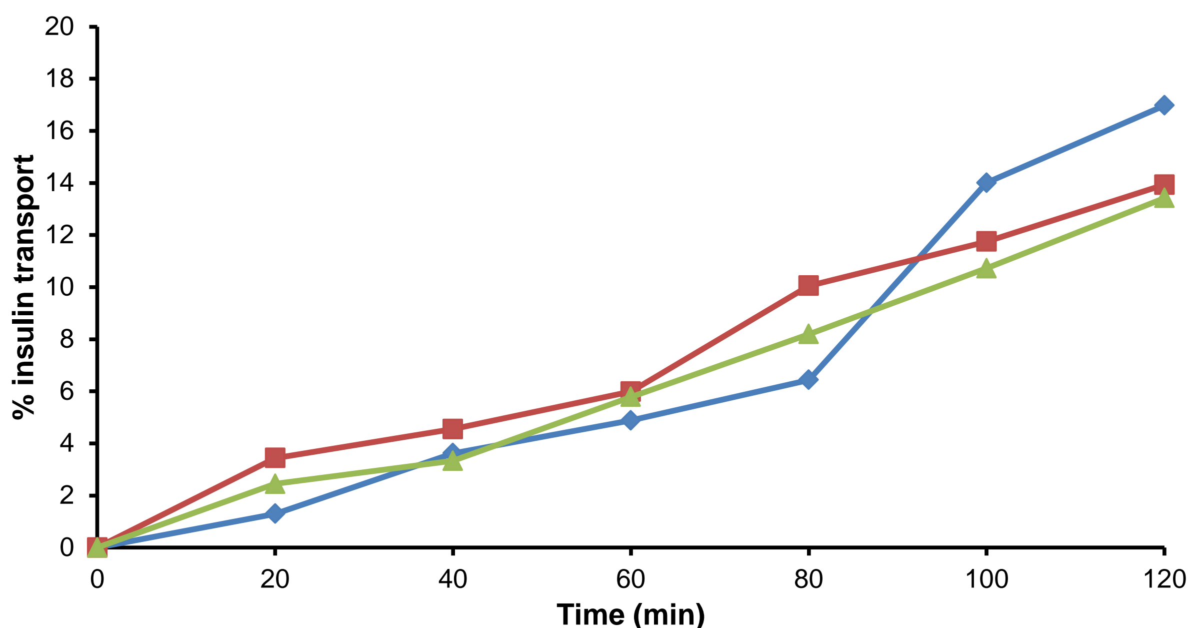

Figure 4.12: Graph of the percentage insulin transport across excised pig intestinal tissue plotted as a function of time for an insulin containing bead formulation after pre-exposure to beads containing *Aloe vera* whole leaf material

Figure 4.12 clearly indicates that pre-exposure of excised pig intestinal tissue with beads containing *A. vera* whole leaf material exhibited enhanced transport of insulin (14.77% ± 1.56%) when compared to the control group (insulin solution, Figure 4.10). This enhanced transport of insulin is also in accordance with the TEER reduction obtained when beads containing *A. vera* whole leaf material was applied to excised pig intestinal tissue. The reduction in TEER indicated that *A. vera* whole leaf material caused the opening of tight junctions which may have resulted in the improved insulin transport likely due to improved paracellular movement across intestinal tissue.

As mentioned before, a bioavailability of 5% has been considered as acceptable systemic delivery for a potent drug such as insulin. Furthermore, it has been shown previously that *A. vera* whole leaf material can enhance *in vitro* insulin transport when applied in solution (Chen *et al*., 2009:593), but this study confirms that inclusion of the *A. vera* whole leaf material in a solid oral dosage form such as beads can produce sufficient delivery of insulin.

4.4.4 Beads containing Sodium glycocholate

Figure 4.13 illustrates the percentage insulin transport obtained when applying the bead formulation containing insulin for a period of 120 min after the excised pig intestinal tissue was pre-exposed to the sodium glycocholate bead formulation for 60 min.

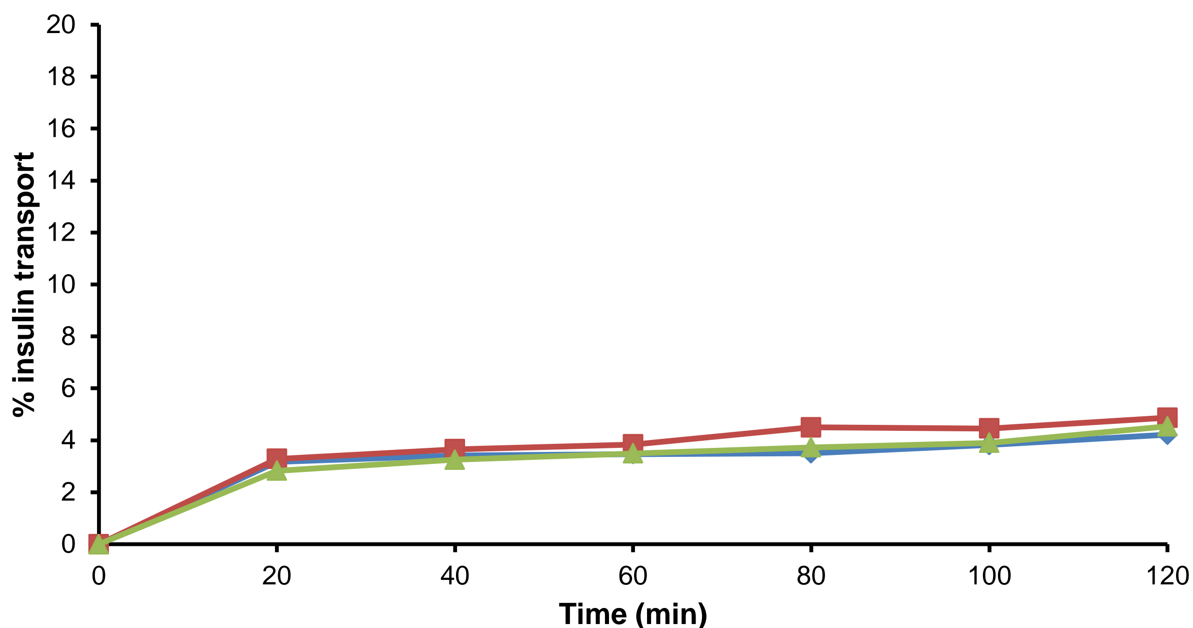

Figure 4.13: Graph of the percentage insulin transport across excised pig intestinal tissue plotted as a function of time for an insulin containing bead formulation after pre-exposure to beads containing sodium glycocholate

Figure 4.13 indicates that pre-exposure of excised pig intestinal tissue to beads containing sodium glycocholate exhibited enhanced transport of insulin (4.54% ± 0.27%) when compared to the control group (insulin solution, Figure 4.10). This improved transport correlates with the reduction in TEER, which was obtained when beads containing sodium glycocholate was applied to excised pig intestinal tissue and corresponds with previous findings (Lindhardt & Bechhaard, 2003:182).

Although it has been shown previously that sodium glycocholate can enhance *in vitro* of transport of mannitol (Lindhardt & Bechhaard, 2003:183), this study confirmed that inclusion of sodium glycocholate in a solid oral dosage form such as beads can promote the delivery of insulin, although to a lesser extent than that obtained for *A. vera* gel and whole leaf materials.

4.4.5 Beads containing Bile salt mixture

Figure 4.14 illustrates the percentage insulin transport obtained when applying the bead formulation containing insulin for a period of 120 min after the excised pig intestinal tissue was pre-exposed to the bile salt mixture bead formulation for 60 min.

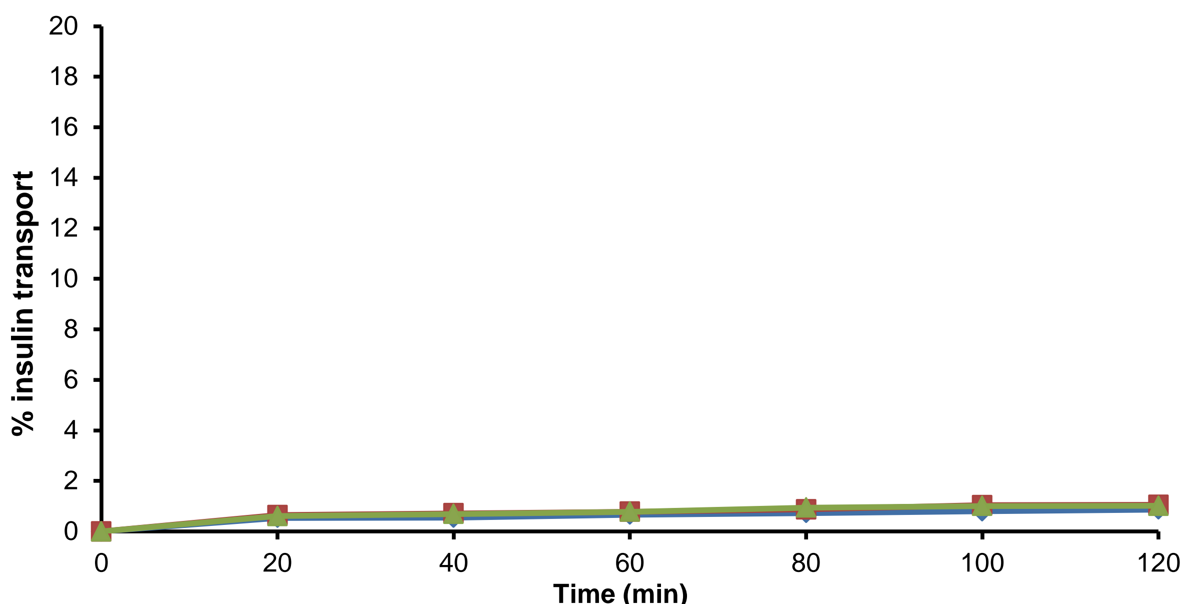

Figure 4.14: Graph of the percentage insulin transport across excised pig intestinal tissue plotted as a function of time for an insulin containing bead formulation after pre-exposure to beads containing bile salt mixture

Figure 4.14 indicates some improvement in transport of insulin across excised pig intestinal tissue when pre-exposed to beads containing bile salt mixture, which exhibited enhanced transport of insulin (0.97% ± 0.08%) when compared to that of the control group (insulin solution, Figure 4.10). This enhanced transport of insulin is in accordance with the TEER reduction obtained when beads containing bile salt mixture was applied to excised pig intestinal tissue.

4.4.6 Beads containing MicroceLac®100 and chitosan

Figure 4.15 illustrates the percentage insulin transport obtained when applying the bead formulation containing insulin for a period of 120 min after the excised pig intestinal tissue was pre-exposed to the MicroceLac®100 and chitosan bead formulation for 60 min.

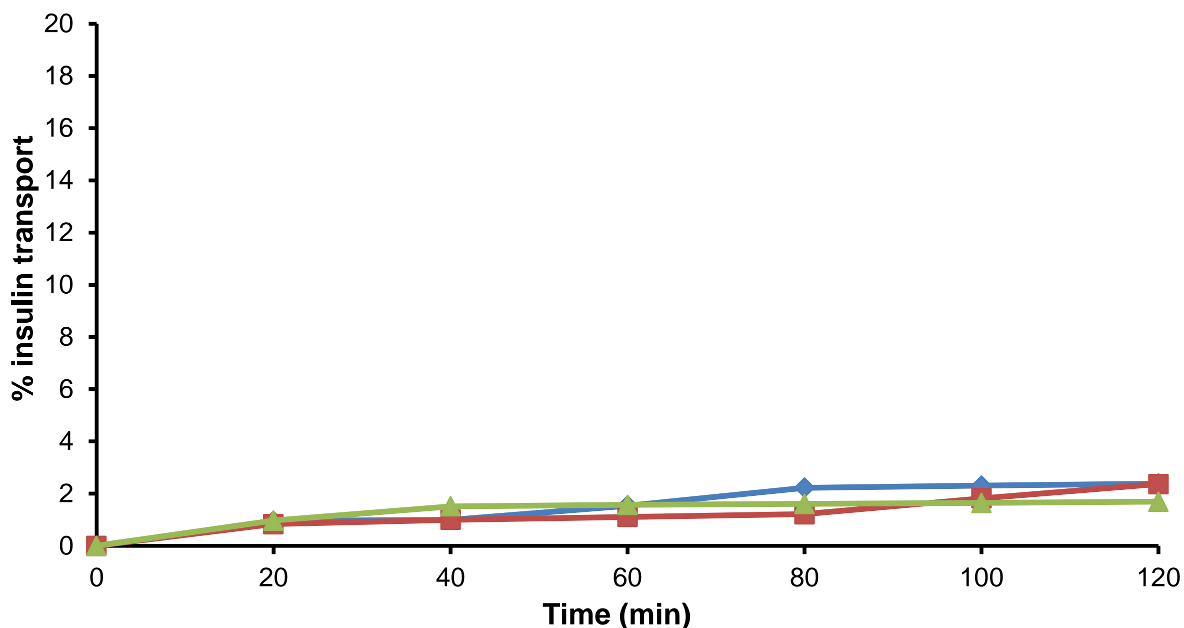

Figure 4.15: Graph of the percentage insulin transport across excised pig intestinal tissue plotted as a function of time for an insulin containing bead formulation after pre-exposure to beads containing MicroceLac®100 and chitosan

Figure 4.15 indicates enhanced transport of insulin after exposure to a control group where the "absorption enhancing" bead formulation contained no absorption enhancer (only MicroceLac®100 which is the filler used in all the formulations and chitosan as muco-adhesive agent) yet it is still clear that there was enhanced transport of insulin (2.14% ± 0.32%) when compared to the insulin solution control group (insulin solution, Figure 4.10). The chitosan, which was incorporated into the insulin bead formulation for its muco-adhesive effects, was likely the cause of this improved transport of insulin due to chitosan's ability to increase the paracellular permeability of peptide drugs across the intestinal epithelium (Thanou *et al.*, 1999:74; Thanou *et al.*, 2001:117).

4.4.7 Comparing insulin delivery from the different bead formulations

The average percentage transport obtained from the transport studies was used to calculate the apparent permeability coefficient (P_{app}) values for each transport study. The P_{app} values for insulin transport across excised pig intestinal tissue after pre-exposure to the bead formulations containing different drug absorption enhancers can be seen in Figure 4.16.

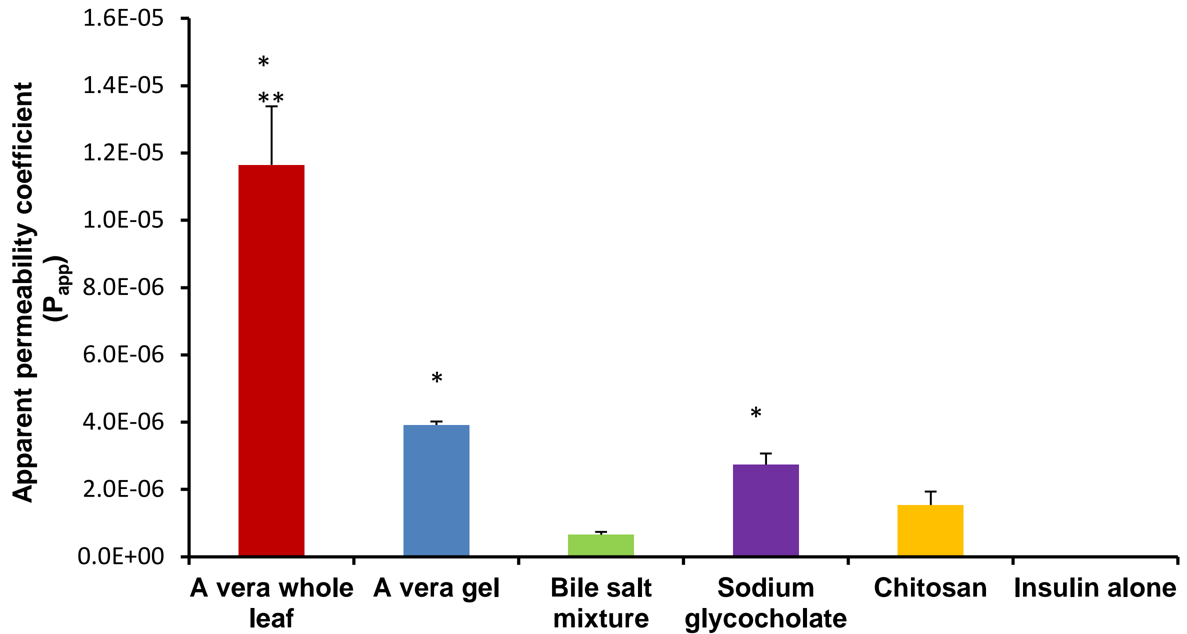

Figure 4.16: Apparent permeability coefficient (P_{app}) values for insulin after pre-exposure to bead formulations containing different drug absorption enhancers. * denotes a statistically significant difference from the control based on an ANOVA analysis, ** denotes a statistically significant difference from the control based on a Dunn's post-hoc test

From Figure 4.16 it is clear that pre-exposure of the excised pig intestinal tissue to beads containing *A. vera* whole leaf material had the greatest effect on the insulin P_{app} value, which is statistically significantly different ($p <0.05$) from the control group based on both the ANOVA analysis as well as Dunn's post-hoc (non-parametric statistics) test. Pre-exposure of the excised pig intestinal tissue to beads containing *A. vera* gel and sodium glycocholate also caused a statistically significant ($p < 0.05$) enhancement of insulin transport (as expressed by P_{app} values) when compared to the control group, but this was only based on the ANOVA analysis.

It is interesting to note that the bead formulations containing aloe leaf materials had a higher effect on insulin transport than the beads containing bile salts or chitosan (which are well-known drug absorption enhancers). Furthermore, it is important to explain that the beads containing chitosan (which was included in the bead formulation specifically as muco-adhesive agent) was included in the transport study as an additional control group to show that the insulin transport enhancement effects were indeed caused by pre-exposure to the beads containing the selected drug absorption enhancers and not due to the fact that chitosan was included in the insulin containing beads.

Pre-exposure of the excised intestinal tissue to beads containing sodium glycocholate resulted in a lower insulin transport enhancement effect as compared to that of the beads containing *A. vera* gel and *A. vera* whole leaf material, but higher than the bile salt mixture containing beads.

The results obtained from the transport studies therefore confirmed that the idea of a double phase drug delivery system is achievable by combining two different bead formulations in one dosage form such as a hard gelatine capsule (i.e. a mixture of a bead formulation that contains the drug absorption enhancer and a bead formulation that contains the active ingredient). From the results obtained in this study, the *A. vera* whole leaf material seems to be the most promising drug absorption enhancer for such a double phase bead drug delivery system. If formulated correctly, the one portion of the beads containing the drug absorption enhancer will be able to open the tight junctions followed by the other portion of the beads carrying the insulin to the site of absorption.

4.5 VALIDATION OF THE CHROMATOGRAPHIC ANALYTICAL METHOD

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

The lowest concentration of insulin that could be detected with a RSD ≤ 15% from six replicates was 0.3 µg/ml, which was noted as the HPLC method's LOQ. The lowest insulin concentration peak that was found to be equivalent to three times the average baseline noise was 0.05 µg/ml, which was noted as the HPLC method's LOD.

4.5.2 Specificity

Figures 4.17 to 4.22 illustrate the ability of the HPLC method to distinguish between insulin and other compounds that could be present in the transport samples. The compounds which

were tested for specificity include all the absorption enhancers used in the study, namely *A. vera* gel and whole leaf materials, bile salt mixture, sodium glycocholate, chitosan and Krebs-Ringer Bicarbonate buffer (which was used as the transport medium). Insulin eluded at a retention time of 5.9 min in the HPLC method used for this study.

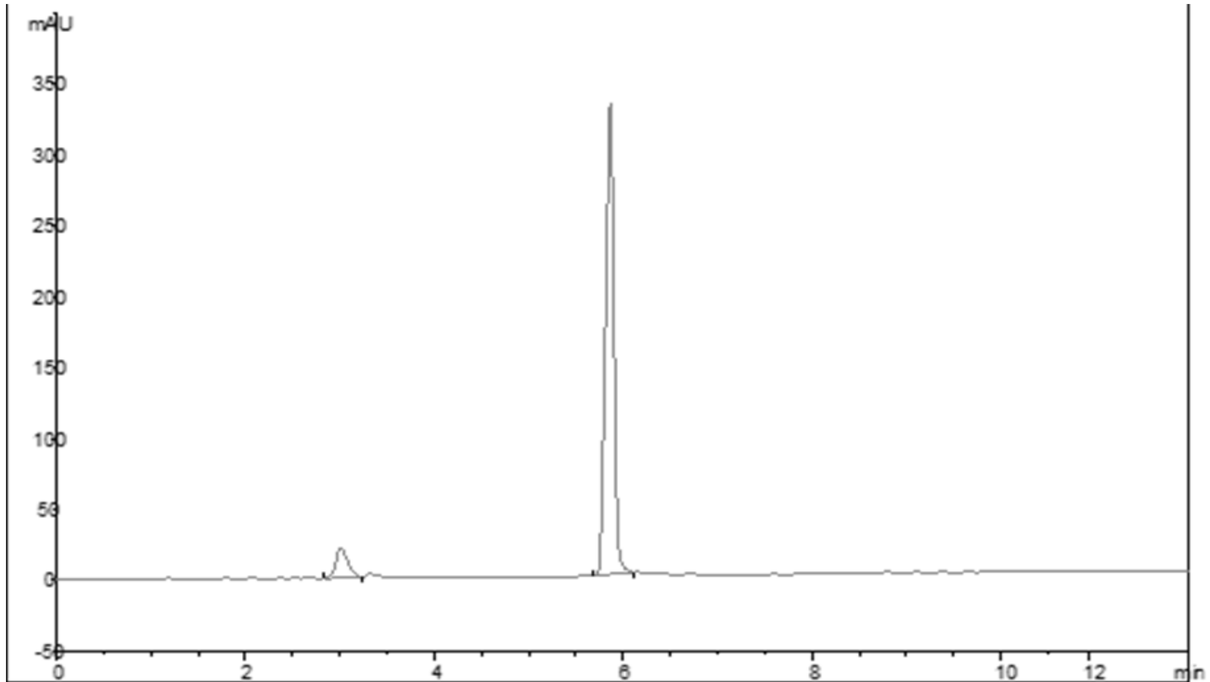

Figure 4.17: Chromatogram of insulin in the presence of Kreb's Ringer Bicarbonate buffer

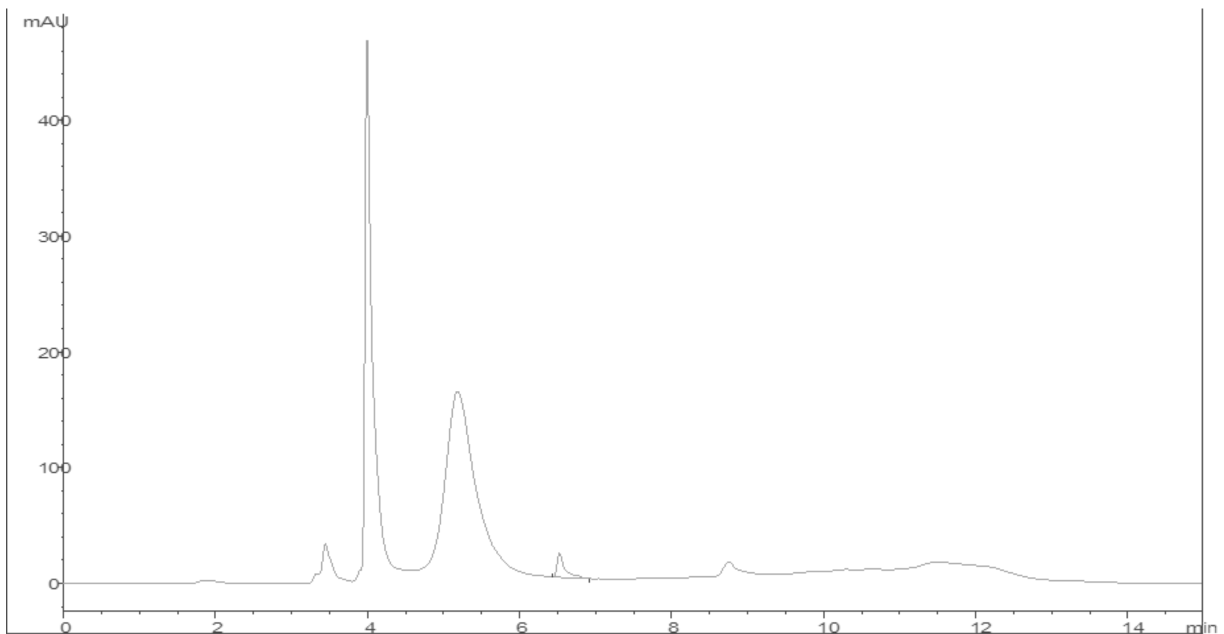

Figure 4.18: Chromatogram of insulin in the presence of *Aloe vera* gel

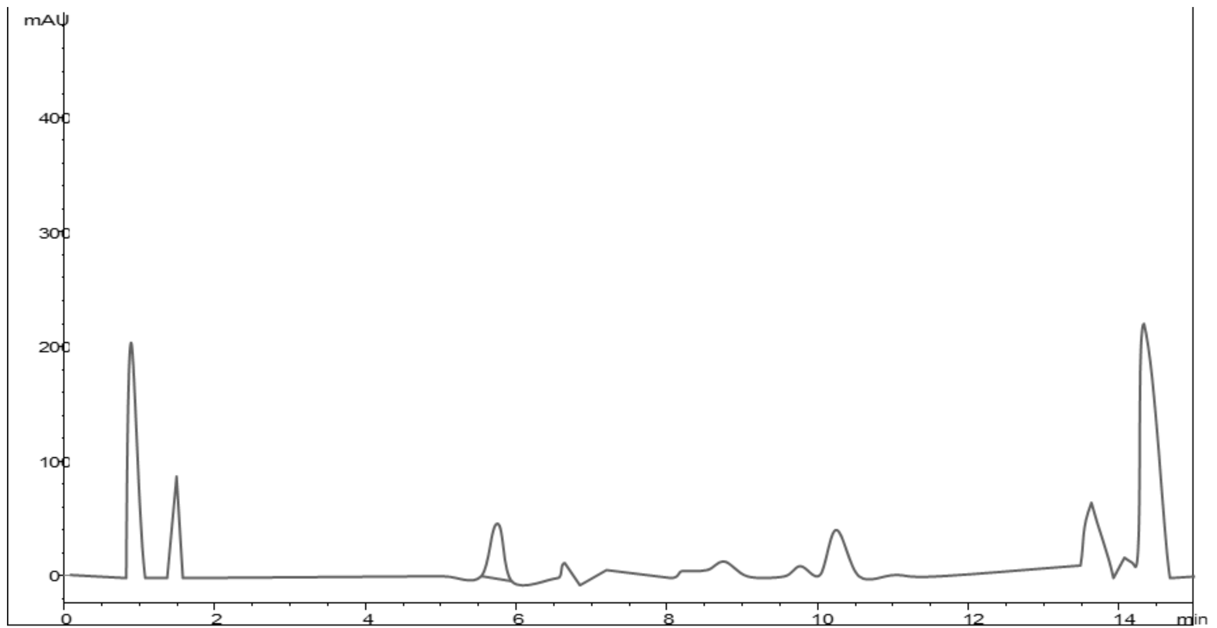

Figure 4.19: Chromatogram of insulin in the presence of *Aloe vera* whole leaf

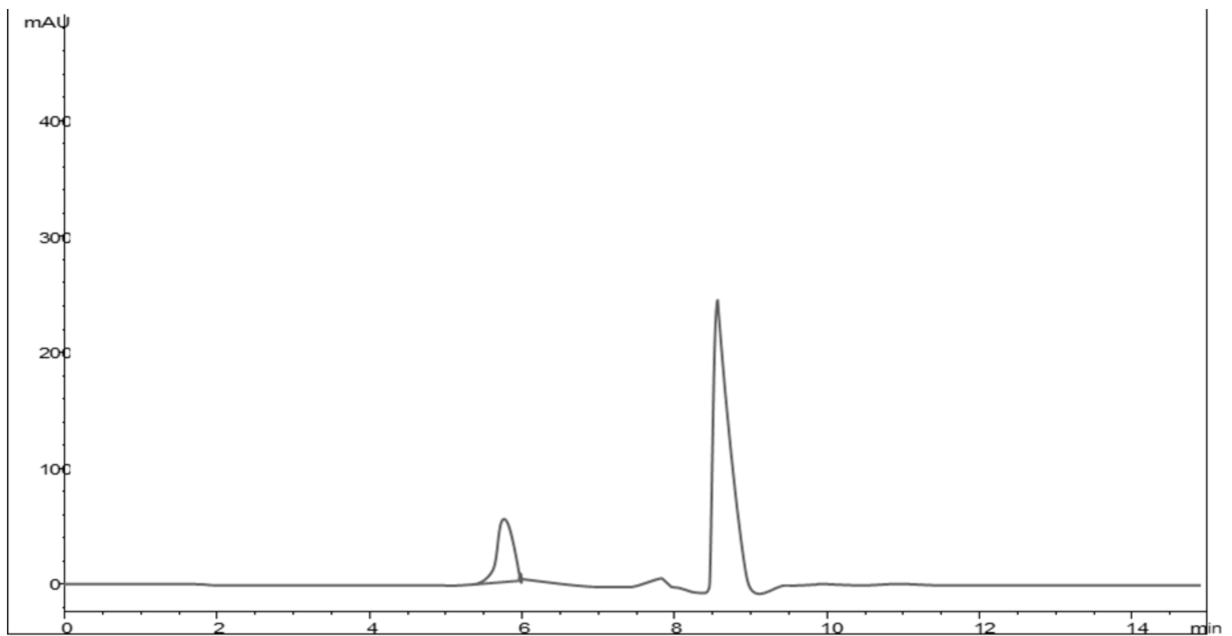

Figure 4.20: Chromatogram of insulin in the presence of Sodium glycocholate

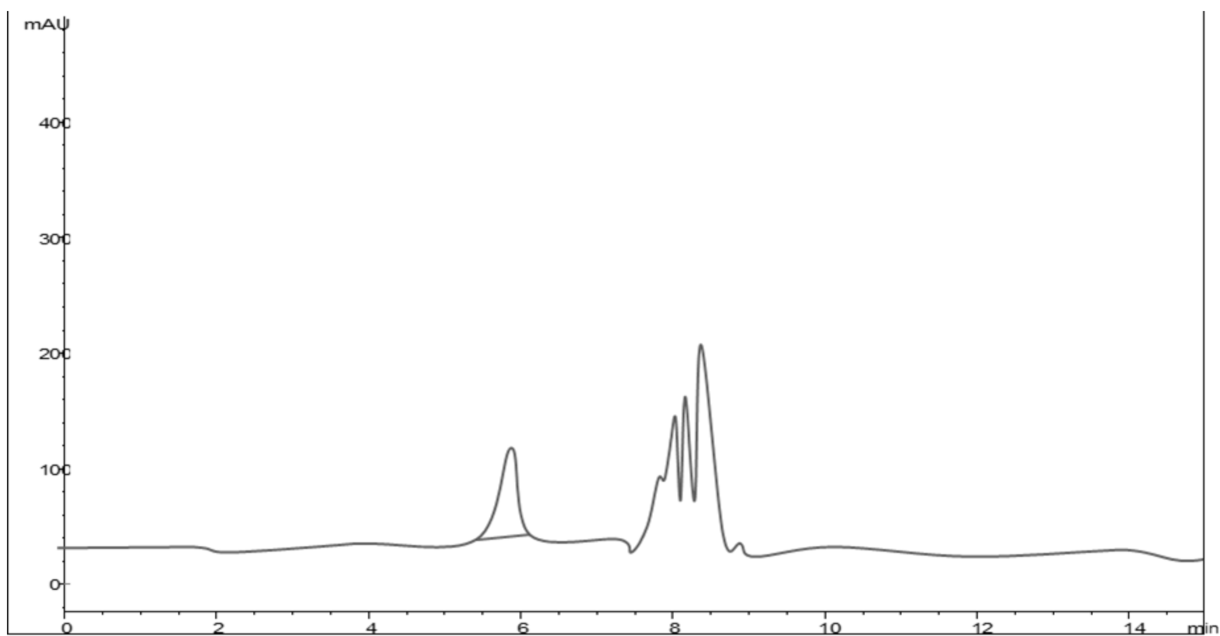

Figure 4.21: Chromatogram of insulin in the presence of Bile salt mixture

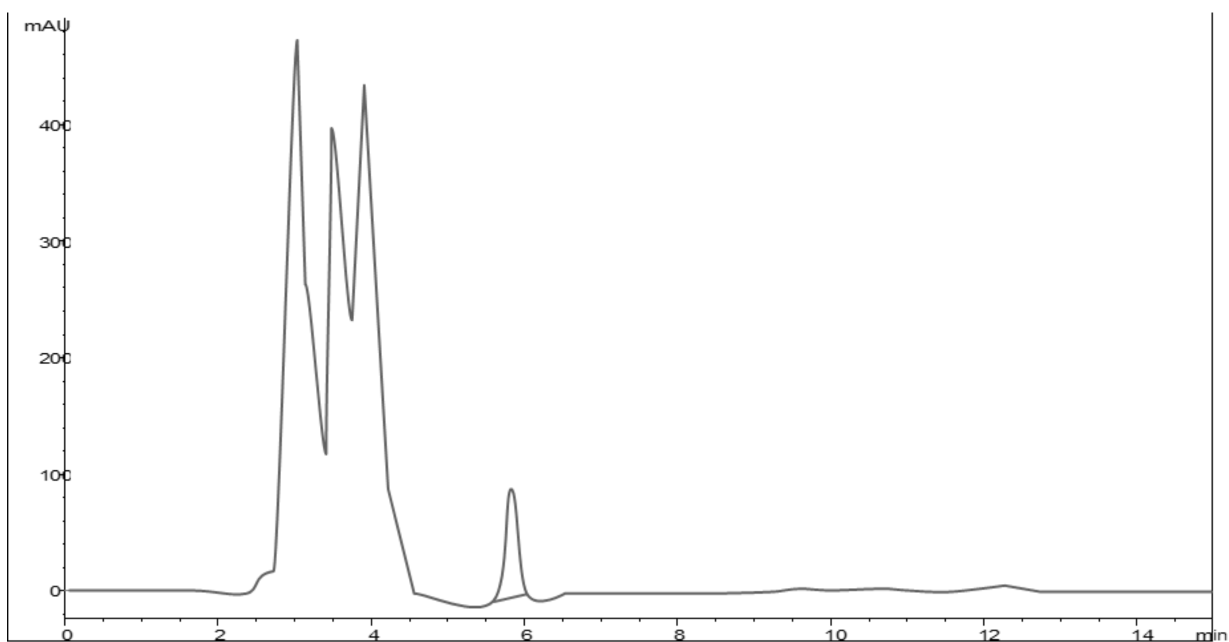

Figure 4.22: Chromatogram of insulin in the presence of chitosan

From these chromatograms, it is evident that the HPLC method could be successfully used to analyse insulin in the transport samples without interference from overlapping peaks of other compounds.

4.5.3 Linearity

Linearity was determined where a standard or calibration curve was obtained with the peak area being plotted as a function of insulin concentration (Figure 4.23). The intercept, slope and regression coefficient are shown in Table 4.8. The regression coefficient (R^2) was 0.9917 for the insulin standard curve, which indicates that the HPLC method used in this study complied with the requirements of the USP (2014:1160). The analytical method complied with the requirement for linearity over the concentration range of 0.3 to 170 μg/ml.

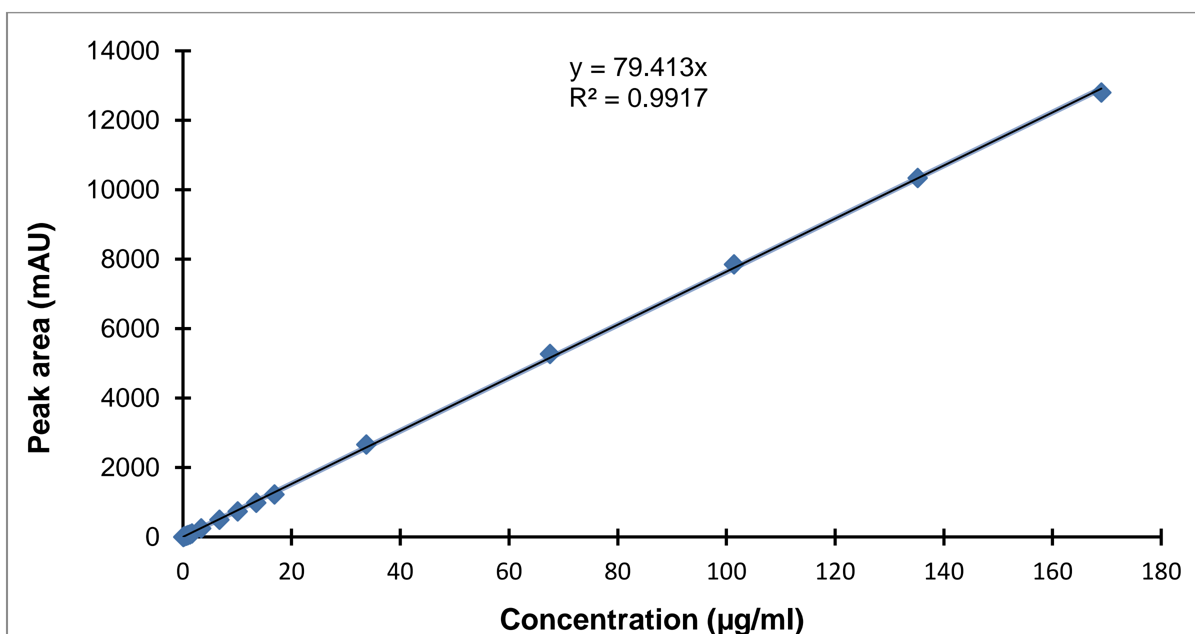

Figure 4.23: Standard curve for insulin on which linear regression was applied

Table 4.3: Regression values for linearity of insulin standard curve

Regression coefficient (R^2)	0.9917
Intercept	0
Slope	79.413

4.6 CONCLUSIONS

From the results of this study it can be concluded that a double phase drug delivery system for insulin oral delivery consisting of different types of beads was formulated with acceptable physical characteristics and drug delivery properties. The beads complied with the requirements of the physical tests and the coated beads released the insulin at a slower rate than the uncoated beads. However, this coating needs to be optmised in future studies in order to delay the insulin release by providing a lag phase. Incorporation of chitosan showed extremely good muco-adhesive properties when compared to the other bead formulations. The beads containing different drug absorption enhancers showed the ability to decrease the TEER of excised pig intestinal tissues to varying extents, which indicate their potential to open tight junctions in order to allow for paracellular drug transport. When excised pig intestinal tissues were pre-exposed to the beads containing drug absorption enhancers, it was shown that insulin transport was enhanced statistically significantly in the apical-to-basolateral direction by *A. vera* whole leaf material containing beads ($p < 0.05$ for ANOVA and Dunn's post-hoc test). The results from this study therefore confirmed the potential of the double phase drug delivery system to effectively deliver insulin across the gastro-intestinal epithelium, albeit this was shown with an *in vitro* model only. Follow-up *in vivo* studies are needed to make any conclusions regarding the efficacy of this delivery system in human subjects, but this research has made a contribution towards finding potential novel drug delivery systems for protein and peptide drugs via the oral route of administration.

CHAPTER 5

SUMMARY OF RESULTS, FINAL CONCLUSIONS AND FUTURE RECOMMENDATIONS

5.1 SUMMARY OF THE PHYSICAL PROPERTIES OF THE BEADS

All the bead formulations complied with the requirements for mass variation as determined by the USP (2014:492), i.e. none of the formulations deviated from the average mass of the bead filled capsules by more than ± 7.5%. This indicates that the extrusion-spheronisation technique was capable of producing relatively uniform beads when using MicroceLac® 100 as filler, irrespective of the other ingredients that were also included (e.g. *A. vera* gel, *A. vera* whole leaf material, bile salt mixture, sodium glycocholate, insulin and chitosan). Since acceptable mass variation was obtained, the double phase dosage form developed in this study should therefore also have acceptable distribution of active ingredient between dosage units.

All the bead formulations showed friability of less than 1%, which means that the formulations have met the requirements as determined by the USP(2014:1146). This indicates that the beads should have sufficient strength to withstand mechanical stresses during packaging and handling.

All the bead formulations prepared in this study showed relatively narrow particle size distribution, which indicates that the extrusion-spheronisation production technique is capable of producing uniform beads irrespective of the ingredients that were added.

5.2 SUMMARY OF THE EFFECT OF COATING ON INSULIN DISSOLUTION

Film coating of insulin beads was done with Eudragit® S/L with a rotating pan coating technique to orchestrate a delayed release of insulin from the double phase delivery system. The coating caused a slower insulin release from the beads as indicated by the dissolution profiles, but it did not provide a lag phase with delayed release. The coating therefore did not provide the desired results for the intended purpose and it is therefore recommended that

future studies should investigate ways to overcome this by applying a thicker layer of coating or by using other coating polymers.

5.3 SUMMARY OF MUCO-ADHESION STUDIES

The beads containing the chitosan (10% w/w) exhibited the strongest muco-adhesive properties of all the bead formulations with 74% retention of the beads during a rinsing technique executed on excised pig intestinal tissues mounted at a 15° slope. The beads containing drug absorption enhancing agents showed much lower retention rates (*A. vera* gel (41%) > *A. vera* whole leaf (25%) > Bile salt mixture (18%) > Sodium glycocholate (16%)). This indicates that after the double phase drug delivery system is administered, the beads containing the absorption enhancer will probably reach the small intestine before the beads containing the insulin due to slower movement of the latter down the gastro-intestinal tract as a result of increased muco-adhesion. This is a desirable outcome, because the idea with the double phase drug delivery system was to get the beads containing the drug absorption enhancer firstly to the site of absorption in order to open the tight junctions and then the insulin beads should reach the site to allow the insulin to get absorbed via the already modulated paracellular pathway.

5.4 SUMMARY OF TRANSEPITHELIAL ELECTRICAL RESISTANCE (TEER) STUDIES

It is clear from the TEER results that all of the absorption enhancer containing beads caused opening of tight junctions in the excised pig intestinal tissues as indicated by a TEER reduction when compared to the control group (beads consisting of MicroceLac®100 only). Beads containing *A. vera* whole leaf material caused the highest TEER reduction after 60 min exposure (51.6% of initial TEER value), while the other drug absorption enhancer containing beads also reduced the TEER to varying extents after 60 min exposure as follows: *A. vera* gel (66% of initial TEER value) > Sodium glycocholate (74% of initial TEER value) > Bile salt mixture (83% of initial TEER value). This indicated the potential of the beads containing drug absorption enhancing agents to increase insulin transport if the intestinal tissue is pre-exposed to these beads. It cannot be excluded that simultaneous exposure of the beads containing drug absorption enhancers with the beads containing insulin may also result in enhanced insulin delivery. However, based on the TEER reduction curves over a period of 60 min, it is evident that a longer period of pre-exposure will probably provide a more pronounced effect on the insulin transport as the TEER reduced constantly over the entire period of exposure to the beads.

5.5 SUMMARY OF *IN VITRO* TRANSPORT STUDIES

It is clear from the *in vitro* transport results that pre-exposure of the excised pig intestinal tissues to the beads containing the drug absorption enhancement agents resulted in increased insulin transport for all the formulations tested. The beads containing *Aloe vera* whole leaf material proved to be the most effective in terms of insulin transport enhancement (P_{app} = 11.6 x 10^{-6} cm/s) followed by *Aloe vera* gel (P_{app} = 3.9 x 10^{-6} cm/s), sodium glycocholate (P_{app} = 2.7 x 10^{-6} cm/s) and the bile salt mixture (P_{app} = 0.7 x 10^{-6} cm/s).

5.6 FINAL CONCLUSIONS

It is clear from the results that the double phase dosage form should be designed in such a way as to allow the beads containing the drug absorption enhancer to reach the site of absorption first in order to open the tight junctions (i.e. the first phase) during a pre-exposure time period (preferably 60 min). This was accomplished with all the selected drug absorption enhancer containing bead formulations prepared in this study. The beads containing insulin should reach the absorption site at a later time point (i.e. the second phase), which can potentially be achieved by inclusion of a muco-adhesive component such as chitosan. The beads containing chitosan showed pronouncedly higher muco-adhesive properties compared to the other beads. The insulin should also ideally be released at this later time point, which can be achieved by applying a film coating onto the beads. The film coating applied to the beads in this study slowed insulin release down, but did not delay it. *In vitro* insulin transport was enhanced by pre-exposure of excised pig intestinal tissues to beads containing the selected drug absorption enhancing agents, but only the bead formulation containing *A. vera* whole leaf material was able to provide statistically significant higher insulin transport (as shown by ANOVA and Dunn's post hoc test). The transport enhancement effect correlated with the reduction in TEER of the excised pig intestinal tissues, indicating opening of tight junctions as the mechanism of drug transport enhancement.

Although proof of concept of a double phase drug delivery system for insulin was shown in this study, it is important to state that the dosage form needs to be optimised in future and also be tested *in vivo* in order to make final conclusions regarding its effectiveness in terms of oral insulin delivery.

5.7 RECOMMENDATIONS FOR FUTURE STUDIES

The double phase drug delivery system provided promising results with regards to peptide drug delivery, which warrants further investigation as recommended below.

- Since there are variations between peptide drugs it would be recommended that other peptide drugs (e.g. buserelin, vasopressin or calcitonin) also be investigated in similar types of drug delivery systems. Other drug absorption enhancers should also be investigated to find optimum drug transport enhancement effects.
- It could add value to the transport results if more than one *in vitro* model and tissues from more than one species of animals are used to evaluate the transport of the peptide drug.
- In order to determine whether the double phase drug delivery system with the selected absorption enhancers can provide clinically significantly insulin bioavailability, *in vivo* evaluation should be done.
- The enteric coating in this study did not provide a sufficient delay in drug release, this should be investigated further in order to optimise the coating formulation and method. Coating can also be manipulated to create various controlled release systems.
- Different concentrations of chitosan and possibly other muco-adhesive agents can be investigated to improve the muco-adhesive properties of the peptide drug containing beads.
- Further modifications can be made to this delivery system to also address other challenges that peptide drugs are faced within the gastrointestinal tract such as enzymatic degradation.

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ADDENDUM A

In vitro Transport data

Table A.1: Insulin transport data after pre-exposure to beads containing *A. vera* gel

Chamber	Inj. Vol. (µl)	Peak area	Correction	Drug transport (µg)	Concentration (µg/ml)	%Transport
M 1					501.4543	100
0	50	0	0	0	0	0
20	50	57.48	57.48	0.86	17.24	3.44
40	50	61.61	63.25	0.91	18.28	3.65
60	50	76.56	78.32	1.05	20.99	4.19
80	50	94.85	97.04	1.22	24.36	4.86
100	50	106.16	108.87	1.32	26.48	5.28
120	50	121.39	124.42	1.46	29.28	5.84
M 2					499.7877	100
0	50	0	0	0	0	0
20	50	49.51	49.51	0.79	15.81	3.16
40	50	89.14	90.55	1.16	23.19	4.64
60	50	90.83	93.38	1.18	23.70	4.74
80	50	94.44	97.04	1.22	24.36	4.87
100	50	110.55	113.25	1.36	27.27	5.46
120	50	123.66	126.82	1.49	29.71	5.94
M 3					500.1082	100
0	50	0	0	0	0	0
20	50	26.1	26.1	0.6	11.6	2.3
40	50	53.7	54.4	0.8	16.7	3.3
60	50	70.5	72.0	1.0	19.9	4.0
80	50	77.7	79.7	1.1	21.2	4.2
100	50	102.9	105.2	1.3	25.8	5.2
120	50	115.9	118.8	1.4	28.3	5.7

Table A.2: Insulin transport data after pre-exposure to beads containing *A. vera* whole leaf material

Chamber	Inj. Vol. (µl)	Peak area	Correction	Drug transport (µg)	Concentration (µg/ml)	%Transport
M 1					1001.1138	100
0	50	0	0	0	0	0
20	50	12.80	12.80	0.65	12.95	1.29
40	50	36.00	36.37	1.81	36.24	3.62
60	50	48.10	49.13	2.44	48.86	4.88
80	50	63.50	64.87	3.22	64.42	6.44
100	50	139.70	141.51	7.01	140.18	14.00
120	50	167.60	171.59	8.50	169.91	16.97
M 2	50				1001.88	100.00
0	50	0.00	0.00	0.00	0.00	0.00
20	50	34.50	34.50	1.72	34.40	3.43
40	50	44.80	45.79	2.28	45.55	4.55
60	50	59.10	60.38	3.00	59.98	5.99
80	50	99.90	101.59	5.04	100.71	10.05
100	50	115.90	118.75	5.88	117.68	11.75
120	50	137.60	140.91	6.98	139.58	13.93
M 3	50				1001.24	100.00
0	50	0.00	0.00	0.00	0.00	0.00
20	50	24.50	24.50	1.23	24.51	2.45
40	50	32.73	33.43	1.67	33.34	3.33
60	50	57.37	58.31	2.90	57.93	5.79
80	50	81.04	82.68	4.10	82.02	8.19
100	50	105.95	108.27	5.37	107.31	10.72
120	50	132.58	135.61	6.72	134.34	13.42

Table A.3: Insulin transport data after pre-exposure to beads containing Sodium glycocholate

Chamber	Inj. Vol. (µl)	Peak area	Correction	Drug transport (µg)	Concentration (µg/ml)	%Transport
M 1					638.7040545	100
0	50	0	0	0	0	0
20	50	576.36	576.36	1.01	20.28	3.17
40	50	630.16	646.63	1.09	21.86	3.42
60	50	641.26	659.26	1.11	22.14	3.47
80	50	649.97	668.29	1.12	22.35	3.50
100	50	742.02	760.59	1.22	24.42	3.82
120	50	851.31	872.51	1.35	26.94	4.22
M 2					639.28	100.00
0	50	0.00	0.00	0.00	0.00	0.00
20	50	609.01	609.01	1.05	21.01	3.29
40	50	696.11	713.51	1.17	23.36	3.65
60	50	746.25	766.14	1.23	24.55	3.84
80	50	932.02	953.34	1.44	28.75	4.50
100	50	914.89	941.52	1.42	28.49	4.46
120	50	1032.16	1058.30	1.56	31.11	4.87
M 3					636.99	100.00
0	50	0.00	0.00	0.00	0.00	0.00
20	50	475.75	475.75	0.90	18.02	2.83
40	50	582.89	596.48	1.04	20.73	3.25
60	50	646.81	663.46	1.11	22.24	3.49
80	50	712.24	730.72	1.19	23.75	3.73
100	50	759.78	780.13	1.24	24.86	3.90
120	50	937.69	959.40	1.44	28.89	4.54

Table A.4: Insulin transport data after pre-exposure to beads containing Bile salt mixture

Chamber	Inj. Vol. (µl)	Peak area	Correction	Drug transport (µg)	Concentration (µg/ml)	%Transport
M 1					460.0670667	100
0	50	0	0	0	0	0
20	50	20.90	20.90	0.12	2.47	0.54
40	50	22.45	23.05	0.13	2.51	0.55
60	50	51.57	52.21	0.15	3.06	0.67
80	50	65.35	66.82	0.17	3.34	0.73
100	50	82.02	83.89	0.18	3.66	0.80
120	50	98.26	100.60	0.20	3.98	0.86
M 2					463.96	100.00
0	50	0.00	0.00	0.00	0.00	0.00
20	50	48.99	48.99	0.15	3.00	0.65
40	50	63.79	65.19	0.17	3.31	0.71
60	50	79.43	81.25	0.18	3.61	0.78
80	50	102.88	105.15	0.20	4.07	0.88
100	50	142.21	145.15	0.24	4.82	1.04
120	50	144.11	148.17	0.24	4.88	1.05
M 3					462.46	100.00
0	50	0.00	0.00	0.00	0.00	0.00
20	50	39.07	39.07	0.14	2.81	0.61
40	50	58.18	59.30	0.16	3.20	0.69
60	50	79.43	81.09	0.18	3.61	0.78
80	50	119.42	121.69	0.22	4.38	0.95
100	50	133.62	137.03	0.23	4.67	1.01
120	50	134.75	138.57	0.23	4.70	1.02

Table A.5: Insulin transport data after pre-exposure to beads containing chitosan

Chamber	Inj. Vol. (µl)	Peak area	Correction	Drug transport (µg)	Concentration (µg/ml)	%Transport
M 1					542.5818462	100
0	50	0	0	0	0	0
20	50	57.56	57.56	0.26	5.19	0.96
40	50	59.54	61.18	0.27	5.45	1.00
60	50	100.85	102.55	0.42	8.35	1.54
80	50	153.03	155.91	0.61	12.10	2.23
100	50	157.59	161.96	0.63	12.53	2.31
120	50	163.55	168.05	0.65	12.96	2.39
M 2					541.27	100.00
0	50	0.00	0.00	0.00	0.00	0.00
20	50	48.22	48.22	0.23	4.54	0.84
40	50	59.57	60.95	0.27	5.43	1.00
60	50	67.37	69.07	0.30	6.00	1.11
80	50	75.38	77.30	0.33	6.58	1.22
100	50	121.81	123.96	0.49	9.86	1.82
120	50	162.72	166.20	0.64	12.83	2.37
M 3					535.89	100.00
0	50	0.00	0.00	0.00	0.00	0.00
20	50	57.56	57.56	0.26	5.19	0.97
40	50	97.59	99.23	0.41	8.12	1.52
60	50	100.66	103.45	0.42	8.42	1.57
80	50	103.38	106.26	0.43	8.61	1.61
100	50	106.35	109.30	0.44	8.83	1.65
120	50	109.64	112.68	0.45	9.07	1.69

Table A.6: Insulin transport data for insulin solution without any pre-exposure

Chamber	Inj. Vol. (µl)	Peak area	Correction	Drug transport (µg)	Concentration (µg/ml)	%Transport
M 1					0	0
0	50	0	0	0	0	100
20	50	0	0	0	0	0
40	50	0	0	0	0	0
60	50	0	0	0	0	0
80	50	0	0	0	0	0
100	50	0	0	0	0	0
120	50	0	0	0	0	0
M 2					0	0
0	50	0	0	0	0	100
20	50	0	0	0	0	0
40	50	0	0	0	0	0
60	50	0	0	0	0	0
80	50	0	0	0	0	0
100	50	0	0	0	0	0
120	50	0	0	0	0	0
M 3					0	
0	50	0	0	0	0	100
20	50	0	0	0	0	0
40	50	0	0	0	0	0
60	50	0	0	0	0	0
80	50	0	0	0	0	0
100	50	0	0	0	0	0
120	50	0	0	0	0	0

ADDENDUM B

Conference Proceedings

ADDENDUM B.1: Abstract

Development of a double phase dosage form for enhanced peptide drug delivery

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Purpose: Parenteral administration remains the most utilised route of administration for therapeutic peptides due to low intestinal epithelial permeability. The purpose of this study is to develop and evaluate a double phase dosage form for effective oral peptide drug delivery.

Methods: Spherical beads were prepared by means of extrusion spheronisation containing insulin and chitosan as mucoadhesive agent. Various other batches of beads were prepared each containing a different drug absorption enhancing agent, which included *Aloe vera* whole leaf, *Aloe vera* gel, bile salt mixture and sodium glycocholate. Mixtures of the insulin containing beads with each of the absorption enhancing agent containing beads were loaded into hard gelatin capsules to prepare four different double phase drug delivery systems. The physical and mucoadhesion properties of the different bead preparations were evaluated, while the insulin delivery performance of the double phase delivery systems was evaluated across excised porcine intestinal tissues in Sweetana-Grass diffusion chambers.

Results: All the bead preparations complied to the specified requirements for physical properties and showed relatively narrow size distribution values. Inclusion of chitosan pronouncedly improved the mucoadhesion properties of the bead formulations. All the double phase drug delivery systems showed enhanced transport of insulin across excised porcine intestinal tissues. *Aloe vera* whole leaf proved to be the most effective absorption enhancer (P_{app} = 11.6 x 10^{-6} cm/s) followed by *Aloe vera* gel (P_{app} = 3.9 x 10^{-6} cm/s), sodium glycocholate (P_{app} = 2.7 x 10^{-6} cm/s) and the bile salt mixture (P_{app} = 0.7 x 10^{-6} cm/s) in the double phase drug delivery systems.

Conclusions: The double phase delivery systems developed in this study proved successful in terms of *in vitro* insulin delivery across excised intestinal epithelial tissues.

ADDENDUM B.2: Report

Development of a double phase dosage form for enhanced peptide drug delivery

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1. Background

Protein and peptide drugs rely mostly on parenteral administration such as subcutaneous or intramuscular injections due to poor oral bioavailability. However, the oral route of administration has many advantages over the parenteral route, which include no need for dosage forms to be manufactured under sterile conditions with reduced production cost as well as avoidance of discomfort, pain and infections normally associated with injections (Fassano, 1998).

There are many barriers that need to be overcome for protein and peptide drugs to be successfully delivered via the oral route of administration. These barriers include enzymatic and chemical degradation, poor aqueous solubility, low intrinsic membrane permeability and pre-systemic metabolism (Chen *et al*., 2009). Oral bioavailability of protein and peptide drugs may be improved by co-administration of chemical absorption enhancers amongst other techniques. Selective and reversible opening of tight junctions between epithelial cells remains one of the most promising strategies to deliver macromolecular drugs systemically by means of the oral route of drug administration (Rosenthal *et al*., 2012).

2. Aim

The aim of this study is to develop and evaluate double phase drug delivery systems that consist of mixtures of beads loaded with an absorption enhancer and a peptide drug in a combined multiple-unit dosage form. The dosage form is designed to make the absorption enhancer available to interact with the intestinal epithelium directly after administration (first phase) followed by the insulin beads (second phase) that moves relatively slowly along the gastrointestinal tract due to mucoadhesion.

3. Methods

3.1 Preparation and evaluation of beads

Beads were prepared by extrusion spheronisation, which can be divided into five steps namely mixing of the powders, wetting of the powder mass, extrusion through a screen, spheronisation and drying. The following bead formulations were prepared: beads

containing insulin (active ingredient, 0.01% w/w) and chitosan (mucoadhesion agent, 15% w/w), four formulations of beads each containing one of four selected drug absorption enhancing agents namely *Aloe vera* whole leaf, *Aloe vera* gel, bile salt mixture and sodium glycocholate. All the beads were prepared with Microcelac® as the filling material and water/ethanol mixture (90:10) was used as wetting agent. An assay was performed on the beads containing the active ingredient and the concentration of insulin was determined by means of a validated high performance liquid chromatgraphy method.

The mucoadhesion was measured with an adapted method as previously described (Vasir *et al.*, 2003).

3.2 Preparation of double phase drug delivery systems

Different mixtures of the beads were loaded into hard gelatine capsules to form five different double phase drug delivery systems as indicated in Table B2.1.

Table B2.1: Composition of the double phase drug delivery systems prepared and investigated for their drug absorption enhancing effects in this study

Formulation	Composition
Double phase drug delivery system: Formulation 1	Beads containing *A. vera* whole leaf + beads containing insulin and chitosan
Double phase drug delivery system: Formulation 2	Beads containing *A. vera* gel + beads containing insulin and chitosan
Double phase drug delivery system: Formulation 3	Beads containing sodium glycocholate + beads containing insulin and chitosan
Double phase drug delivery system: Formulation 4	Beads containing bile salts + beads containing insulin and chitosan
Double phase drug delivery system: Formulation 5	Beads containing insulin and chitosan (without absorption enhancer)

3.3 Evaluation of *in vitro* insulin delivery

The different double phase drug delivery systems were evaluated in terms of insulin delivery across excised pig intestinal tissues in Sweetana-Grass diffusion chambers. The pig intestinal tissues were prepared and mounted in the chambers of the diffusion apparatus as previously described (Beneke *et al.*, 2013).

In order to mimic the *in vivo* situation, a suspension of the beads (0.78 g/7 ml) containing the absorption enhancer was applied to the donor chamber for a period of 1 h while the transepithelial electrical resistance (TEER) was measured at intervals of 20 min. The contents of the donor chambers were then replaced with a suspension of beads containing insulin and chitosan. Samples (200 µl) were withdrawn from the acceptor chambers at 20 min intervals for a period of 2 h. The insulin content of the samples was determined by means of a validated high performance liquid chromatography method. The apparent permeability coefficient (P_{app}) values were calculated from the transport data.

4. Results and Discussion

4.1 Mucoadhesion

The insulin beads containing the chitosan exhibited the highest mucoadhesion of all the bead formulations with 74% retention of the beads on the excised intestinal tissue. This indicates that the beads containing the absorption enhancer will reach the small intestine before the beads containing the insulin. The mucoadhesive properties of the different beads containing absorption enhancing agents were in the following order: *A. vera* gel (41%) > *A. vera* whole leaf (25%) > Bile salt mixture (18%) > Sodium glycocholate (16%).

4.2 *In vitro* insulin delivery

The apparent permeability coefficient (P_{app}) values of insulin delivered across excised pig intestinal tissues from the different formulations are shown in Figure B2.1.

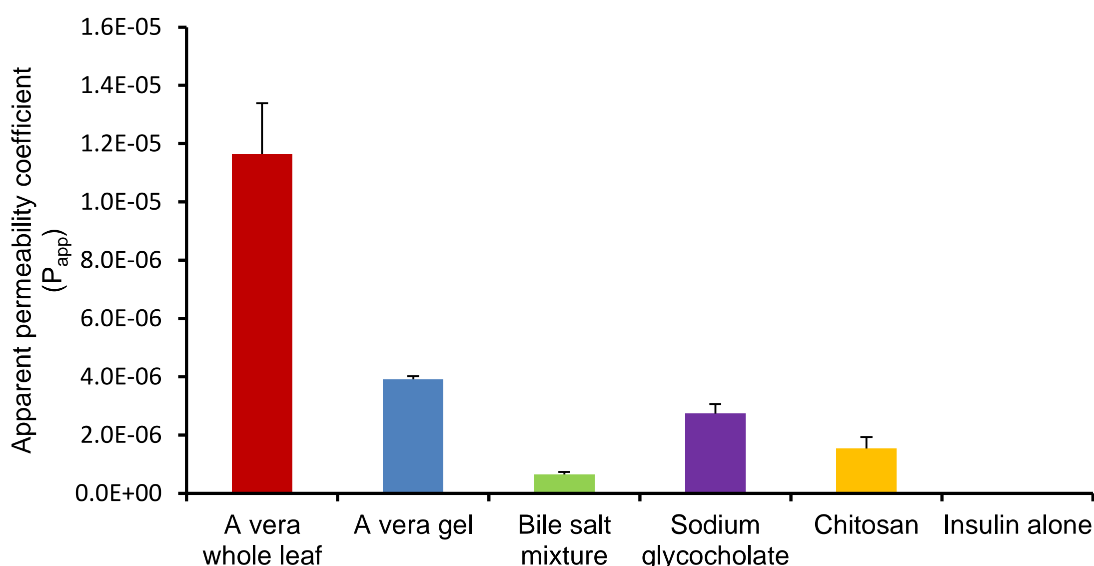

Figure B2.1: Apparent permeability coefficient (P_{app}) values of insulin delivered across excised intestinal tissues from the different double phase drug delivery systems

It is clear from Figure 1 that exposure of the excised intestinal tissues to the beads containing the drug absorption enhancement agents resulted in increased insulin transport for all the formulations tested. The beads containing *Aloe vera* whole leaf proved to be the most effective in terms of insulin transport enhancement (P_{app} = 11.6 x 10^{-6} cm/s) followed by *Aloe vera* gel (P_{app} = 3.9 x 10^{-6} cm/s), sodium glycocholate (P_{app} = 2.7 x 10^{-6} cm/s) and the bile salt mixture (P_{app} = 0.7 x 10^{-6} cm/s).

5. Conclusions

All the double phase drug delivery systems developed in this study showed *in vitro* potential to successfully deliver insulin across intestinal epithelia. The *A. vera* leaf materials showed higher insulin absorption enhancement potential than the bile salts. Inclusion of chitosan caused acceptable mucoadhesion.

6. References

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VASIR, J.K., TAMBWEKAR, K., GARG, S. 2003. Review: Bioadhesive microspheres as a controlled drug delivery system. *International Journal of Pharmaceutics.* 255: 13-32.

ADDENDUM C

HPLC chromatograms (examples)

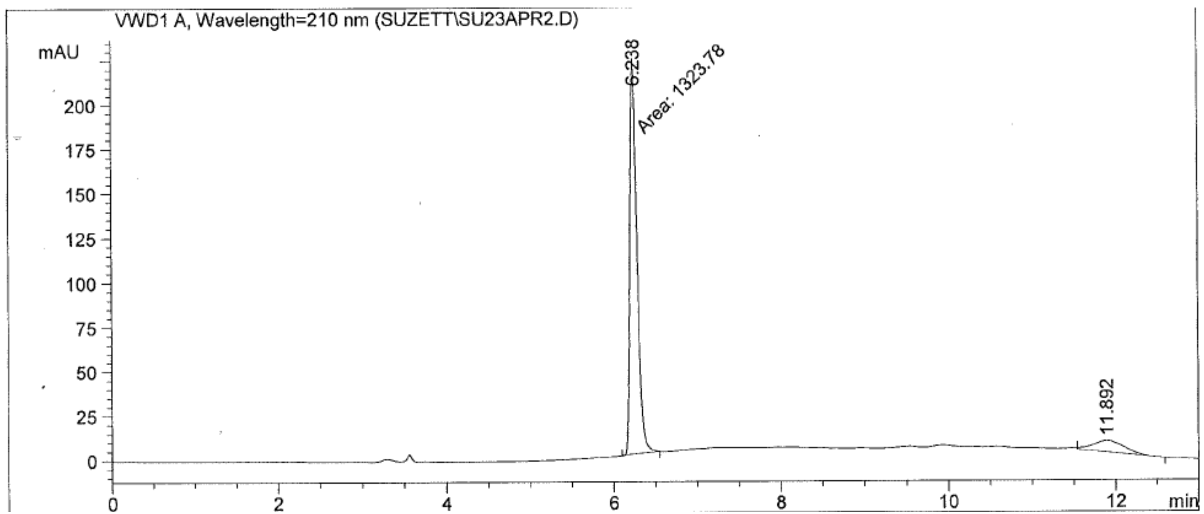

Figure C.1: Chromatogram of insulin for standard curve (10 µl injection volume)

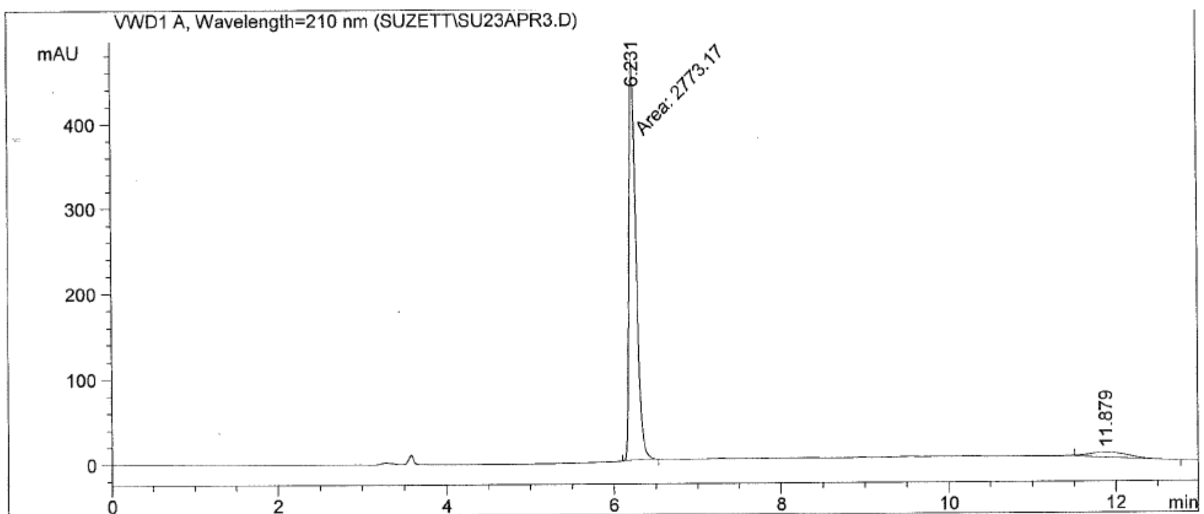

Figure C.2: Chromatogram of insulin for standard curve (20 µl injection volume)

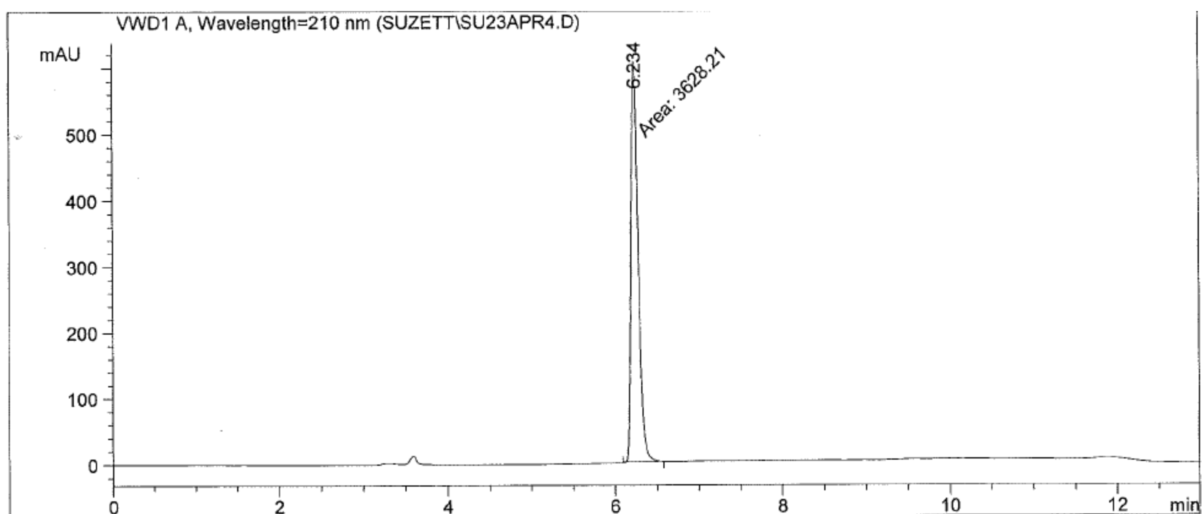

Figure C.3: Chromatogram of insulin for standard curve (30 µl injection volume)

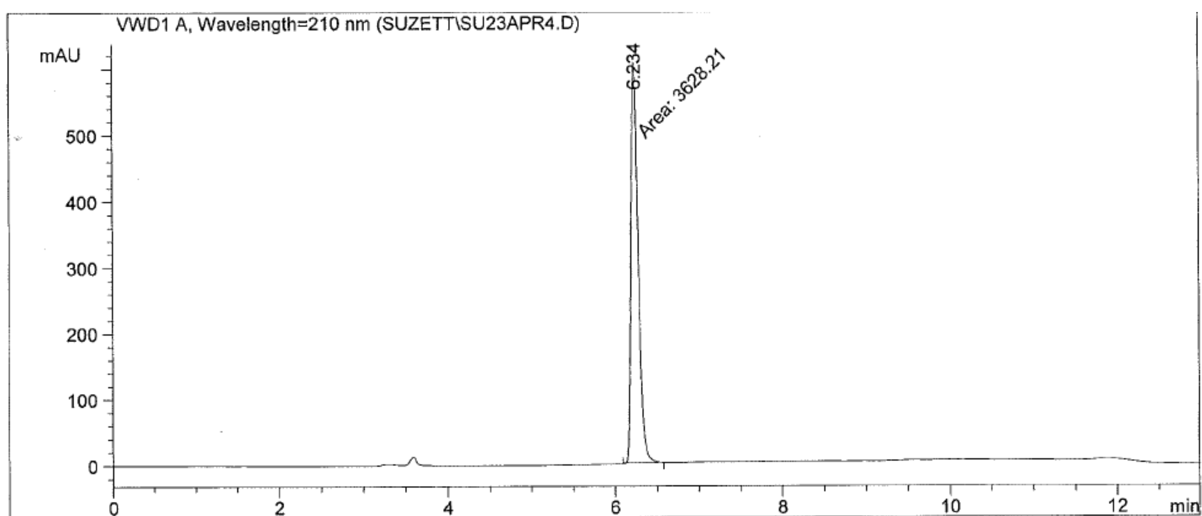

Figure C.4: Chromatogram of insulin for standard curve (40 µl injection volume)

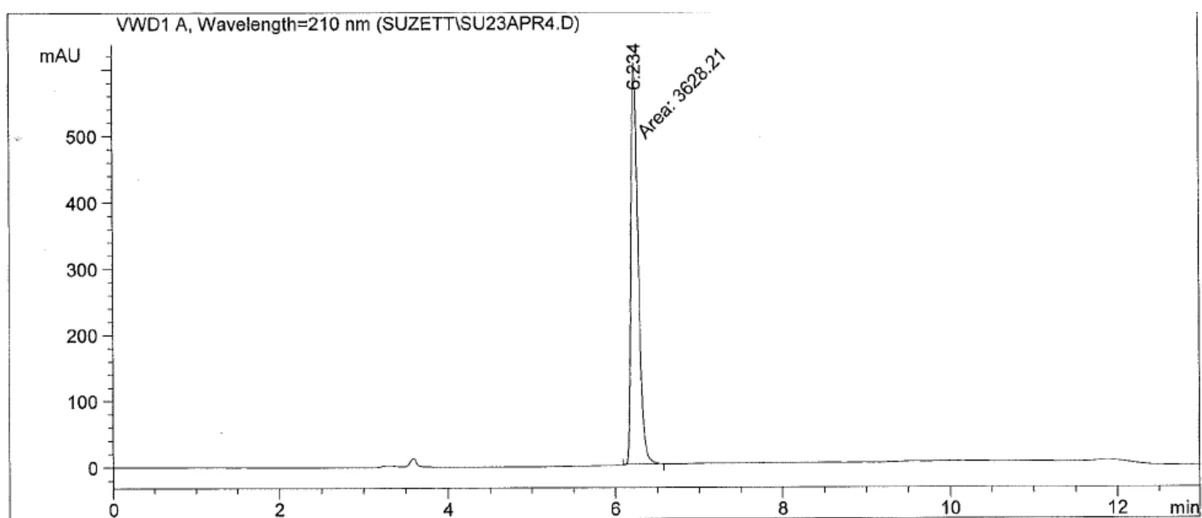

Figure C.5: Chromatogram of insulin for standard curve (50 µl injection volume)

ADDENDUM D

Statistical Analysis

Table D.1: Descriptive statistical data of the transport results

Treatment	2-Way Tables of Descriptive Statistics (T N=18 (No missing data in dep. var. list) Pap Means	Pap N	Pap Std.Dev.
Insulin sol (control)	0.000000	3	0.000000
Aloe vera gel	0.000004	3	0.000000
Aloe vera whole leaf	0.000012	3	0.000002
Sodium glycocholate	0.000003	3	0.000000
Bile mixture	0.000001	3	0.000000
Chitosan	0.000002	3	0.000000
All Grps	0.000003	18	0.000004

Table D.2: ANOVA analysis of the transport results

Variable	Analysis of Variance (Transport data vir statistiek (Sias - Suzette de Bruyn)) Marked effects are significant at p < .05000 SS Effect	df Effect	MS Effect	SS Error	df Error	MS Error	F	p
Pap	0.000000	5	0.000000	0.000000	12	0.000000	98.49755	0.000000

Table D.3: Tukey post-hoc test results (parametric statistical analysis based on ANOVA)

Treatment	Tukey HSD test; Variable: Pap (Transport data vir statistiek (Sias - Suzette de Bruyn)) Marked differences are significant at p < .05000 {1} M=0.0000	{2} M=.00000	{3} M=.00001	{4} M=.00000	{5} M=.00000	{6} M=.00000
Insulin sol (control) {1}		0.000479	0.000159	0.007414	0.882705	0.189659
Aloe vera gel {2}	0.000479		0.000159	0.428392	0.001928	0.020008
Aloe vera whole leaf {3}	0.000159	0.000159		0.000159	0.000159	0.000159
Sodium glycocholate {4}	0.007414	0.428392	0.000159		0.044751	0.410780
Bile mixture {5}	0.882705	0.001928	0.000159	0.044751		0.695150
Chitosan {6}	0.189659	0.020008	0.000159	0.410780	0.695150	

Table D.4: Dunn's (Kruskal-Wallis) post-hoc test results (non-parametric statistical analysis)

	Multiple Comparisons p values (2-tailed); Pap (Transport data vir statistiek (Sias - Suzette de Bruyn)) Independent (grouping) variable: Treatment Kruskal-Wallis test: H (5, N= 18) =16.64767 p =.0052					
Depend.: Pap	Insulin sol (control) R:2.0000	Aloe vera gel R:14.000	Aloe vera whole leaf R:17.000	Sodium glycocholate R:11.000	Bile mixture R:5.0000	Chitosan R:8.0000
Insulin sol (control)		0.088581	0.008686	0.584212	1.000000	1.000000
Aloe vera gel	0.088581		1.000000	1.000000	0.584212	1.000000
Aloe vera whole leaf	0.008686	1.000000		1.000000	0.088581	0.584212
Sodium glycocholate	0.584212	1.000000	1.000000		1.000000	1.000000
Bile mixture	1.000000	0.584212	0.088581	1.000000		1.000000
Chitosan	1.000000	1.000000	0.584212	1.000000	1.000000	