Comparison of drug permeability in rat, pig and human *in vitro* models

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It all starts here ™

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ABSTRACT

A crucial step in the drug discovery and development process is the assessment of membrane permeability properties of new chemical entities and researchers are constantly searching for cost-effective, high through-put models with as high as possible predictive value. In addition, a thorough understanding of the membrane permeability pathways and metabolism mechanisms are required when evaluating drug disposition and pharmacokinetics. Various *in vitro* methods/techniques are available to measure the rate of permeation of compounds across epithelial cell membranes to estimate oral drug absorption in humans.

The aim of this study is to compare three *in vitro* models (i.e. excised rat intestinal tissue, excised pig intestinal tissue and Caco-2 human cell cultures) in terms of drug permeability characteristics by means of different techniques including the Ussing type Sweetana-Grass diffusion chamber apparatus, everted sac glass apparatus and the Transwell® plate apparatus. The transport of abacavir sulphate was determined in two directions (i.e. apical-to-basolateral or AP - BL and basolateral-to-apical or BL - AP) across excised rat intestinal tissue, excised pig intestinal tissue and Caco-2 cell monolayers. The test solution was applied to the donor side and samples (200 μl) were drawn from the acceptor side at 20 min intervals for a period of 2 h. The concentration of abacavir in the samples was then measured by means of a validated high performance liquid chromatography (HPLC) method. The transepithelial electrical resistance (TEER) was measured before and after each transport experiment to give an indication of the integrity of the cell membranes. The apparent permeability coefficient (P_{app}) and efflux ratio (ER) values were calculated and used to compare the different methods and techniques in terms of drug permeation characteristics.

All three of the *in vitro* methods, in all of the techniques employed, showed higher transport of abacavir in the BL - AP direction than in the AP - BL direction. This indicates that all three *in vitro* methods had intact active efflux transporters over the entire study period. The excised rat intestinal method showed similar drug permeability characteristics in both techniques compared to that of the Caco-2 cell monolayers. In contrast, the excised pig intestinal method only showed similar drug permeability characteristics in the Sweetana-Grass diffusion apparatus when compared to the Caco-2 cell monolayers. This phenomenon can possibly be explained by the relatively large surface area of the pig tissue used in the everted sac technique where the role of physiological and other factors take effect. These factors may include the thickness of the membrane and mucus layer as well

as variables such as diet, age, gender and size of the pigs obtained from the abattoir that cannot be controlled.

Key words: *In vitro* model, Sweetana-Grass diffusion chambers, everted sac technique, Caco-2 cell monolayers, high through-put models, abacavir.

UITTREKSEL

'n Kritiese stap in die proses van geneesmiddelontdekking en -ontwikkeling is die studie van membraandeurlaatbaarheidseienskappe van nuwe chemiese entiteite en navorsers is konstant opsoek na koste-effektiewe, hoë-deursettingsmodelle met die hoogste moontlike voorspellingswaarde. Daarmee saam, is 'n deeglike begrip van die onderskeie membraandeurlaatbaarheids-meganismes en metaboliese-weë noodsaaklik vir die evaluering van geneesmiddelfarmakokinetika. Verskeie *in vitro* metodes/tegnieke is beskikbaar om die tempo van geneesmiddeldeurlaatbaarheid oor epiteelmembrane te bepaal in mense na orale geneesmiddelabsorpsie.

Die doel van hierdie studie was om drie *in vitro* modelle te vergelyk (nl. gedissekteerde varkjejenumweefsel, gedissekteerde rotjejenumweefsel en Caco-2 menslike selkulture) in terme van geneesmiddel-deurlaatbaarheidseienskappe met behulp van verskillende tegnieke wat onder andere die "Ussing" tipe Sweetana-Grass diffusie apparaat, "evereted sac" glasapparaat, en die Transwell®-plaat apparaat insluit. Die beweging van abacavir sulfaat oor bogenoemde membrane was getoets in twee rigtings (nl. apikaal-na-basolateraal of AP - BL en basolateraal-na-apikaal of BL - AP). Die toetsoplossings was toegedien aan die skenkerkant en monsters (200 µl) was ontrek vanaf die ontvangerkant met 20 min intervalle oor 'n tydperk van 2 ure. Die abacavir konsentrasie in die monsters was bepaal met behulp van 'n gevalideerde hoë-druk vloeistof chromatografie (HDVC) metode. Die transepiteel elektriese weerstand (TEEW) was gemeet voor en na elke eksperiment as aanduiding van die integriteit en lewensvatbaarheid van die selmembrane. Die deurlaatbaarheidskoeffisiënt (P_{app}) en effluksverhouding (EV) waardes was bereken en gebruik om die verkillende modelle en tegnieke met mekaar te vergelyk in terme van hul geneesmiddel-deurlaatbaarheidseienskappe.

Al drie *in vitro* metodes, in beide die tegnieke, het meer beweging van abacavir getoon in die BL - AP rigting as in die AP - BL rigting. Dit dui daarop dat al drie *in vitro* metodes oor intakte aktiewe efflukstransporters beskik het gedurende die studieperiode. Die gedissekteerde rotjejenumweefselmetode het soortgelyke deurlaatbaarheidseienskappe getoon in beide tegnieke in vergelyking met die Caco-2 selmonolae. In kontras, het die gedissekteerde varkjejenumweefsel slegs soortgelyke deurlaatbaarheidseienskappe getoon in die Sweetana-Grass diffusie apparaat in vergelyking met die Caco-2 selmonolae. Hierdie verskynsel kan moontlik toegeskryf word aan die relatiewe groot oppervlakarea van die varkjejenumweefsel wat gebruik was vir die "everted sac" tegniek waar fisiologiese en ander faktore 'n rol gespeel het. Hierdie faktore mag insluit die dikte van die membraan en

mukuslae sowel as veranderlikes soos dieët, ouderdom, geslag en grootte van die varke wat verkry was vanaf die *abattoir* wat nie beheer kan word nie.

Sleutelwoorde: *In vitro* model, Sweetana-Grass diffusieapparaat, “everted sac” tegniek, Caco-2 selmonolae, hoë-deursettingsmodelle, abacavir.

CONGRESS PROCEEDINGS

1.1 Congress proceedings

Comparison of drug permeability in rat, pig and human *in vitro* models. Presented at the 35th Conference of the Academy of Pharmaceutical Sciences held from 12–14 September 2014 at the Summerstrand Hotel in Port Elizabeth hosted by the Department of Pharmacy at the Nelson Mandela Metropolitan University. (See Appendix A)

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LIST OF ABBREVIATIONS

% RSD	Percentage relative standard deviation
3 R's	Reduce, Replace, Refine
ABC	ATP-binding cassette
ANOVA	Analysis of variance
AP	Apical
AP - BL	Apical to basolateral
ATP	Adenosine triphosphate
BCS	Biopharmaceutics Classification System
BL	Basolateral
BL - AP	Basolateral to apical
Caco-2	Human Caucasian colon adenocarcinoma
CYP 3A4	Cytochrome P450 3A4
df	Degrees of freedom
DMEM	Dulbecco's Modified Eagle's Medium
ECACC	European Collection of Cell Cultures
ER	Efflux ratio
EV	Effluksverhouding
F	Ratio
FBS	Fetal bovine serum
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric acid
HDVC	Hoë-druk vloeistof chromatografie
HEPES	[n-(2-hydroxyethyl), piperazine-N-(2-ethanesulfonic acid)]
HIV	Human immunodeficiency viral
HPLC	High performance liquid chromatography
ICH	International Conference of Harmonisation

KRB	Krebs-Ringer bicarbonate
LOD	Limit of detection
LOQ	Limit of quantitation
MDCK II	Madin-Darby canine kidney II
MS	Mean squares
NCE	New chemical entities
NEAA	Non-essential amino acids
NRTI	Nucleoside reverse transcriptase inhibitor
P_{app}	Apparent permeability coefficient
PBS	Phosphate buffered saline
PCDDP	Pre-clinical drug development platform
PD	Potential difference
P-gp	P-glycoprotein
R^2	R squared
RSD	Relative standard deviation
SD	Standard deviation
SS	Sum of squares
TEER	Transepithelial electrical resistance
TEEW	Transepiteliale elektriese weerstand
USP	United States Pharmacopoeia

CHAPTER 1: INTRODUCTION

1.1 Background and justification

1.1.1 Screening of biopharmaceutical properties of compounds

A crucial step in the drug discovery and development process is the assessment of membrane permeability properties of new chemical entities (NCE) and researchers are constantly searching for cost-effective, high-throughput models with high predictive value (Balimane & Chong, 2005:335). In addition, a thorough understanding of the mechanisms of membrane transport is required when evaluating drug disposition and pharmacokinetics (Mudra *et al.,* 2011:750). High throughput, less predictive models are often utilised for primary screening purposes followed by low throughput, more predictive models for secondary screening purposes (Balimane *et al.,* 2006:E1).

1.1.2 Nomenclature

In this dissertation, differentiation will be made between different models, methods and techniques. The term "model" refers to a study within one of the following categories: *in vivo*, *in situ*, *in vitro* or *in silico*. The term "method" refers to different options within a model such as cell cultures or other excised animal tissues (from different species). The term "technique" refers to different ways or apparatuses that will be used to conduct a transport study with a specific method (e.g. Transwell® membrane plates, Sweetana-Grass diffusion chambers or everted sac apparatus).

1.1.3 Models for evaluating drug absorption

Various models, methods and techniques are available to measure the rate of permeation of compounds across epithelial cell membranes to estimate oral drug absorption in humans. These methods are broadly categorised into computational, physicochemical and biological models (Ashford, 2008:304). The biological models are further sub-divided into the following types:

- *In vitro* (a term which refers to experiments outside the living organism in a controlled environment and includes artificial membranes, cell cultures and excised tissues used in everted sacs and in Ussing type chambers).

- *Ex vivo* (a term which refers to excised tissues removed from organisms used in techniques such as everted sacs and in Ussing type chambers).

- *In situ* (a term which refers to experiments in an organ as part of a living organism, e.g. single pass perfusion through segments of the intestinal tract).

- *In vivo* (a term which refers to experiments in a living organism, e.g. various animal models such as rats or humans) (Volpe, 2010:672; Dixit *et al.,* 2012:13; Reis *et al.,* 2013:781).

In vivo bioavailability studies in vertebrates are time-consuming, require relatively large amounts of the test compounds and can exhibit great variability due to differences in luminal contents, mucus layer thickness, hepatic clearance and animal-to-animal or species-to-species variation (Hämäläinen & Frostell-Karlsson, 2004:397).

In vitro approaches are commonly used to evaluate biopharmaceutical properties of compounds, especially to screen large numbers of lead compounds where financial considerations are important. Models that closely mimic conditions in the human intestinal mucosa produce absorption data, which can help to select lead compounds that are more likely to be successful in clinical development. In some cases, the data may be sufficiently predictive for regulatory approval of a generic product depending on the biopharmaceutical classification of the specific drug. However, one drawback with all *in vitro* models is the fact that physiological factors such as disease state, hepatic or renal dysfunctions and age are not reflected in the results (Sarmento *et al.,* 2012:607). *In vitro* models for predicting intestinal drug absorption include the determination of the partition coefficient or log P values, measuring drug permeability by means of artificial membranes (e.g. immobilised artificial membrane columns or parallel artificial membrane permeation assays), measuring drug transport across cultured cell monolayers (e.g. Caco-2 cells), measuring drug transport across excised animal tissues in Ussing type chambers and surface plasma resonance biosensor analysis (Ashford, 2002:217; Li, 2001:357).

One of the most commonly used *in vitro* cell culture based permeability models to estimate a compound's absorption in humans, after oral administration, is the human adenocarcinoma cell line (i.e. Caco-2). Although a good correlation exists between Caco-2 cell monolayer permeability and drug absorption in humans, this approach has certain limitations. When drugs have very low membrane permeability or when drugs are absorbed through carrier-mediated pathways, discrepancies may arise. Furthermore, it does not account for the dose or the solubility of the drug in the intestinal fluids (Cao *et al.,* 2006:1675).

1.1.4 Abacavir as model compound for transport studies

Due to abacavir's high solubility and high intestinal permeability, it is classified as a Class I compound according to the Biopharmaceutics Classification System (BCS) (Wu & Benet, 2005:13). Abacavir is a weak base (pK_a= 5.01) and solubility is therefore higher in intestinal fluids with pH values of 5 to 6.5 (more soluble in an acidic environment) (Kumar *et al.* 2010:66). Abacavir, a nucleoside reverse transcriptase inhibitor (NRTI), is used for the treatment of human immunodeficiency viral (HIV) infection and is readily absorbed (> 83%) after oral administration and freely partitions into most tissues (Kumar *et al*., 1999:603; Chittick *et al*., 1999:933 & 938). Abacavir is primarily metabolised by alcohol dehydrogenase to form two metabolites: glucuronide conjugate and a carboxylic acid derivative. These metabolites do not have any anti-HIV or cytotoxic effects. Both water soluble metabolites (glucuronide conjugate and carboxylic acid derivative) are excreted from the body mainly by the kidney in the urine (Chittick *et al*., 1999:933).

A previous study showed abacavir is effluxed from the Madin-Darby canine kidney II (MDCK II) cell monolayers with higher transport in the basolateral (BL) to apical (AP) direction than in the AP to BL direction. With the addition of a P-gp inhibitor (e.g. verapamil) the efflux of abacavir was decreased (Shaik *et al.*, 2007:2082-2084).

1.2 Problem statement

Although several *in vitro* models already exist for evaluating drug permeation and metabolism, researchers are constantly searching for new models that are more cost-effective and highly predictive of the *in vivo* scenario. As mentioned before, Caco-2 human cell cultures are commonly used as a method for screening drug permeability, but this method has drawbacks such as the relatively high cost of the Transwell® membrane plates, the possibility of infections over a relatively long growth period and morphological changes that may occur during culturing of the cells. The excised rat intestinal tissue model has been used frequently as a model for *in vitro* transport studies, but showed different absorption rates than in humans (Jung *et al*., 2000:147).

Pig intestinal tissue is available as a by-product from the slaughtering of pigs for meat production, which makes it an attractive model for drug permeation studies. Although the excised pig intestinal model has been used before (Pietzonka *et al.,* 2002:39), relatively little information is available about how it compares with other *in vitro* models in terms of drug permeation. By comparing the drug permeability properties of the mentioned *in vitro* animal methods (i.e. rat and pig) with a human method (i.e. the Caco-2 cell monolayers), it will give

an indication of their usefulness as *in vitro* models in both the diffusion chambers and everted sac techniques for drug permeation and metabolism screening.

1.2.1 General aim

The aim of this study is to compare three different *in vitro* models (i.e. the Caco-2 cell monolayer, pig intestinal tissue and rat intestinal tissue) in terms of their drug permeability properties.

1.2.2 Specific objectives

- To culture Caco-2 cells in confluent monolayers on 6-well Transwell® membrane plates to provide a suitable human cell culture method for transport studies.
- To develop standardised glass apparatuses for everted sac experiments for each of the excised rat and pig intestinal methods.
- To compare the bi-directional transport of abacavir as model compound across three different *in vitro* methods, namely Caco-2 cell monolayers, excised rat intestinal tissue and excised pig intestinal tissue.
- To compare the bi-directional transport of abacavir across the excised tissue methods (i.e. rat and pig) between the Sweetana-Grass diffusion chamber technique and the everted sac technique.
- To develop and validate a high performance liquid chromatography (HPLC) analytical method for abacavir.

1.3 Ethics

An ethics application was submitted to the Ethics Committee of the North-West University. The study and all experimental procedures were approved and an Ethical approval number was issued (NWU-0030-13-A5) (Appendix B).

1.4 Dissertation layout

Chapter 1 describes the rationale and motivation for this study as well as the aim and objectives. A literature review on the topic of intestinal drug absorption evaluation is given in Chapter 2. The materials being used in this study are listed and a full description of the methods followed is given in Chapter 3. In Chapter 4, the results and statistical analyses are presented in the form of tables and figures and an explanation and conclusion of the results

achieved is discussed. A final conclusion and recommendations for future studies are summarised in Chapter 5.

CHAPTER 2: LITERATURE REVIEW ON INTESTINAL DRUG ABSORPTION EVALUATION

2.1 Introduction

2.1.1 Anatomy and physiology of the gastrointestinal tract

Four main layers can be distinguished in the wall of the gastrointestinal tract: the serosa, muscularis externa, submucosa and the mucosa, as shown in Figure 2.1.

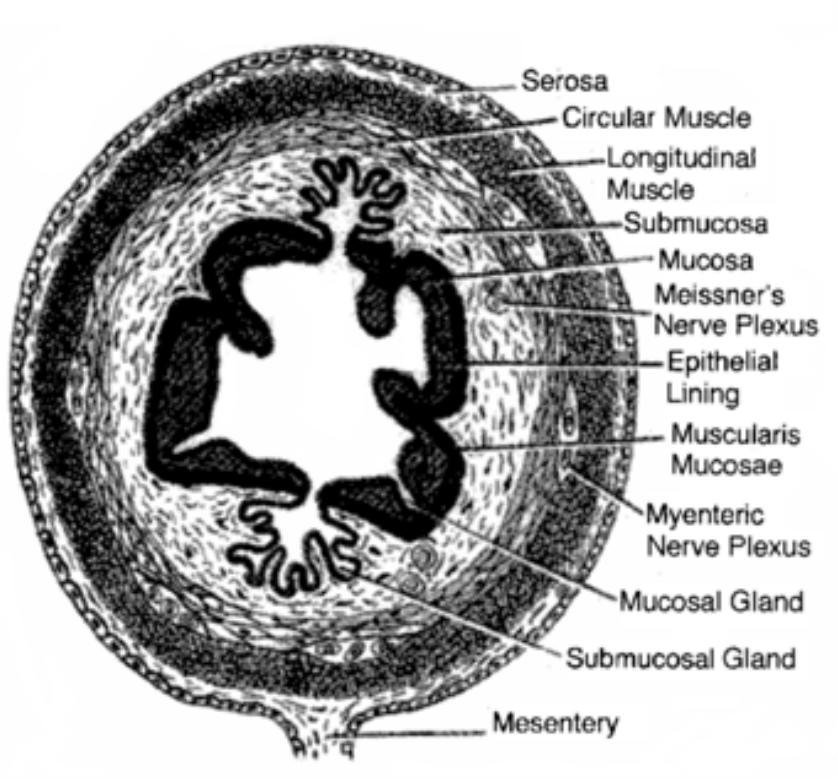

Figure 2.1: Cross-sectional view of the small intestine anatomy (Deferme *et al*., 2008:184)

The serosa is the most outer layer of the gastrointestinal tract, providing connective tissue to attach and keep the intestine intact. The muscularis externa consists of two smooth muscle layers which cause peristalsis when they contract and relax. The outer layer of muscle is thinner and longitudinally orientated, whilst the inner layer is much thicker and in a circular formation. The sub-mucosa is richly supplied with blood vessels and nerve cells. The nerve cell network within this layer is called the sub-mucosal plexus. A mucosal layer (on the apical side) contains the muscularis mucosa, lamina propria and the epithelium, which are responsible for providing a physical barrier to control the movement of compounds and to

protect the organism against pathogens changing the shape of the intestine and acts as connective tissue (Aulton, 2007:271).

The epithelial surface consists of villi, microvilli and intestinal folds, which are in contact with the luminal contents. The villi and microvilli form the so called crypt-villus axis as shown in Figure 2.2 and are composed of differentiating, proliferating, functional and absorptive cells. These crypts enlarge the surface area of the gastrointestinal tract which in turn ensures longer residence times which promotes absorption of ingested substances to a great extent (Engman, 2003:9).

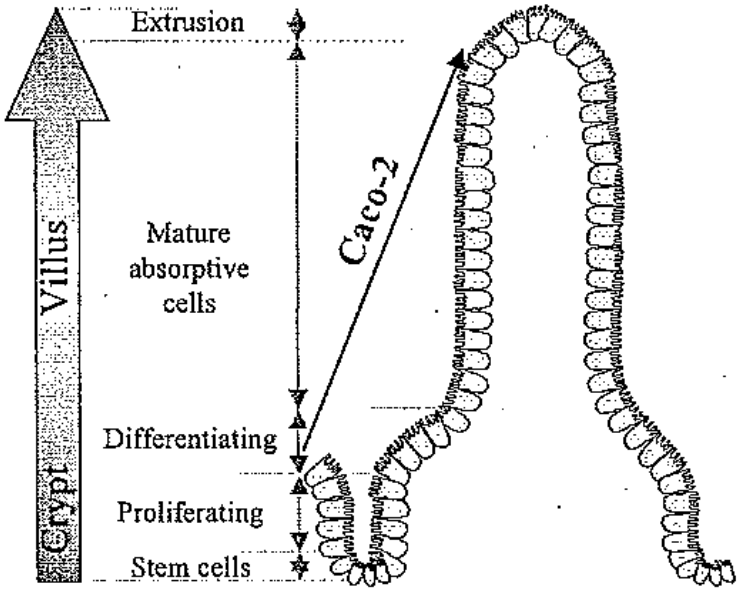

Figure 2.2: Schematic illustration of the intestinal epithelium showing the crypt-villus functional unit (Engman, 2003:9)

The gastrointestinal tract secretes a great amount of fluids such as bicarbonates, enzymes, mucus, surfactants, electrolytes, hydrochlorides and water. These secretions may cause fluctuations in surfactant concentrations, pH, buffer capacities and the luminal content volumes. Fluctuations in secretion composition may limit solubility and the rate of dissolution of compounds and should be taken into consideration when performing dosage corrections (Dressman *et al*., 1998:14-15).

2.1.2 Comparison between rat, pig and human gastrointestinal anatomy

The rat, pig and human gastrointestinal tract is approximately 0.15 m, 47.00 m and 8.75 m in length, respectively, and has a tube-like structure with differing diameters (Kararli, 1995:353-356). It stretches from the oral cavity all the way to the anus with four major anatomical areas namely the oesophagus, stomach, small intestine (duodenum, jejunum and ileum) and the large intestine/colon (Figure 2.3).

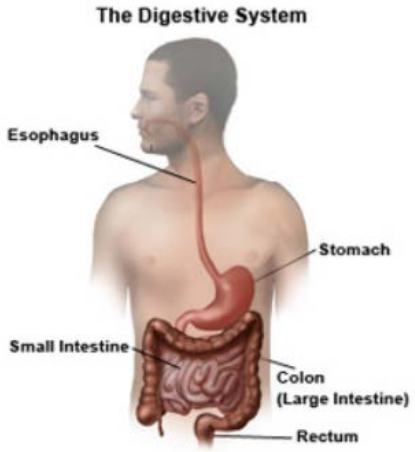

Figure 2.3: Four major anatomical areas including the oesophagus, stomach, small intestine and the large intestine/colon (digestivehealthreno.com, 2013)

2.1.2.1 Oral cavity

The oral cavity has a neutral pH and is lined with salivary glands which secrete saliva. A volume of approximately 1500 ml of saliva is secreted daily from the salivary glands. Salivary amylase is a major component of saliva and is required for the digestion of starch. A glycoprotein, namely mucin, is the component of saliva which is responsible for lubrication of the compounds that are taken orally and may have some interaction with certain chemicals (Shargel *et al.,* 2005:384).

2.1.2.2 Oesophagus

The oesophagus is a muscular tube, approximately 0.25 m in length, which connects the oral cavity to the stomach. The lumen is lined with a multilayer of epithelial cells. The oesophageal wall has two muscular layers, namely the outer muscle layer, with longitudinal fibres that runs over the length and the inner muscle layer with circular fibres that form rings around the oesophagus. These muscle layers help with peristalsis, which aids in the downward movement of swallowed substances (Meiring *et al*., 2006:248).

2.1.2.3 Stomach

A human's stomach can be divided into a glandular and non-glandular portion. The first mentioned portion of the stomach is thick walled, whilst the second portion is relatively thinner. Furthermore, these two portions are lined with different types of epithelium which distinguishes each with its own set of properties and functions. The glandular portion is covered by columnar epithelia. The tubular gastric glands, with mucus-secreting neck cells, pepsinogen secreting chief cells and hydrochloric acid (HCl)-secreting parietal cells can be found here. The non-glandular portion on the other hand is lined with keratinised stratified squamous epithelia which contribute to the digestion and storage of food (Stevens, 1977:216). The stomach of humans is composed mainly of the glandular type. In rats, parietal and chief cells occupy the lower third of the lamina propria and it is considerably smaller than that of the human and pig (Kararli, 1995:351-352). The difference between the pig and human stomach is the former's is three times larger and the cardiac mucosa lines the greater portion of the stomach (Kararli, 1995:352). From the stomach, the contents pass through the pylorus sphincter into the small intestine.

2.1.2.4 Small intestine

The small intestine forms the major site of absorption in the gastrointestinal tract for nutrients and drugs. Data from post mortem investigations showed the average length of the small intestine is 1.02 m for the rat, 18.00 m for the pig and 6.25 m for the human (Stevens, 1977:217). The villi, which cover 90% of the luminal surface of columnar absorptive cells and enterocytes, create a large surface area which promotes absorption of various substances (Carr & Toner, 1984:12; Kararli, 1995:354-356). This area is enlarged even more by micro-villi which are situated on the luminal surface of the enterocytes. The expression of micro-villi per unit area of villi surface in rats is estimated to be 65 micro-villi per square micrometre (μm^2) of villi surface. The amount of micro-villi differs from species to species, but by taking the size of micro-villi surface area into account, it becomes a constant value of 25 per μm^2. The villi and micro-villi are known as the brush border (Robinson *et al*., 1977:190; Kararli, 1995:356). The small intestine is further divided into three sections with varying lengths, the duodenum, jejunum and ileum. Comparing these three sections, the duodenum is the shortest followed by the jejunum and ileum. The transit time of contents is faster in the duodenum and proximal jejunum and increases in time as the contents approach the ileum. The duodenum also has less villi present than the jejunum and ileum. Furthermore, the pH of the duodenum, jejunum and ileum also differ with values ranging from 4.6 - 6.0, 6.3 - 7.3 and 7.6 respectively. The diameter of the rat, pig and human small intestine is 0.30 to 0.50 cm, 2.50 to 3.50 cm and 5.00 cm, respectively and it empties into the

large intestine (colon) (Kararli, 1995:3560; Stevens, 1977:218). From the literature and previous studies it is clear that the major sites of absorption are the duodenum and proximal jejunum (Kararli, 1995:356; DeSesso & Jacobson, 2001:215). The jejunum is the most suitable region of the gastrointestinal tract to perform transport studies on as it is longer than the duodenum and therefore more tissue can be obtained from a single animal and more replicates can be done in the same animal (to prevent inter-subject variation).

2.1.2.5 Colon

The average length of the human colon is approximately 0.90 to 1.50 m and can be divided into ascending, transverse, descending and sigmoid sections. The colon of rats and pigs differs from humans, as their average colon length is 0.90 to 1.10 m and 4.00 m, respectively (Kararli, 1995:357). On the luminal surface of the colon there are no villi, but it is divided into geographical areas by transverse furrows. Microvilli can be found in the colon, but they are not as densely expressed as in the small intestine. Another difference between the three species is that the colon of the human and pig has a saclike appearance (Taylor, 1972:443; Kararli, 1995:357). Furthermore, the ceacal region of humans is poorly defined and is a protrusion that continues from the colon, the ceacum of the pig is much longer, whilst the rat's ceacum is large compared to its colon and does not have a saclike appearance (Kararli, 1995:357).

2.1.2.6 Rectum

In lower mammals, the rectum is aligned "straight" in contrast with the human rectum, which is curved and fits exactly in the sacrum (Heald & Moran, 1998:66). The viscous fluid of 1 to 3 ml within the rectum has a pH 7.5 to 8 and an average temperature of ± 37°C. It is well supplied with blood by three veins (superior, middle and inferior rectal veins). The superior rectal vein drains into the portal circulation, which leads to the liver and chemical entities absorbed from this region may experience first pass effects. The middle and inferior rectal veins will directly enter the systemic circulation by draining into the inferior vena cava and thereby bypassing the liver. Compounds within the rectum may be absorbed by means of transcellular and paracellular mechanisms. The extent of drug absorption from the rectum is governed by various factors such as spreading over the surface area, drug release, absorption rate, drug residence time (in its unchanged form) and the molecular weight of the drug(De Boer & Breimer, 1997:230-231).

2.1.3 Absorption mechanisms within the gastrointestinal tract

For a chemical entity to become bioavailable after oral administration, it needs to cross the intestinal lining. This can be accomplished by passive diffusion through the epithelial cells (i.e. transcellular), through the interstitial spaces (i.e. paracellular) or by vesicular uptake or by active transport (Dressman *et al*., 1998:11-12).

Passive diffusion is the process by which molecules spontaneously diffuse from a region of higher concentration to a region of lower concentration. This mechanism does not require energy to accomplish transport of molecules and is driven by the concentration gradient (Shargel *et al.,* 2012:326-327).

Active transport, in contrast, is energy dependant since diffusion of compounds can occur against a concentration gradient (i.e. from a lower concentration to a higher concentration). For a compound to be transported by active transporters, it needs to bind to the carrier molecule to form a carrier-compound complex, which will then traverse across the membrane and dissociate on the other side thereby releasing the compound and reactivating the carrier molecule (Shargel *et al.,* 2012:328-329).

The most common transport mechanisms for drug molecules across the intestinal epithelium are illustrated in Figure 2.4 and discussed in further detail below.

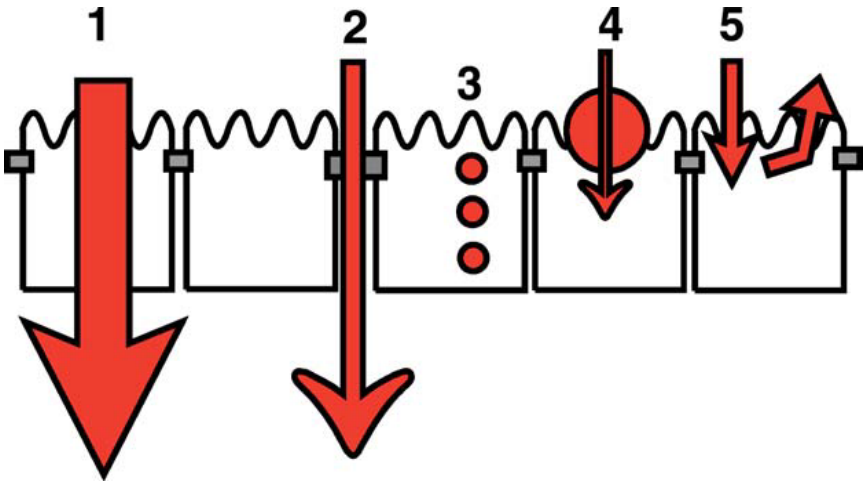

Figure 2.4: Pathways of drug transport across the intestinal mucosa. (1) Passive transcellular transport, (2) Passive paracellular transport, (3) Transcytosis, (4) Carrier-mediated transport and (5) Efflux transport (Deferme *et al.,* 2008:185)

2.1.3.1 Passive transcellular transport

The transcellular pathway is the main route by which most compounds are absorbed. Lipophilic compounds intended for absorption from the gastrointestinal tract are almost

exclusively absorbed by this pathway. These compounds can partition into the apical enterocyte membrane, diffuse through the membrane and thereafter through the cytoplasm to cross the basolateral membrane and then move into the blood circulation (Li. 2001:357).

2.1.3.2 Passive paracellular transport

Hydrophilic compounds will not easily partition into the membranes because of the charge they carry and the energy needed to break the hydrogen bonds with surrounding water molecules. These compounds will rather be absorbed by means of other pathways, such as between adjacent cells through the interstitial spaces into the blood circulation. However, the tight junctions prevent free movement of molecules through the paracellular pathway (Kumar *et al*., 2010:67; Li, 2001:357).

2.1.3.3 Transcytosis

Transcytosis is the transport of molecules across cells. The mechanism of trancytosis can be explained by a few steps. Firstly the molecules are engulfed by the plasma membrane and pinched off to form a material filled intra-cellular vesicle. This is termed endocytosis and can be subdivided into pinocytosis (engulfment of small droplets) and phagocytosis (engulfment of macromolecules). The material is then transported into the cell and transferred to other vesicles or lysosomes (to be digested). Some of these materials escape digestion and move through the cell to be released at the BL side, known as exocytosis (Di Pasquale & Chiorini, 2006:506; Ashford, 2007:283).

2.1.3.4 Carrier-mediated transport

This is an active transport method which needs energy in the form of adenosine triphosphate (ATP). Hydrophilic chemical entities which mimic the chemical structures of certain naturally occurring nutrients can be absorbed and transported across the intestinal wall to the systemic circulation by means of carrier-mediated transport. Carrier-mediated transport is prone to saturation at high concentrations of the substrate which has a detrimental effect on drug absorption (Artursson *et al.*, 2012:282).

Facilitated diffusion is a carrier-mediated transport mechanism which does not require energy to take place, but needs a concentration gradient. Like active transport, this transport mechanism is saturable, but in general it plays a minor role in drug absorption (Ashford, 2007:282-283).

2.1.3.5 Efflux transport

Efflux of compounds is mediated by active transporters in the secretory direction (i.e. from the basolateral to the apical side of the intestine). The most common and well documented active transporter of drug efflux is P-glycoprotein (P-gp).

P-gp forms part of the human ATP-binding cassette (ABC) superfamily of active transporters, which include membrane transporters, ion channels and receptors" (Chan *et al.*, 2004:27). P-gp is found in many organs, which include the apical membranes of the intestinal epithelium of humans and other animal species. Efflux has a negative effect on the absorption and bioavailability of drugs which are P-gp substrates (Bergren *et al.*, 2007:252-253).

2.1.4 *In vitro* pharmacokinetic screening models: Considerations and challenges

The pharmaceutical industry and academia are constantly striving to develop and implement predictive *in vitro* methods for pharmacokinetics, which are more cost-effective and less time consuming (Deferme *et al.*, 2008:184). The development of these methods entails the consideration of various factors which are discussed below.

2.1.4.1 Physiological factors

2.1.4.1.1 Gastric motility and emptying

Gastric motility is the contraction of the stomach muscles in such a manner that food is ground into smaller particles and mixed with gastric juices. This is a forward and backward movement of all the contents within the stomach, which is later emptied into the duodenum. There are noticeable physiological differences between the fasted and fed states of the stomach.

During the fasting state, the peristaltic cycle consists of four different phases with duration of about two hours (Klausner, 2003:145). Phase one consists of low amplitude contractions for 45 to 60 min. In Phase two, intermediate contractions continue for a further 30 to 45 min and involve the secretion of bile. The third phase lasts for 5 to 15 min with high amplitude contractions, occurring in a frequency of 4 - 5 per min and the maximum opening of the pyloric sphincter. This phase, also termed "housekeeper wave," enables efficient evacuation of the stomach contents (Kararli, 1995:367-370). The fourth phase is the shortest, which lasts for only 5 min and connects the maximum amplitude and the initial phase.

The second scenario is the fed state of the stomach and its motor activity starts 5 to 10 min after the ingestion of a meal. The larger the meal ingested, the longer the time of fed activity. The phasic contractions are the same as in phase two of the fasted state. The stomach churns and mills whilst particles with a size of less than 1 mm are emptied into the duodenum every twenty seconds. This will continue for 3 to 4 hours, or until all stomach contents have been emptied into the small intestine (Klausner, 2003:145).

2.1.4.1.2 Volume and composition of intestinal fluids

The volume of fluid available in the intestinal tract can affect the rate of dissolution of a drug which is administered orally. The fluids present are either from co-administered fluids, secretions and/or water flux into the intestine. In the stomach, an average of 20 to 30 ml of fluid is present as wet mucus. In the small intestine, a baseline volume of 120 to 350 ml was noted.

The presence of surfactants in the gastrointestinal tract may play a role in drug absorption. Surfactants lower the surface tension of gastric fluid to 35 to 45 $mN.m^{-1}$, which is lower than that of water. This can lead to micelle formation and may influence drug absorption in a positive or negative way (Dressman *et al*., 1998:14 - 15).

The pH of the gastrointestinal tract fluids plays an important role in dissolution, solubilisation and absorption of ionisable drugs. In the ionised form, the drug is more soluble and the dissolution rate is higher. For the diffusion of drug molecules across membranes, the unionised form is favourable. After food ingestion, the pH of the human stomach increases due to a buffering action caused by the food. After about one hour the pH levels returns to a lower value. The opposite occurs in the small intestine. As the pyloric sphincter opens and the acidic content of the stomach enters the duodenum, the pH of the duodenum contents lowers, followed by secretion of an alkaline fluid from the pancreas, which neutralises the pH in the duodenum (Kararli, 1995:357-360).

2.1.4.1.3 Mucus

The epithelium contains mucus secreting goblet cells. The mucus is a viscous and elastic type of gel containing mucin. The physicochemical properties of this mucus layer have a great influence on the absorption of drug molecules at the absorption site (Le Ferrec *et al*., 2001:650-651). Mucin is made up of a large protein core with oligosaccharide side chains. The sugars incorporated in this oligosaccharide side chains are anionic, which can lead to a prolonged transit time in the gastrointestinal tract (Kararli, 1995:360-365). This highly viscous mucus layer can be seen as the first absorption barrier (Le Ferrec *et al*., 2001:651).

2.1.4.1.4 Metabolism and efflux

Cytochrome P450 3A4 (CYP 3A4) is the most abundant enzyme found in mature enterocytes lining the villus of the small intestine. CYP 3A4 in the intestinal epithelial cells has the same catalytic properties as those found in the liver cells and it is the enzyme primarily responsible for first pass metabolism of most drugs (Watkins, 1997:161; Boüer *et al.*,1999:495), which contributes to decrease the bioavailability of drugs.

Efflux transporters, e.g. P-gp, are highly expressed on the apical side of the small intestine and are responsible for limiting the absorption of drugs. These transporters use an energy source such as ATP to pump substrates against a concentration gradient back into the lumen of the intestine. This efflux mechanism lowers the bioavailability of drugs and may sometimes lead to sub-therapeutic drug concentrations (Estudante *et al.* 2013:1342-1344).

Some drugs may be susceptible to both CYP 3A4 metabolism and P-gp mediated efflux, which may lead to a larger decrease in drug bioavailability due to a synergistic action between the two mechanisms (Estudante *et al.* 2013:1348-1349).

2.1.4.1.5 Lymphoid tissues such as Peyer's patches

Within the gastrointestinal tract epithelium are organised structures of lymphoid cells. These lymphoid structures are called Peyer's patches, which can differentiate into M cells able to sample and transport certain materials across the lumen as an immunological response. However, they occupy a relatively small surface area of the gastrointestinal tract and differ morphologically from normal epithelial cells and should preferably not be included in an *in vitro* transport method as they can skew the transport results (Daugherty *et al*., 1999:145).

2.1.4.2 Physicochemical properties

In recent years, the discovery and development of NCE's has improved markedly. Thousands of NCE's can now be synthesised with automated equipment. To screen and test all of these entities is time consuming and expensive. Lipinski (2012:7) has proposed four physicochemical parameters for NCE's required to achieve acceptable solubility and membrane permeability. These requirements are termed the 'rule of 5' as the cut-off values for each of them is close to 5, or a multiple of 5. The four parameters of the 'rule of 5' include the following: (1) molecular weight, (2) Log P, (3) the number of H-bond donors and (4) acceptors respectively. If an entity's molecular weight is more than 500, its Log P value over 5, there are more than 5 H-bond donors or more than 10 H-bond acceptors, poor solubility or permeation can be expected. If two or more of the parameters are out of the

proposed range for a NCE, it should be eliminated from the drug development programme and not considered for further pre-clinical testing (Lipinski *et al.*, 2012:7 - 8).

2.1.4.3 Dosage form

Drugs are often formulated into solid dosage forms for oral administration and drug absorption can be hindered by various mechanisms such as:

- Lack of protection against metabolism and chemical degradation of drugs before it can reach the systemic circulation.
- Insufficient permeability of drug molecules in the apical to basal direction across the intestinal wall.
- The formation of non-absorbable drug complexes.
- Insufficient drug release from the dosage form, i.e. poor pharmaceutical availability (Dressman *et al*., 1998:12).

2.1.4.4 Biopharmaceutical classification system

During the 1970's and 1980's, it was realised that a drug classification system needed to exist to help pharmaceutical scientists in their study designs. Amidon and co-workers recognised aqueous solubility and membrane permeability (within the gastrointestinal tract) as the fundamental parameters that determined the rate and extent of drug absorption. They developed a classification system, known as the Biopharmaceutical Classification System (BCS), which divided compounds and drug products into four classes (illustrated in Figure 2.5). According to this classification system, compounds are assigned to specific classes based on their aqueous solubility and membrane permeability (Wu & Benet., 2005:11; Amidon *et al.*, 1995:413).

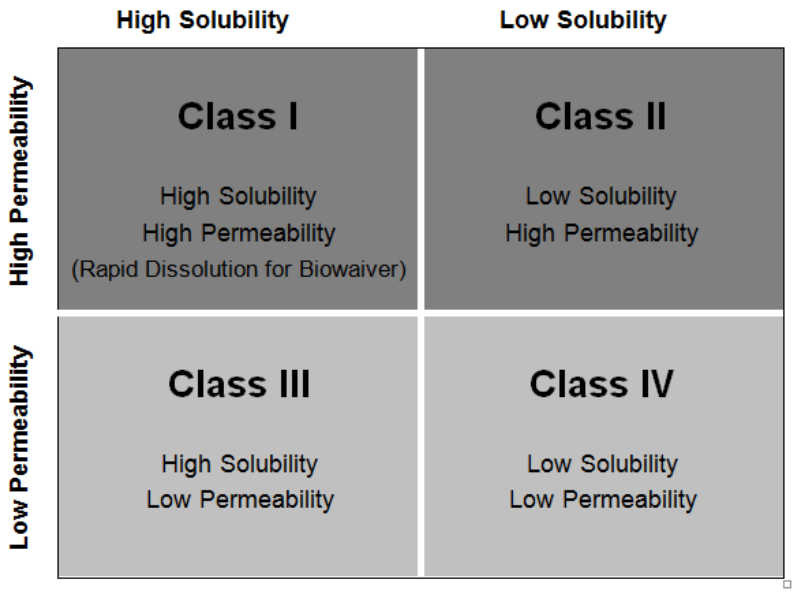

Figure 2.5: The BCS as defined by the FDA (Wu & Benet., 2004:12)

Class I compounds permeate readily through the intestinal membranes due to their small molecular weight and non-polar properties. The high solubility of this class leads to high concentrations of drug within the lumen, which creates a large concentration gradient. These high concentrations can saturate the absorptive and efflux transporters (Estudante *et al.* 2013:1350).

Intestinal uptake transporters are generally less important for class II compounds due to rapid partition of these lipophilic compounds into cell membranes. Therefore, passive transcellular diffusion is the major pathway of absorption. Due to the low solubility, the concentration is noticeably lower and will not easily cause saturation of the efflux transporters, which may play a role in their rate of absorption (Estudante *et al.* 2013:1350).

For class III drugs, there will be a sufficient concentration of compound in solution and available for absorption, since they are highly soluble in aqueous solutions. Due to their poor membrane permeability properties, an active transport mechanism is required to transport these drugs across the epithelial membranes, which may also apply to some class IV drugs as solubility increases to a satisfying level within the natural surfactant contents of the gastrointestinal tract (Estudante *et al.* 2013:1350; Maldonado-Valderrama *et al.* 2011:36).

2.2 Prediction of drug absorption

2.2.1 The need for *in vitro* screening models

There are several steps involved in the drug development process, which includes aspects such as target identification and screening, lead compound generation and optimisation, pre-clinical and clinical studies and finally the registration of the drug. The average development time of a NCE, up to registration, is 5 to 10 years (8 years being typical for most entities) at an average cost of 1 billion USD per NCE. Of the 5000 entities being tested, only 1 will be approved to be marketed (Cunha *et al*., 2014:936). The failure of NCE's was mainly attributed to a shortage of adequate metabolic and pharmacokinetic data (Branczewski *et al.,* 2006:453).

A crucial step in the drug discovery and development process is the assessment of membrane permeability properties of NCE's and researchers are constantly searching for cost-effective, high-throughput methods with as high as possible predictive value (Balimane & Chong, 2005:335). Identification of the permeability and metabolic profile of a NCE in the early stages of drug development has a major advantage associated with the use of *in vitro* methods. Secondly, the data generated from these methods are more relevant and important to predict human *in vivo* situations. The use of *in vitro* methods are also time and cost effective and the failure rate of NCE's had been reduced by 10% since implementation of *in vitro* screening methods in 2002 (Baranczewski *et al.,* 2006:453).

In addition, a thorough understanding of the mechanisms of action of membrane permeability and metabolism is required when evaluating drug disposition and pharmacokinetics (Mudra *et al*., 2011:750). High throughput, but less predictive methods are often utilised for primary screening purposes followed by low throughput, but more predictive methods for secondary screening purposes (Balimane *et al*., 2006:E1).

2.2.2 Classification of models

2.2.2.1 In vivo

In vivo models refer to experiments which take place within a living organism, e.g. in vertebrates such as mice and rats. During *in vivo* studies, a compound is usually administered extravascularly and its permeation through the intestinal wall into the blood, as well as its distribution into tissue compartments, is measured by means of blood sampling or tissue biopsies (Hildago, 2001:389).

Animals have been used by mankind for food, transport and companionship since the beginning of existence. In ancient Greece, as the development of medicine started, animals became a popular model for experimentation and up until the 17th century, they were used without any great moral dilemmas (Baumans, 2004:S64).

After the discovery of anaesthesia and the publication on *Origin of Species* in 1859 by Darwin, where he defended the similarities that exist between humans and animals, an increase in the number of animals used in experiments was noted. This number kept increasing during the centuries. Even in the 20th century, this number increased more rapidly due to the development of biomedical disciplines e.g. pharmacology and toxicology. From the 1980's public awareness, strict legislation on animal use and the development of ethical committees led to a decrease in the usage of animals in experiments (Van Zutphen., 1993:2 - 5).

Today, about 75 to 100 million animals are used each year for research and medical testing. Major areas where animals are being utilised are drug research, vaccination testing and cancer research, in which mice and rats are the most popular species. The use of this large number of animals contributed to the development of Laboratory Animal Science. With the origin of Laboratory Animal Science, Russell and Burch proposed specific guiding principles to be used by researchers and ethic committees worldwide, including replacement, reduction and refinement which are commonly known as the three R's. Replacement refers to the replacing of animals by other *in vitro* (e.g. Caco-2 cell monolayers) and computerised methods. Reduction focuses on a decrease in the amount of animals used during experimentation. By using power analysis prior to the experiment (a statistical method), researchers can accurately calculate the least amount of animals required per group for the study. Refinement implies a reduction in any discomfort an animal may endure and it includes adequate housing, providing the correct amount of anaesthesia, analgesia, care by trained personnel and researchers and the determining of a humane end point, such as euthanasia to prevent the animal from unnecessary suffering (Baumans, 2004:S64 - S66).

In vivo methods have several desirable attributes such as the presence of a mucosal layer, blood circulation and other essential important biological factors. Another advantage is the ability to study the kinetics, pharmacological and toxicological properties of the administered drug (Hildago, 2001:389).

Certain drawbacks are also associated with the use of *in vivo* methods such as the fact that the rate-limiting factor during absorption cannot be identified, a large amount of the test compound is required, a large number of animals is required, analysis of drug in the blood

plasma can be complex and the mechanism of absorption is not clearly revealed during the use of *in vivo* methods (Hildago, 2001:389; LeFerrec *et al*., 2001:653).

Furthermore, pharmaceutical companies had to revolutionise their approach to drug development due to high costs and the need to reduce the rate of NCE failure. An increase in the number of promising compounds due to advances in chemical synthesis of lead compounds has placed great pressure on the evaluation process used to select compounds that will enter the next evaluation phase (Spalding *et al.,* 2000:74).

Previously, failure of compounds to move on to the next phase of development could be mainly ascribed to poor pharmacokinetic properties. Therefore, the previous low-throughput approaches needed to be replaced by high-throughput screening techniques to optimise the process (Spalding *et al.,* 2000:74).

2.2.2.2 In situ

The term *"in situ"* refers to experiments which take place in an organ as part of a living organism, e.g. single pass perfusion, recirculating perfusion, closed-loop and/or oscillating perfusion through parts of the intestinal tract. The model has certain advantages which include the presence of an intact mucosal layer, blood perfusion and the nerve system is connected, as well as the expression of enzymes and transporters (Holmstock *et al.,* 2012:1473-1474). These advantages make it a useful model for evaluating drug absorption and metabolism. However, highly sophisticated instruments and surgical procedures performed by trained personnel are necessary, which make this a less suitable option for screening of large numbers of compounds (Luo *et al*., 2013:209).

2.2.2.3 In silico

The term "*in silico"* refers to computational models that check the drug-likeness of NCE's, but software programmes exist that can predict pharmacokinetics of NCE's based on their chemical structures and *in vitro* data. This is becoming more popular since it is cost-effective, has high-throughput and may not require physical sampling. One example of an *in silico* model is a computational programme that eliminates NCE's based on the requirements of Lipinski's rule of 5 (Spalding *et al*., 2000:71).

2.2.2.4 Ex vivo

The term "*ex vivo*" refers to whole organs which are removed from living organisms, but is often also used to describe studies that utilise excised tissues such as everted sacs and excised tissues pieces in Ussing chambers (which overlaps partly with "*in vitro*" experiments)

(Dahan & Hoffman., 2007:97). *Ex vivo* methods differ from certain *in vitro* methods (e.g. synthetic membranes and some cell lines) and can be separated by a number of distinctive features which include the presence of transport proteins, presence of drug metabolising enzymes, the presence of a mucosal layer and availability of the paracellular pathway. However, shortcomings include a lack of a nervous supply and blood perfusion which can give a false indication of the extent of drug permeation. Despite the shortcomings, this method is widely used in the design and testing of NCE's due to its simplicity (Luo *et al*., 2013:209).

2.2.2.5 In vitro

The concept of "*in vitro* models" refers to experiments that take place in a controlled environment outside a living organism, e.g. artificial membranes, cell cultures and excised tissues, such as everted sacs and in Ussing type chambers (Volpe, 2010:672; Dixit *et al*., 2012:13; Reis *et al*., 2013:781). *In vitro* methods are generally preferred over *in vivo* and *in situ* methods for ethical reasons and time constrains. Although widely used, shortcomings include the absence of gastric emptying, gastrointestinal transit rate, absence of a mucus layer and absence of region specific pH fluctuations. Despite these shortcomings, *in vitro* model has already been widely applied to permeation studies with different levels of success (Deferme *et al*., 2008:187, Balimane *et al*., 2005:335).

The most commonly used *in vitro* cell methods for permeability studies are the Caco-2 and Madin-Darby canine kidney (MDCK) cell lines. Caco-2 cells, derived from human colon adenocarcinoma tissue, are one of the most appropriate methods for the prediction of oral drug absorption in humans since they are of human origin. Recent studies have shown that MDCK cells have little difference in permeability compared to those found in Caco-2 cells. The MDCK cells have an advantage over Caco-2 cells because they can be cultured more quickly, which is more favourable for high-throughput. The high-throughput rate can be increased even more by automated dispensing and sampling techniques. This can be achieved by growing the cell lines on miniaturised formats or trans-wells and using mass spectrometric detection which can detect the smallest concentration of compound within the sample every 2 min. Considering only a single component of the pharmacokinetic parameters is analysed *in vitro* (i.e. membrane permeation), there can be discrepancies when compared to *in vivo* bioavailability data. However, this screening method has already been used successfully to screen compounds/hits and eliminate NCE's with insufficient pharmacokinetic properties from further evaluation (Spalding *et al.,* 2000:73).

2.2.3 Excised animal tissue-based *in vitro* (or *ex vivo*) techniques

2.2.3.1 Ussing chamber technique and apparatus

In earlier years, it was not possible to study the transport mechanisms of ions (e.g. NaCl) with *in vitro* techniques. This challenge prompted the Danish inventor Hans H. Ussing (1911 - 2000) to develop a device called the Ussing chambers, which would make it possible to study the various mechanisms of transport across the intestinal epithelium. He used frog skin, mounted on spikes, to separate two halves of a chamber which was perfused by an electrolyte. He postulated that if the potential difference (PD) between the two chambers was set to zero, by applying an external current, the flux transport ratio would also be zero since the concentration on both sides of the epithelium was the same. The reverse is also true, if an ion is transported by the active route then the flux ratio of transport would be different from unity (Boudry, 2005: 220).

Studies performed on intestinal mucosa using Ussing type chambers provided a clear understanding of the transepithelial transport processes on a molecular level. This technique was used to elucidate the processes of electrogenic Cl^- secretion, electrogenic Na^+-coupled glucose absorption and electroneutral NaCl absorption (Clarke, 2009: G1151 - G1152).

There are two types of Ussing chambers currently in use, namely the circulating chamber and the continuously perfused chamber as depicted in Figures 2.6 and 2.7, respectively. Each of these chambers consists of two half-chambers, where freshly collected tissue is mounted in between as a flat sheet to provide an apical and a basolateral side. The heat can be adjusted to a specific temperature (e.g. 37°C) by placing the chambers in a water jacket heating system if necessary. The PD is measured by placing two electrodes on each side of the tissue. A short-circuit current can be applied by another pair of electrodes so that the PD can be nullified. Gas flow is introduced to these chambers for oxygenation of the tissue as well as to maintain stirring of the solution (Boudry, 2005: 220).

2.2.3.1.1 Circulating Ussing type chamber

This type of chamber is usually made out of glass tubing and shaped as a “U”. The U-shaped tubing is filled with the experimental solution (e.g. Kreb’s Ringer Bicarbonate buffer/drug solution) and heated if required, and supplied with a gas (e.g. air, CO_2, O_2 or N_2). Damage to the tissue can be minimised by keeping an equal hydrostatic pressure on both sides of the chamber. A drug may be added to either or both sides of the tubing in a sequential manner at any time during the experimental procedure (Li *et al.*, 2004:123-124).

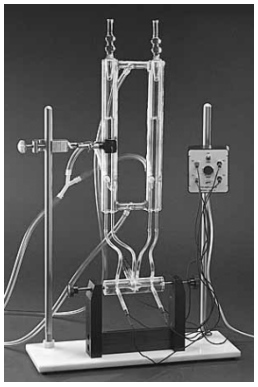

Figure 2.6: Circulating Ussing chamber

2.2.3.1.2 Continuously perfused Ussing type chamber

The two half-chambers of this apparatus are specially designed to minimise hydrostatic pressure in order to prevent tissue damage during perfusion. A reservoir containing buffer/drug solutions, mounted 20 to 50 cm above, is connected to the chamber by polyethylene tubes, which are used to supply the two sides of the chamber with the relevant test solution. The flow of the solution into the chambers can be regulated by means of a valve. The flow rate can also be altered by changing the diameter of the tubes or the hydrostatic pressure. The temperature can also be adjusted (Li *et al.*, 2004:123-124).

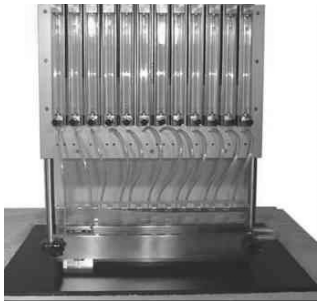

Figure 2.7: Continuously perfused Ussing chamber

Ussing type chambers offer an ideal way to study drug transport across excised animal tissue and have various advantages (Le Ferrec *et al*., 2000:265). These advantages include the fact that the trans epithelial electrical resistance (TEER) of the membrane can easily be measured to ensure viability, excised tissue from the animal of choice can be used to examine membrane permeation characteristics, a controlled environment can be created to mimic *in vivo* circumstances, only a small amount of drug and buffer is required and collected samples can easily be analysed to produce quantitative results without extraction from protein containing plasma. However, this technique has some shortcomings such as

the absence of blood supply and nerve innervations, changes in the morphology and functionality of the drug transporter proteins during preparation of the tissue and a relatively rapid loss of tissue viability after dissection may occur (Balimane *et al*., 2000:305-306).

2.2.3.2 Everted sac technique

Wilson and Wiseman described a technique in 1954, known as the everted sac. This can be used to evaluate the permeability of various compounds and the performance of novel drug delivery systems (Dixit *et al*., 2012:13; Wilson & Wiseman, 1954:116). In short, a section of intestine is removed from the animal and flushed with buffer. The mesenteric border is then removed from the membrane by means of blunt dissection. The intestine is then everted and mounted over a rod or tube, filled with oxygenated buffer and tied off with silk thread, which is then placed in a container containing the compound/buffer solution. After a set time (e.g. 20 min) samples are withdrawn and analysed. This technique can also be performed in the opposite direction where the intestine is not everted (Volpe, 2010:673; Wilson & Wiseman, 1954:116).

There are well known limitations to this technique such as morphological damage to the intestine during preparation (Balimane *et al*., 2000:305-306), the presence of an intact muscularis mucosa (Le Ferrec *et al*., 2000:265) and small volume of the serosal compartment (Dixit *et al*., 2012:13). Another great limitation is the fact there is no standardised method in place to measure the TEER (Dixit *et al*., 2012:13). As for all *in vitro* techniques the lack of blood flow and nerve innervations is another shortcoming (Balimane *et al*., 2000:305-306).

Although the TEER cannot be measured when using the everted sac technique, previous studies have shown the intestinal tissue will remain viable for up to 2 h under strictly monitored, ideal, experimental conditions (Dixit *et al*., 2012:13; LeFerrec *et al*., 2000:265). A glass apparatus was developed in an effort to optimise and standardise everted sac studies as illustrated in Figure 2.8.

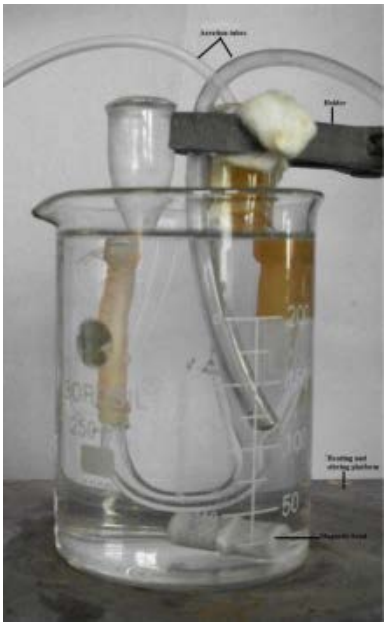

Figure 2.8: Example of the everted sac apparatus used by Dixit and colleagues (Dixit *et al*., 2012:14)

2.2.4 Cell culture-based *In vitro* models

Various types of cell monolayers, which mimic the human intestine, have been developed to study intestinal permeation. The permeation across some of these cell monolayers correlate well with *in vivo* human pharmacokinetic studies. The cells have the advantage of growing quickly into monolayers and spontaneously differentiating to provide a near ideal system for drug permeation testing. Currently Caco-2, HT-29, T-84, MDCK and LLC-PK1 are the most popular cell lines being used for drug permeation studies (Balimane *et al*., 2000:306).

2.2.4.1 Caco-2 cell line

The Caco-2 cell line, originating from human colorectal adenocarcinoma tissue, is the most popular cell line being used in studies which investigate drug absorption. Caco-2 cell monolayers are cultured and grown under strictly regulated conditions, on specially designed semi-porous membrane filters. The monolayer mimics the morphological and functional attributes of the human intestinal epithelium. The membrane filters are placed in the wells of Transwell® plates, providing an apical (AP) and basolateral (BL) side as illustrated in figure 2.9. The compound is applied to either the AP or BL side depending on the required direction of transport. At pre-determined time intervals, samples can be taken from either the AP or BL chamber and the apparent permeability coefficient values can be calculated from the transport data (Le Ferrec *et al*., 2000:655).

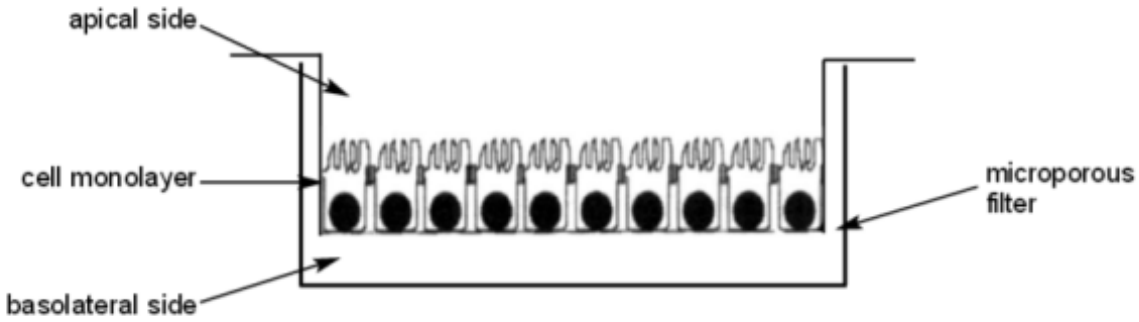

Figure 2.9: Caco-2 cell monolayer cultured on the Transwell® plate (Le Ferrec *et al*., 2000:658)

Limitations of the cell culture method include: a lower expression of pharmaceutically important active transporters than that encountered in the human intestine, a lack of blood flow and nerve innervations is encountered with this technique (Balimane *et al*., 2000:306) and it lacks the mucus layer which is present in the *in vivo* scenario (Le Ferrec *et al*., 2000:655).

2.3 Conclusion

From the literature, it is clear that new *in vitro* methods are necessary to screen NCE's as soon as possible in the drug discovery and development process. These methods need to be of a high-throughput and cost effective nature so that the evaluation of the NCE's can be performed in great volumes, as quickly as possible. Various *in vitro* methods and techniques do exist, such as the everted sac technique and Ussing type chambers which require excised animal tissues (e.g. rat intestine). Another method and technique which has been used to evaluate the epithelial permeability of NCE's is cell monolayers that are cultured on membranes in Transwell® plates.

In vitro models normally contribute to the implementation of the 3 R's concept, however, when excised animal tissues are used, it is important to consider ethical aspects. Although the excised pig intestinal model has been used before (Pietzonka *et al.,* 2002:39), very little information is available about how it compares with other *in vitro* models for purposes of drug permeation. Therefore further studies on pig intestinal tissue are necessary.

CHAPTER 3: MATERIALS AND METHODS

3.1 Introduction

In vitro transport studies across excised intestinal tissues from mammals are commonly used to study drug permeation. This technique entails the isolation of intestinal tissue from an animal, dissecting it into correctly sized pieces, clamping it in between two half cells of a suitable diffusion apparatus and measuring the drug transport across the tissue in a controlled environment (Balimane *et al*., 2000:305). Various animal methods have been used in these excised tissue *in vitro* techniques to assess the intestinal permeability of NCEs such as rats, rabbits, monkeys, dogs and pigs. The successful use of *in vitro* methods to predict drug absorption depends on how accurately it mimics the *in vivo* situation (Balimane *et al*., 2000:305; Tarirai *et al*., 2012:255). Growing epithelial cells in monolayers is another popular *in vitro* method to screen the permeability properties of NCEs. The Caco-2 cell line is recommended by the World Health Organisation as well as the Food and Drug Administration of the United States of America as the method of choice to classify drugs according to the BCS (Abalos, 2012:224).

In this study, the Sweetana-Grass diffusion chamber technique and the everted sac technique were used to determine the bi-directional *in vitro* transport of abacavir sulphate (abacavir) across excised rat and pig intestinal tissues. The Caco-2 cell line was used as a representative method from human origin to determine the bi-directional transport of abacavir. The different *in vitro* methods were compared statistically to each other in terms of transport (P_{app} values), efflux ratio (ER values) and variability (inter- and intra-method experiments).

3.2 Materials

Abacavir (batch number CAD0770029) was kindly donated by Prof Wilna Liebenberg from North-West University, which was bought from Sri Sai Nikitha Pharma ((Pty) Ltd.) (See Appendix C for certificates of analysis). Krebs-Ringer bicarbonate (KRB) buffer (product no. K4002), Phosphate buffered saline (PBS) and Trypan Blue Solution (0.4%) was purchased from Sigma-Aldrich (Johannesburg, South Africa). Spraque-Dawley rats were obtained from the pre-clinical drug development platform (PCDDP) *vivarium* at North-West University. Pig intestine was collected at the local abattoir in Potchefstroom. Caco-2 cells were purchased from the European Collection of Cell Cultures (ECACC) (Cell Line Name: CACO-2; Description: Human Caucasian colon adenocarcinoma; Growth mode: Adherent) (Sigma-Aldrich, Johannesburg, South Africa). HEPES [n-(2-hydroxyethyl), piperazine-N-(2-

ethanesulfonic acid)] buffer solution (1M) (50x) (Biochrom), Amphotericin B (250 µg/ml) (Biochrom), Hank's Balanced Salt Solution (HBSS), without phenol red, containing 0,35g/l $NaHCO_3$ (Biochrom) and fetal bovine serum (FBS) Superior - heat inactivated (Biochrom) were purchased from The Scientific Group (Randburg, South Africa). HYCLONE Dulbecco's Modified Eagle's Medium (DMEM) with high glucose, 4.0 mM L-glutamine, sodium pyruvate (Thermo Scientific) was purchased from Separations (Randburg, South Africa). HYCLONE Penicillin/Streptomycin Solution (Thermo Scientific) was purchased from Separations (Randburg, South Africa). L-glutamine (200mM) (Lonza), Trypsin-Versene (EDTA) mix (1x) (Lonza) and Non-essential amino acids (NEAA, 100x) (Lonza) were purchased from Whitehead Scientific (Cape Town, South Africa). Transwell membrane plates (24 mm inserts, 6 well plates with a 4.67 cm^2 membrane surface area) (Costar®) for the Transport study was purchased from Corning Costar® Corporation, USA.

3.3 HPLC method validation

3.3.1 High performance liquid chromatography analysis method

The HPLC instrument and conditions used to determine the abacavir concentration in the transport samples are summarised in Table 3.1.

Table 3.1: Analytical instrument and chromatographic conditions

Analytical conditions	Description
Analytical instrument	HP1100 series HPLC equipped with a pump, auto sampler, UV detector and Chemstation Rev. A.10.01 [1653], Agilent® Technologies data acquisition and analysis software
Column	Column #79, Venusil XBP C18(2), 5 µm, 100Å, 4.6x150 mm Cat no: VX951505-2 SIN: V9510515BM 0027b
Mobile phase	40:60, acetonitrile : 0.005M Heptane-11sulfonic acid sodium salt, pH 3.5
Flow rate	1 ml/min
Sample volume injection	10 µl
Detection wavelength	210.8 nm
Retention time	4.8 min
Stop time	8 min
Solvent	Krebs-Ringer bicarbonate buffer

3.3.2 Specificity

Specificity is the ability to clearly assess the analyte in the presence of components which are also expected to be present (ICH, 1996:4). All possible components that may be present were individually analysed to ensure there would be no interference with the key compound, abacavir. A placebo sample was prepared by diluting 10 ml of KRB buffer (used as solvent during the *in vitro* transport studies) to 25 ml with a mixture of 50:50 methanol and distilled water. An HPLC vial was then filled with the placebo which was then injected into the HPLC and analysed.

An abacavir sample was constituted by dissolving 51.2 mg of abacavir in 200 ml of methanol. Then 10 ml of the solution was diluted to 25 ml with a mixture of 50:50 methanol and distilled water to give a final concentration of 102.4 µg/ml. The samples were injected into the HPLC and analysed.

3.3.3 Linearity

The ability of an analytical procedure to determine the concentration of an analyte in the sample by means of a well-defined mathematical calculation is known as the linearity (USP, 2014:1160).

In order to determine the correlation between the response of the detector of the HPLC and the concentration of abacavir, a range of standard solutions of abacavir was prepared. A stock solution of abacavir was prepared by dissolving 12.21 mg of abacavir in 100 ml methanol. This stock solution had an abacavir concentration of 122.1 µg/ml. One millilitre of this solution was then diluted to 10 ml with methanol to achieve the second solution with a concentration of 12.21 µg/ml. For the third solution, the same procedure was followed and a concentration of 1.221 µg/ml was achieved. Different volumes of these diluted solutions (1 µl, 2.5 µl, 5 µl, 10 µl, 15 µl, 20 µl, 30µl, 40 µl and 50 µl) were injected in duplicate and analysed with the HPLC.

The peak areas obtained for the standard solutions were plotted as a function of concentration and linear regression was performed on this calibration curve. Linear regression analysis should yield a regression coefficient (R-squared) value of at least 0.99. An R-squared value of 0.99 (or closer to unity) is therefore necessary to describe a linear concentration-response relationship (USP, 2013a:985).

3.3.4 Limit of detection (LOD) and limit of quantitation (LOQ)

LOD can be defined as the lowest amount of analyte (known concentration) that can be detected, not always quantitated, in a sample by means of the particular analytical method (USP, 2014:1159).

The LOQ is defined as the determination of the lowest amount of analyte in a sample, using the particular analytical method, within acceptable precision and accuracy (USP, 2014:1160).

Four volumes (10 µl, 20 µl, 30 µl and 50 µl) of a 11.4 µg/ml concentration abacavir was injected in six fold and the mean % relative standard deviation calculated. The LOD and LOQ were then determined by these values.

3.3.5 Accuracy

The accuracy of an analytical method indicates how close the value which is expected and the experimental value obtained are to each other (ICH, 1996:4). To determine the accuracy of the analytical method, two abacavir solutions were prepared namely 56 µg/ml and 280

µg/ml, which were injected into the HPLC at five different injection volumes each. Three different abacavir solutions (approximately 10 µg/ml, 100 µg/ml and 250 µg/ml) were prepared and triplicate samples of these solutions were injected into the HPLC to determine the amount of abacavir that could be recovered from each sample.

An HPLC method is considered accurate when the mean analyte recovery is 100 ± 2% (ICH, 1996:4).

3.3.6 Precision

The precision of an analytical method indicates the familiarity of concurrence between a series of measurements obtained following multiple sampling of the same solution (ICH, 1996:4). Precision can be divided into two categories namely intra-day and inter-day precision.

3.3.6.1 Intra-day precision

Repeatability expresses the precision under the same operating conditions over a short interval of time (ICH, 1996:4). Since no sample preparation was performed during analysis, the data obtained for the accuracy experiment were also used for the determination of precision. Intra-day precision is specified to have a RSD better than 5% (n = 9) (Shabir, 2004:214).

3.3.6.2 Inter-day precision

Inter-day precision was determined by analysing two replicate samples of three different abacavir concentrations on three successive days. Three samples with a concentration of ± 100 µg/ml (prepared according to the same method as described for the accuracy experiment) were analysed on two occasions of each successive day to determine the inter-day consistency of the method.

3.3.7 Stability

The chemical stability of a given analyte should be evaluated under specific conditions over given time intervals. This is necessary to establish if the analyte in the samples did not undergo any degradation over the period of HPLC analysis.

A standard stock solution with a concentration of 105.54 µg/ml was injected into the HPLC and analysed. The % recovery was calculated and compared to the % recovery limits of abacavir (97.0 - 102.0%) given in the United States Pharmacopeia's monograph (USP, 2014:1161).

3.4 Transport studies

3.4.1 Preparation of *in vitro* drug transport solutions

KRB buffer was used as transport medium throughout all the experiments. Stock solutions of abacavir were prepared and diluted with KRB to the desired concentration. KRB buffer solution was applied to either the apical or basolateral side of the excised tissue, depending on the direction of transport being studied. The surface area of the tissue/cell monolayers exposed to the abacavir for absorption differed between the three models and techniques. These exposed surface area differences required an adjustment in the abacavir concentration used in each method, with the purpose of measuring a detectable concentration (within the LOD and LOQ range) by means of the validated HPLC method. An abacavir concentration of approximately 400 µM was accurately prepared and used for the everted sac technique, where an average surface area of 35 cm^2 for the pig intestinal tissue and 4.2 cm^2 for the rat intestinal tissue was available for drug transport, while the concentration was increased by a factor of 10 (i.e. to approximately 4 mM abacavir accurately prepared) for the Sweetana-Grass diffusion chambers where an average surface area of 1.78 cm^2 was available for drug transport. The abacavir concentration was adjusted by a factor of 5 (i.e. to approximately 2 mM abacavir accurately prepared) for the Caco-2 cell monolayers where an average surface area of 4.67 cm^2 was available for drug transport. A summary of the respective surface areas of each species, model and technique as well as the concentrations of abacavir applied are given in Table 3.2.

Table 3.2: Surface areas available for drug transport between the different species, methods and techniques and the abacavir solution concentrations

Technique	Surface area (cm^2)			Abacavir stock solution	Abacavir solution applied
	Rat	Pig	Human		
Everted sac	4.20	35.00	N/A	527.76 mg in 4 L	131.94 µg/ml; 393.42 µM
Sweetana-Grass	1.78	1.78	N/A	1319.40 mg in 1 L	1319.40 µg/ml; 3934.16 µM
Caco-2 cell monolayers	N/A	N/A	4.67	659.70 mg in 1 L	659.70 µg/ml; 1967.08 µM

3.4.2 Preparation of intestinal tissue for transport studies

3.4.2.1 Preparation of pig intestinal tissue

Pig intestinal tissue (jejunum) was collected freshly for each experiment from a local abattoir (Potch Abattoir, Potchefstroom, South Africa) directly after slaughtering of pigs for meat production purposes. A veterinary surgeon was consulted beforehand to ensure proper identification and dissection of the jejunum. The proximal 30% of the small intestine consists of the duodenum, followed by the jejunum (60%) and the ileum (distal 10%). A piece of the jejunum, about 60 cm, was dissected from the pig gastrointestinal tract at the abattoir and rinsed and flushed with ice cold KRB. Thereafter, the piece of jejunum was placed in a cooler box filled with ice cold KRB and transported to the laboratory (approximately 20 min after slaughtering). The cold chain was never interrupted.

Once at the laboratory, the piece of jejunum was rinsed and flushed again with ice cold KRB and pulled over a clean glass rod. The serosa was then removed by blunt dissection (Figure 3.1).

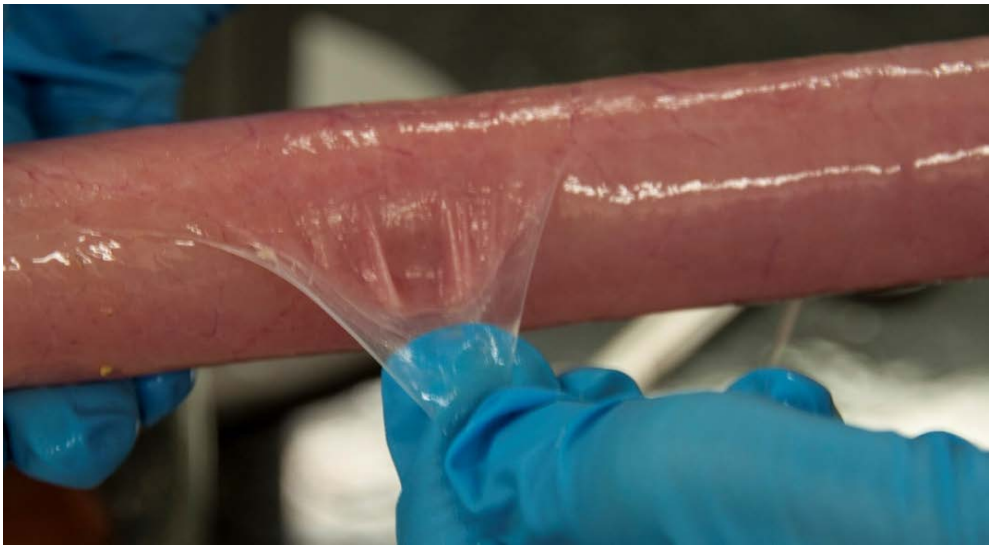

Figure 3.1: Image of an excised section of pig jejunum pulled over a clean glass rod, while removal of a part of the serosa is shown

After completion of this general preparation of the excised jejunum tissue, two different approaches were followed depending on the technique that was used for the transport study.

For the Sweetana-Grass diffusion chamber technique, heavy duty filter paper was placed on top of a glass container, filled with ice, and moistened with ice cold KRB. The jejunum was then cut along the mesenteric border and washed off the glass rod, rendering the jejunum lying flat on the filter paper with the apical side facing upwards (Figure 3.2). The piece of

jejunum, along with the filter paper, was then cut into smaller pieces (approximately 2 cm wide). The filter paper is necessary to protect the segments from being damaged during handling. Throughout the entire procedure, ice cold KRB was applied to the tissue to keep it moist. Strips that contained Peyer's patches (macroscopically visible) were not used during the study (Daugherty *et al*., 1999:145).

Figure 3.2: Image of a piece of excised pig jejunum, on filter paper, being cut into smaller pieces

For the everted sac technique, the section of pig jejunum obtained from the abattoir was cut into smaller pieces (approximately 8 cm), excluding Peyer's patches, and attached to the ends of the U-shaped glass apparatus (Figure 3.3). The jejunum was everted before mounting it to the everted sac apparatus when the AP - BL direction of transport was measured. In the BL - AP directional transport study, the jejunum was not everted before mounting it to the apparatus. The ends were securely tied down with surgical thread to prevent any leakage. The tissue was kept moist with ice cold KRB at all times.

Figure 3.3: Image of a piece of excised pig jejunum mounted on to the everted sac glass apparatus that was kept moist by regularly applying ice cold KRB

3.4.2.2 Preparation of rat intestinal tissue

Intestinal tissue (jejunum) was obtained from Sprague-Dawley rats supplied by the PCDDP of the North-West University (Potchefstroom, South Africa). Approval was obtained from the ethics committee of the North-West University (ethics approval number NWU-0030-13-A5, the letter of approval is given in Appendix A). A training course was attended (Appendix C) to ensure that the rats were euthanised in a humane manner and that dissection of the correct piece of intestine was performed. Directly after euthanisation of the rat, the abdomen was cut open and a piece of the jejunum (about 20 cm in length, starting at about 10 cm from the stomach) was dissected and rinsed with ice cold KRB (Figure 3.4). Thereafter, the piece of jejunum was placed in a cooler box filled with ice cold KRB and transported to the laboratory (approximately 5 min after euthanisation). The cold chain was never interrupted.

Once at the laboratory, the same procedure was followed as described for the pig intestinal tissue in section 3.4.2.1. However, in the case of rat intestinal tissue, a section of approximately 4 cm was mounted on the everted sac glass apparatus.

Figure 3.4: Image illustrating the jejunum being removed from a Spraque-Dawley rat after euthanisation

3.4.3 Transport studies using the Sweetana-Grass diffusion technique

The Sweetana-Grass diffusion chamber apparatus was adapted and modified from the well-known Ussing chambers by Stephanie A. Sweetana and George M. Grass (1988:372). The apparatus can accommodate six cell blocks or diffusion chambers in between a heated front and back plate. The desired temperature can be adjusted by circulating water through these plates from a heated water reservoir. Each cell block consists of two half-cells where the excised intestinal tissue can be clamped in between. Metal pins, in between these half-cells, keep the intestinal tissue in position. A circulation pathway allows gas flow parallel within each half-cell to the tissue surface to ensure maximum exposure of the fluid to the membrane and to prevent the formation of a stagnant layer (Sweetana & Grass, 1993:1). Electrodes were inserted to the half cells to measure the TEER of the inserted tissue pieces as an indication of membrane integrity.

The Sweetana-Grass diffusion apparatus (Warner Instruments, Hamden, Connecticut, USA) that was used in this study is illustrated in Figure 3.5.

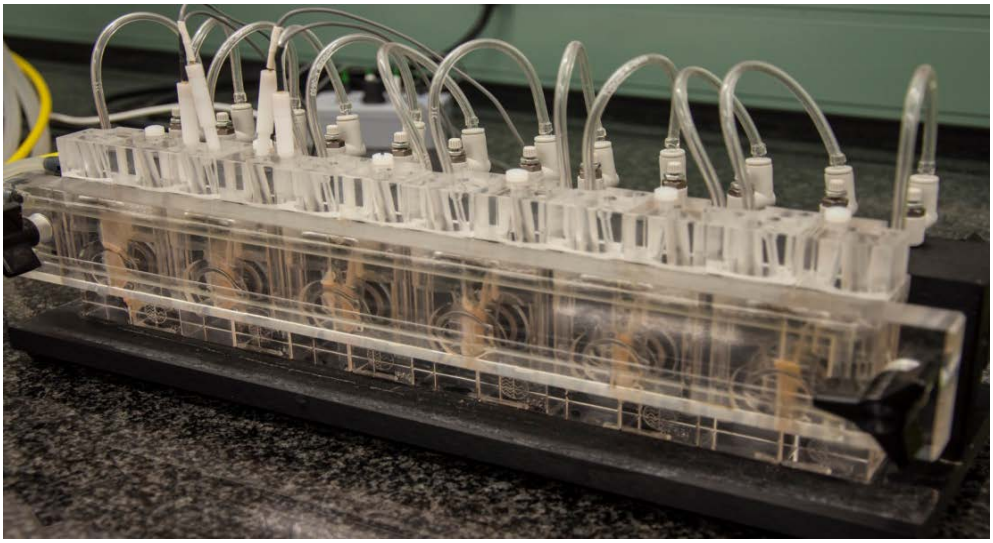

Figure 3.5: Image illustrating the Sweetana-Grass diffusion apparatus used for the transport studies with pig intestinal tissue mounted between the half cells

Pieces of the excised segments of the pig and rat intestinal tissue were each mounted respectively onto the pins of the half-cells with the apical side facing downwards. The upward facing filter paper was then carefully removed to avoid any damage to the tissue after which the second matching half-cell was added and clamped onto the first half-cell (Figure 3.6). The six assembled cell blocks or chambers were then mounted between the two heating blocks of the apparatus. Thereafter, the half-cells of the chambers were simultaneously filled with 7 ml of pre-heated (37 ºC) KRB. The half-cells of each chamber were supplied with gas (95% O_2: 5% CO_2) at a flow rate of 15 to 20 ml/min to ensure circulation of the buffer inside the half-cells. A period of 30 min was then allowed to equilibrate the tissues to this environment before commencement of the transport studies. The elapsed time since collection of the excised animal jejunum until the start of the transport study (including the period of equilibration) did not exceed 60 min.

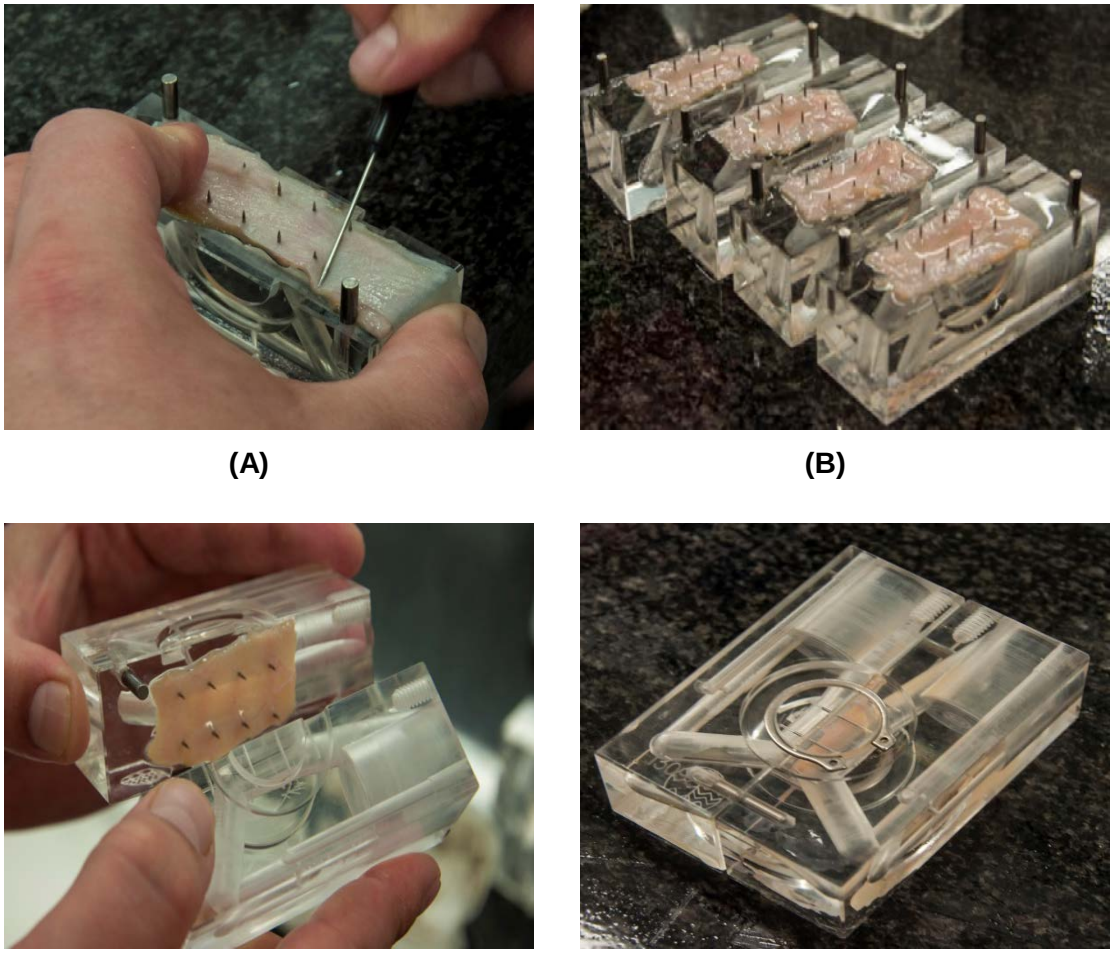

Figure 3.6: Images illustrating the (A) mounting of a piece of excised animal jejunum onto the metal pins of a half-cell, (B) mounted jejunum pieces on half-cells with the filter paper removed, (C) combining of two half-cells with the piece of jejunum mounted on the one half-cell with pins and (D) assembled cell block ready for insertion into the Sweetana-Grass apparatus

After completion of the tissue equilibration period, the buffer was removed by aspiration from the half-cells with an Integra Vacusafe vacuum system (Vacusafe®, model 158 3XX). Immediately thereafter, 7 ml of the pre-heated test solution, containing abacavir, were added to either the apical or basolateral side of each diffusion chamber depending on the direction of the transport study. A volume of 7 ml of KRB, also pre-heated, was added to the other side of each diffusion chamber.

The TEER was measured in each diffusion chamber across the mounted intestinal tissue with a Dual Channel Epithelial Voltage Clamp (Warner Instruments, Hamden, Connecticut, USA) immediately after addition of the test solutions as well as at the end of the transport

study. As mentioned earlier, this was performed to verify the viability and integrity of the intestinal tissue over the period of the transport study.

A 200 µl sample was withdrawn from the acceptor chamber every 20 min over a period of 120 min. The relevant chamber from which the sample was withdrawn was immediately replenished by the same volume of fresh pre-heated KRB buffer solution. The samples were placed into HPLC vials containing inserts and immediately refrigerated. These samples were then analysed by means of HPLC using a validated analytical method for abacavir.

3.4.4 Transport studies using the Everted sac technique

3.4.4.1 Development of glass apparatuses for rat and pig tissues

The everted sac apparatuses were manufactured by the instrument manufacturing department of the North-West University. The dimensions of the rat everted sac apparatus was based on the design that Dixit *et al*. (2012:14) used during their study (Figure 3.7) but with slight modifications.

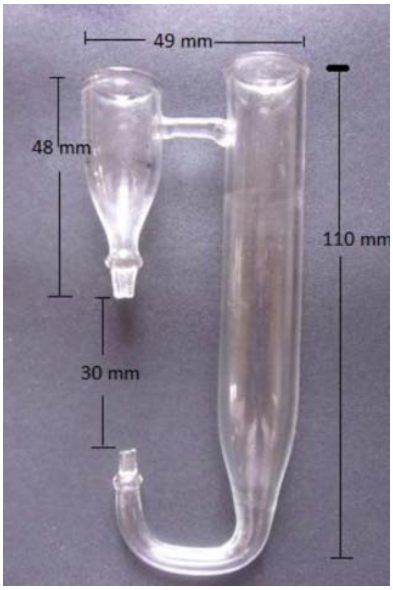

Figure 3.7: Image illustrating the everted sac apparatus used by Dixit *et al.* 2012:14

The everted sac glass apparatus used for the excised rat intestinal tissue consisted of a cylindrical glass tube (110 x 17 mm) joined to another cylindrical glass tube (48 x 17 mm) with a J-shaped end. They are held in position by means of a glass joint, on the upper end. The bottom ends are tapered (8 mm) with a small bulge so that the intestinal tissue could easily be mounted on the ends (Figure 3.8). The dimensions made it possible to fit the apparatus in a 250 ml glass beaker. The excised rat intestinal tissue was mounted (without

everting the intestine) so that the apical side of the intestine was facing inwards and the serosal/basolateral side was facing outwards to perform AP - BL studies (Dixit *et al.,* 2012:14). The excised rat intestinal tissue was everted and then mounted to perform BL-AP studies. The serosal/basolateral side was then facing inwards and the mucosal side outwards.

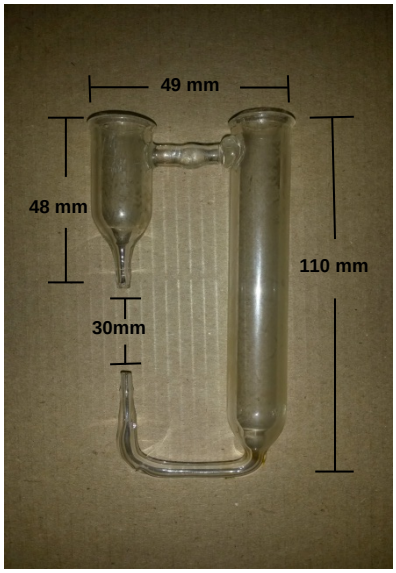

Figure 3.8: Designed everted sac apparatus for rat intestinal tissue

The dimensions for the pig everted sac glass apparatus were adapted from the previous design (Dixit *et al*., 2012) taking into consideration the dimensions of the pig jejunum. The first (longer J-shaped) cylindrical glass tube (130 mm x 25 mm) joined to the second (smaller) tube (45 mm x 25 mm) fitted into a 500 ml glass beaker. The bottom ends were also tapered with a small bulge to keep the intestinal tissue securely mounted (Figure 3.9).

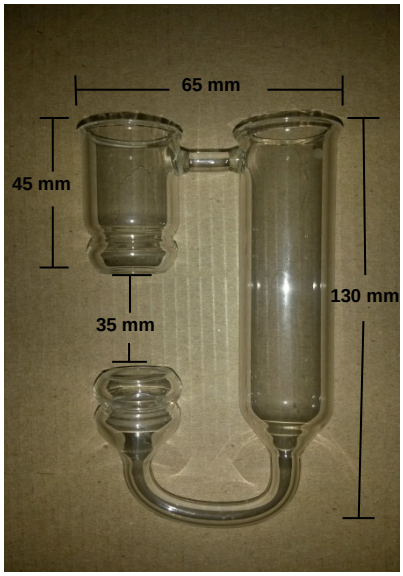

Figure 3.9: Designed everted sac apparatus for pig intestinal tissue

3.4.4.2 Transport studies using the everted sac technique with excised pig and rat intestinal tissues

The glass apparatuses with the mounted excised pig and rat intestinal segments were placed in 500 ml and 250 ml glass beakers, respectively. The beakers were filled to volume with freshly prepared pre-heated (37 °C) abacavir test solutions (131.94 µg/ml as indicated in Table 3.2). Each apparatus was supplied with gas flow (95% O_2: 5% CO_2) at a flow rate of 15 to 20 ml/min to supply the tissue with oxygen and ensure circulation of the buffer (Figure 3.10).

A 200 µl sample was withdrawn every 20 min from the acceptor chamber over the duration of 120 min. The acceptor chamber was immediately replenished after each sample withdrawal by adding the same volume of pre-heated KRB buffer solution. The samples were transferred into HPLC vials containing inserts and immediately refrigerated. The samples were then analysed by means of a validated HPLC analytical method for abacavir.

The TEER of the pig intestinal tissue was repeatedly measured within the Sweetana-Grass diffusion technique where excised pig intestinal tissue was exposed to the same conditions as those in the evreted sac technique. Since the excised pig intestinal tissue was found to be intact over the 2 h period in the diffusion apparatus, it was concidered to be iintact in the everdted sac technique. Furthermore, although the TEER was not measured within the everted sac technique in this study, previous studies have shown that the intestinal tissue remains viable for up to 2 h under strictly monitored, ideal, experimental conditions (Dixit *et al*., 2012:13; LeFerrec *et al*., 2000:265) (as discussed in section 2.2.3.2).

Figure 3.10: Image illustrating the everted sac apparatus with mounted excised pig intestinal tissue, in the circulating heating bath

3.4.5 Transwell®-plate technique

3.4.5.1 Culturing of Caco-2 cells

The Caco-2 cell line was used for the transport studies in the Transwell® plate technique. The cells were grown and then sub-cultured in 75 cm^2 cell growth flasks (Corning Costar Corporation, USA). DMEM was used as culture medium which contained 4.5 g/L D-glucose, 0.11 g/L sodium pyruvate and 4.0 mM L L-glutamine. It was supplemented with 10% fetal bovine serum, 1% Penicillin/streptomycin, 1% amphotericin B and 1% L-glutamine (Separations, Johannesburg, S.A.).

The cell cultures were incubated using a CO_2 incubator (Galaxy 170R, Eppendorf Company, Stevenage, UK) under strictly regulated conditions such as a temperature of 37 °C and an atmosphere of 95% air with 5% CO_2. The culture medium was replaced with freshly prepared growth medium every second day. This was done under aseptic conditions in a horizontal laminar air flow cabinet.

3.4.5.2 Trypsinisation of Caco-2 cell monolayers

Macroscopical inspection was done on the Caco-2 cells in the culture flasks and only flasks with 60 to 80% convergence were used for the trypsinisation procedure (within ± 7 days). HBSS, trypsin-versene solution (Separations Randburg, SA) and culture medium were heated in a water bath at 37 °C. The prepared solutions and the flasks containing the cell cultures were transferred to the horizontal laminar air flow cabinet where the cells were rinsed twice with the HBSS. After rinsing, the cells were covered by adding 0.5 ml of trypsin-

versene solution. It was then transferred to the incubator for 10 min and 2 ml of the heated cell culture medium (37 °C) were added. The cell suspension was then transferred to a 50 ml sterile tube and made up to volume with pre-heated culture medium (37 °C). It was then agitated with the aid of a Pasteur pipette to loosen the cells from each other in order to render a homogenous suspension. The flask was then rinsed with 1 ml of the heated medium (37 °C) to ensure that all the cells were removed from the flask.

3.4.5.3 Seeding of Caco-2 cell monolayers

Counting of the cells in suspension was conducted with a haemocytometer and the cell suspension was diluted to achieve a concentration of 1.77 x 10^4 cells/ml, which were seeded onto membrane filters in Costar Transwell® 6-well plates. The culture medium consisted of DMEM supplemented with 4.5 g/L D-glucose, sodium pyruvate, L-glutamine supplemented with 10% fetal bovine serum, 1% Penicillin/streptomycin, 1% amphotericin B, and 1% L-glutamine (Separations, Randburg, SA). To both the AP and BL compartments, 2.5 ml of pre-heated culture medium were added. The culture medium was changed every second day under aseptic conditions for a total of 21 days.

3.5 Transport studies using Caco-2 cell monolayers

A Millicell® ERS meter (Millipore, USA) connected to chopstick electrodes was used to measure the TEER of the Caco-2 cell monolayers before and after each transport study to ensure the viability of the monolayer. Prior to performing the transport experiment, (AP-BL direction of transport) the BL chamber was filled with fresh DMEM buffered at pH 7.4 with 25 mM HEPES. The AP chamber was filled with 2.5 ml of the abacavir solution with a concentration of 659.7 µg/ml. The reverse of the above procedure was followed when transport in the BL-AP direction was conducted. Every 20 min, a 200 µl sample was withdrawn from the acceptor chamber and replaced with fresh pre-heated DMEM in a horizontal laminar airflow cabinet. After sample collection, the Transwell® plates were placed back in the incubator. The samples were collected over a period of 120 min. The samples were placed in HPLC vials with inserts and analysed with a validated HPLC method as described in 3.3.

(A) **(B)**

Figure 3.10: Image illustrating A) the measurement of TEER across Caco-2 cell monolayers and B) withdrawal of a sample from the apical chamber of a Transwell® plate

3.6 Data processing and statistical analysis

3.6.1 Percentage transport (% Transport)

The percentage transport is calculated over each time interval (Equation 1). These calculated values are then used to plot a percentage transport vs. tme curve for each transport study.

$$\%\ \text{Transport} = \frac{\text{Peak area of sample at specific time interval}}{\text{Peak area of solution applied}} \times 100 \qquad \text{Eq. 1}$$

3.6.2 Apparent permeability coefficient (P_{app})

The apparent permeability coefficient (P_{app}) is an index commonly used and is a primary transport indicator used in the screening process during drug absorption studies. It is defined as the initial flux of a compound through the membrane, normalised by membrane surface area and donor concentration (Equation 2) (Palumbo *et al*., 2008:235).

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A.C_0.60} \qquad \text{Eq. 2}$$

In Equation 2, dQ/dt (g/s) represents the increase in the amount of drug in the acceptor chamber over time, A (cm^2) denotes the effective surface area of the excised tissue or cell monolayer exposed to the transport medium and C_0 (g/ml) represents the initial drug concentration in the donor chamber. The P_{app} values were calculated from the curves where percentage transport was plotted as a function of time (where time was given in minutes). To convert minutes to seconds, the equation includes 60 as a term, because P_{app} is expressed in $cm.s^{-1}$ (Hayeshi *et al*., 2006:72; Tarirai *et al*., 2012:257).

3.6.3 Efflux ratio (ER)

The efflux ratio (ER) was calculated from the transport data obtained in both directions to determine the extent of efflux of abacavir in the different *in vitro* models and methods. ER was calculated with equation 3.

$$ER = \frac{P_{app}(BL\text{-}AP)}{P_{app}(AP\text{-}BL)} \qquad \text{Eq. 3}$$

The apparent permeability in the secretory and absorptive directions is represented by P_{app} (BL-AP) and P_{app} (AP-BL), respectively (Tarirai *et al*., 2012:257).

3.6.4 Statistical analysis of results

Statistical analysis was performed on the transport data obtained across the excised pig and rat intestinal tissues (i.e. from both the Sweetana-Grass diffusion chamber technique and the everted sac technique) and the transport data obtained across the Caco-2 cell monolayers.

Analysis of variance (ANOVA) was performed to determine if there were any statistically significant differences between the P_{app} and mean ER values rendered by different methods and techniques. Tukey's test and Kurskal-Wallis's post-hoc tests were used to analyse non-parametric data. A student's t-test was also applied to all the data. The data was analysed with the aid of Statistica (Statsoft®, 2007). Differences between the data of the different methods and techniques were deemed statistically significant if $p \leq 0.05$.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 Introduction

The results obtained from the validation of the HPLC method are reported to prove that the analytical method, which was used to measure the abacavir concentrations in the transport samples, produced reliable results. The bi-directional transport of the model compound in each *in vitro* model (i.e. excised rat and pig intestinal tissues as well as Caco-2 cells) investigated in this study is reported separately for each technique (i.e. everted gut sac, Sweetana-Grass diffusion chamber and Transwell® plates). This is followed by a statistical comparison between the methods in terms of P_{app} values as well as ER values. The statistical analysis of the data was also used to determine if significant differences exist in terms of drug permeation between the three different species namely rat, pig and human models.

4.2 Validation of HPLC analytical method

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

4.2.1 Specificity

The HPLC chromatograms of an abacavir solution and KRB buffer solution are given in Figures 4.1 and 4.2, respectively.

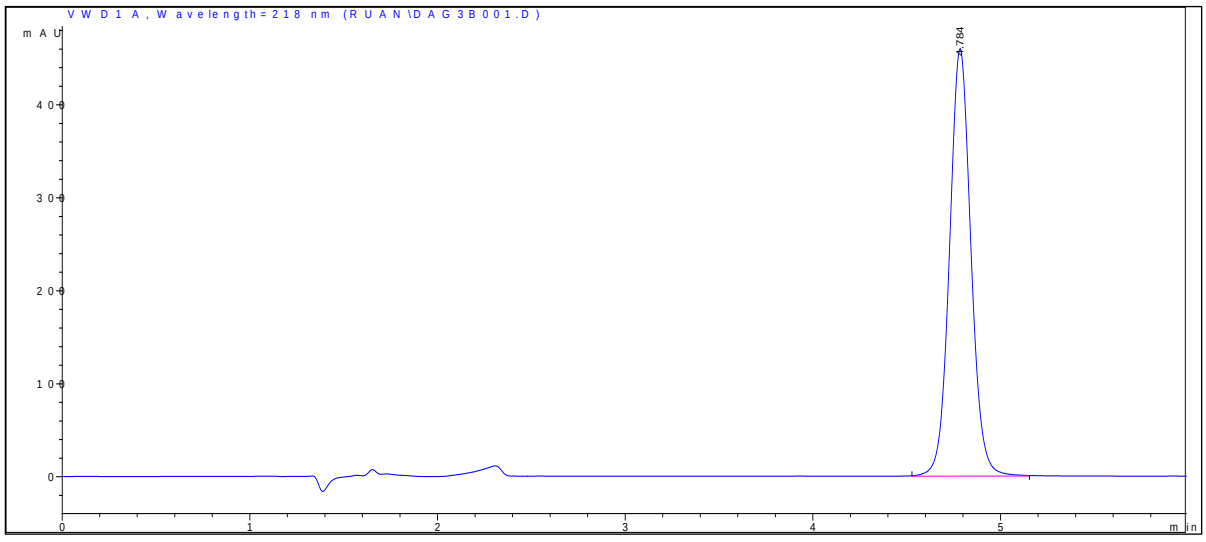

Figure 4.1: High performance liquid chromatography (HPLC) chromatogram of abacavir

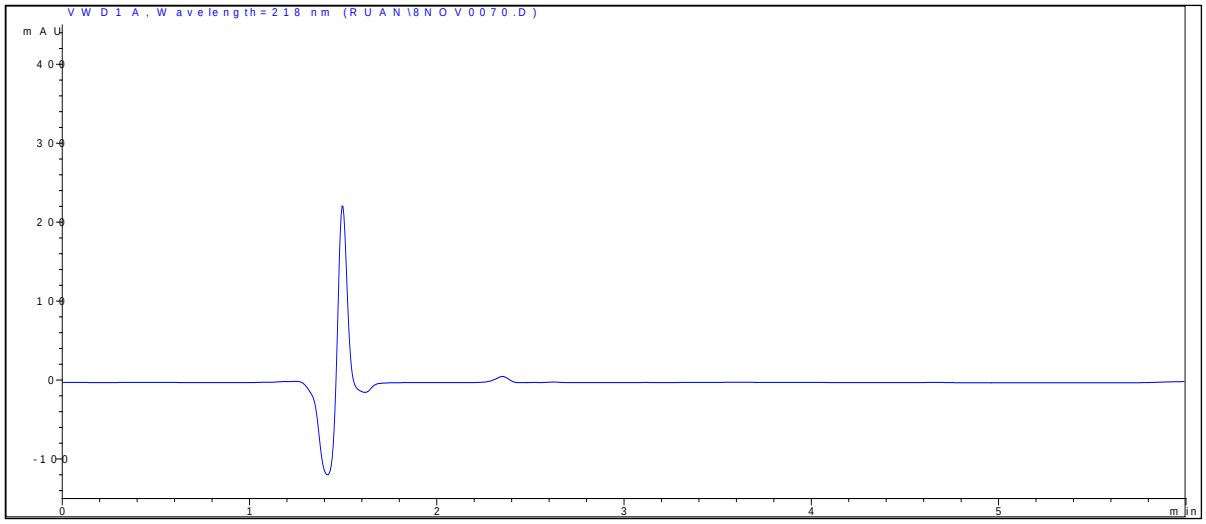

Figure 4.2: High performance liquid chromatography (HPLC) chromatogram of Kreb's Ringer Bicarbonate (KRB) buffer solution (placebo)

From Figures 4.1 and 4.2, it is clear that there were no interferences with the peak of abacavir by using KRB as solvent (or transport medium). Abacavir exhibited a retention time of 4.8 min whereas the chromatogram of the KRB buffer solution showed no peak visible that can cause any interference at that spesific time interval.

4.2.2 Linearity

The peak areas for the standard solutions of abacavir, injected at different volumes, are given in Table 4.1.

As described in the method section, different volumes from each dilution of the standard abacavir solution were injected in duplicate into the HPLC and the peak areas of abacavir were determined. The chromatographic data from this series of abacavir solutions were used to determine if a linear correlation exists between peak area and concentration for the HPLC analytical method used to determine the abacavir concentrations in the transport samples.

Table 4.1: Peak areas obtained for a series of abacavir standard solutions

Standard solution (µg/ml)	Injection volume	Final concentration (µg/ml)	Peak area		Average peak area
1.221	1	0.024	10.7	11.1	10.9
	2.5	0.061	26.6	25.7	26.1
	5	0.122	51.7	50.7	51.2
	10	0.244	101.1	102.0	101.6
	20	0.488	204.5	203.2	203.8
	30	0.733	305.5	306.4	305.9
	40	0.977	408.5	406.6	407.5
	50	1.221	508.7	508.9	508.8
12.21	10	2.442	1040.2	1040.2	1040.2
	20	4.884	2082.5	2082.9	2082.7
	30	7.326	3125.2	3124.2	3124.7
	40	9.768	4164.4	4159.8	4162.1
	50	12.210	5194.5	5197.8	5196.1
122.1	10	24.420	9970.7	9971.7	9971.2
	20	48.840	19513.5	19562.6	19538.1
	30	73.260	27291.1	27245.0	27268.1
	40	97.680	32329.8	32386.3	32358.1
	50	122.100	36133.9	36133.9	36133.9

From Table 4.1 it is clear that the peak areas increased with an increase in abacavir concentration in the standard solutions. The peak area values for the duplicate injections were very similar and showed only slight variations indicating high repeatability.

The linear regression graph was constructed by plotting the peak area as a function of concentration (Figure 4.3). The regression statistical analysis data obtained from this graph is shown in Table 4.2.

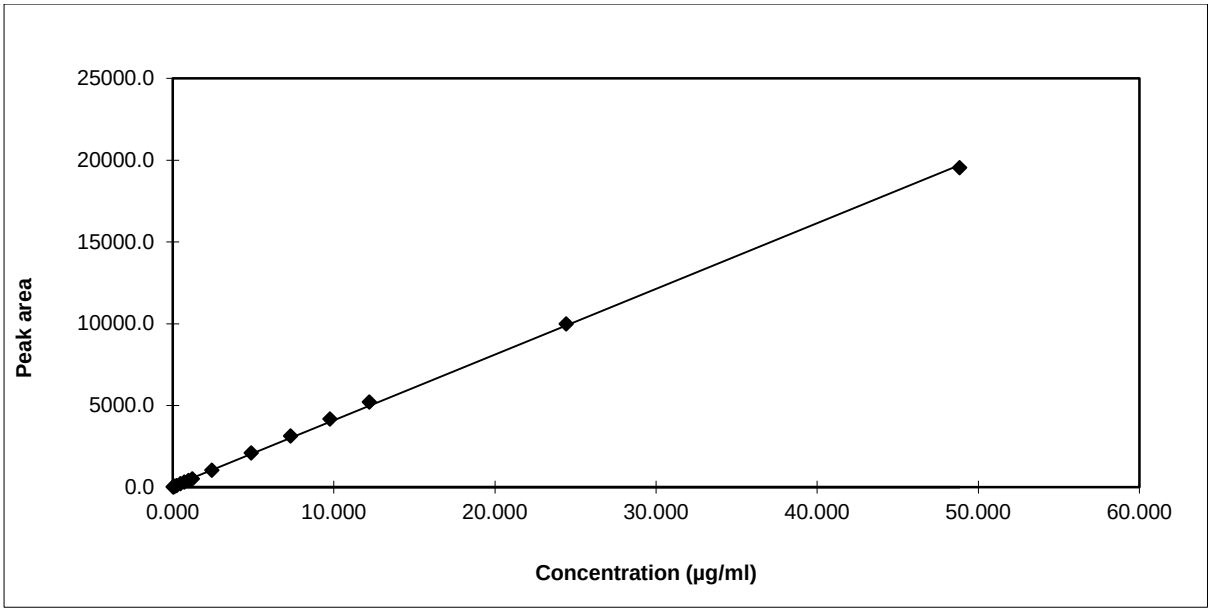

Figure 4.3: Linear regression graph (or standard curve) of abacavir

Table 4.2: Regression statistics obtained from the standard curve of abacavir

R^2	0.9996
Intercept	0
Slope	404.1136

According to the scientific literature on the topic of HPLC method validation, the linear regression analysis between peak area and concentration should yield an R squared (R^2) value of at least 0.99 (Shabir, 2004:30-31; Singh, 2013:29). From Table 4.2, it is clear that the R^2 value for the linear regression graph of the HPLC analytical method used in this study for abacavir is 0.9996 and therefore meets the required criteria. The data given in Table 4.2 and shown in Figure 4.3, indicate linearity for the graph of peak area plotted as a function of concentration over a concentration range of 0.0024 to 0.0122 µg/ml. Based on these results the analytical method was deemed suitable for abacavir analysis in the transport samples.

4.2.3 Limit of quantification and limit of detection

The chromatographic data obtained from the limit of quantification (LOQ) and limit of detection (LOD) evaluation are shown in Table 4.3. From Table 4.3 it is clear that the LOD for abacavir, using this method of analysis, is 0.0024 µg/ml (% RSD ≤ 30). The LOQ is 0.0048 µg/ml with a % RSD ≤ 10.

Table 4.3: Data from limit of quantification and limit of detection measurements

Concentration (µg/ml)	Injection volume	Final concentration (µg/ml)	Peak area						Mean	% RSD
11.4	10	0.0024	1.681	0.949	1.080	1.0111	0.9838	0.805	1.085	25.7
	20	0.0048	1.876	2.012	2.132	2.048	1.852	2.244	2.027	6.7
	30	0.0073	2.851	2.999	3.003	2.806	2.782	2.216	2.776	9.5
	50	0.0122	6.461	7.191	7.006	6.253	7.430	7.913	7.042	8.0
LOQ = 0.0048 µg/ml (% RSD ≤ 10)										
LOD = 0.0024 µg/ml (% RSD ≤ 30)										

4.2.4 Accuracy

The data obtained from the measurements to determine accuracy are given in Table 4.4.

Table 4.4: Data obtained from spiked solutions to determine accuracy

Repeat	Conc. spiked (µg/ml)	Peak area		Mean	Recovery (µg/ml)	% Recovery
		1	2			
1	10.2	887.1415	886.39331	887	10.6	103.3
	102.4	9059.915	9043.3252	9052	108.0	105.5
	256.0	22620.9	22562.2	22592	269.6	105.3
2	10.1	857.7335	856.00018	857	10.2	101.0
	101.2	8675.223	8673.9346	8675	103.5	102.3
	253.0	21636.4	21654.5	21645	258.3	102.1
3	10.0	812.1157	810.88281	811	9.7	96.4
	100.4	8307.162	8292.1123	8300	99.0	98.6
	251.0	20713.4	20721.9	20718	247.2	98.5
Mean % recovery = 101.45						
SD = 2.94						
% RSD = 2.89						

It is recommended by the International Conference of Harmonisation (ICH) that the mean percentage recovery of an analyte should be between 98 to 102% for the method to be considered sufficiently accurate (ICH, 1996:4). This HPLC analytical method yielded a mean percentage recovery of 101.45%, which means that it meets the specific criteria and is therefore acceptable.

4.2.5 Precision

A robust analytical method is required to ensure repeatable measurements of homogenous samples, prepared in a similar way, whenever the analysis is repeated. Therefore intra-day and inter-day precision was measured.

4.2.5.1 Intra-day precision

Intra-day precision was performed with the same solutions as used for the accuracy measurements. A RSD value of 2.89% (Table 4.4) was obtained, which is less than the specified value of 5% (n = 9) (Shabir, 2004:33). The method proved to be precise when subjected to intra-day measurements.

4.2.5.2 Inter-day precision

The chromatographic data obtained for the inter-day precision measurements, one-way analysis of variance (ANOVA) single factor statistics and the ANOVA statistics done on the inter-day chromatographic data are summarised in Tables 4.5, 4.6 and 4.7, respectively.

Table 4.5: Inter-day precision data obtained for abacavir

	Day 1	**Day 2**	**Day 3**	**Average**
Mean % recovery	103.41	104.08	105.98	104.49
SD	0.23	2.15	7.23	3.20
% RSD	0.22	2.07	6.82	3.04

Table 4.6: Variance analysis (ANOVA single factor) of inter-day precision data

SUMMARY				
Groups	**Repeats**	**Sum total peak area**	**Average peak area**	**Variance**
Day 1	3	310.227	103.409	0.078
Day 2	3	308.613	102.871	0.109
Day 3	3	308.870	102.957	0.098

Table 4.7: ANOVA statistics for inter-day precision data

ANOVA					
	Df*	***SS****	***MS****	***F****	***Significance F****
Regression	1	$8.94 \times 10^{+8}$	$8.94 \times 10^{+8}$	673757.20	39.88×10^{-8}
Residual	4	5305.86	1326.47	-	-
Total	5	$8.94 \times 10^{+8}$	-	-	-

SS* denotes the sum of squares, df* the degrees of freedom, MS* the mean squares and F* the ratio.

Inter-day precision as indicated by the % RSD value is required to be less than ± 5% (n = 9). This method showed a % RSD of 3.04 (Table 4.6) for inter-day precision data and is therefore acceptable in terms of inter-day precision.

4.2.6 Stability

Table 4.8: Percentage recovery over 24 h from an abacavir solution to indicate stability

Time (hours)	Peak Area	% Recovery
0	19614.90	100.00
1	19610.90	100.00
2	19573.60	99.80
3	19587.80	99.90
4	19605.00	99.90
5	19574.70	99.80
6	19595.10	99.90
7	19583.60	99.80
8	19641.30	100.10
9	19629.10	100.10
10	19634.40	100.10
11	19639.30	100.10
12	19637.30	100.10
13	19648.60	100.20
14	19665.60	100.30
15	19699.90	100.40
16	19681.60	100.30
17	19668.70	100.30
18	19649.20	100.20
19	19702.60	100.40
20	19705.30	100.50
21	19741.30	100.60
22	19681.20	100.30
23	19721.50	100.50
24	19774.10	100.80
Mean	19650.70	100.20
SD	52.28	0.27
% RSD	0.27	0.27

From table 4.8 the mean percentage recovery from the abacavir sample over a 24 h period was 100.20% with a % RSD of 0.27. Thus abacavir remained stable in solution over a period of 24 h.

4.2.7 Summary of validation results

No interference was encountered from other materials with the abacavir peak on the HPLC chromatogram and therefore the method was found to be specific for the analyte. The HPLC analytical method was proven to be accurate, precise and repeatable and abacavir remained stable in solution over 24 h. The method used in this study to analyse the transport samples for abacavir content is therefore suitable and valid.

4.3 Transport conducted in different *in vitro* methods and with different techniques

For the excised animal jejunum tissues, the transport was investigated by means of two techniques namely the everted sac apparatus and Sweetana-Grass diffusion chambers. For the Caco-2 cell monolayers, the Transwell® plate membrane technique was used.

The transport data was used to calculate the P_{app} and ER values, which were statistically analysed by means of the Kruskal-Wallis test. The p-values were then used to compare the methods and techniques, $p \leq 0.05$ indicates a statistically significant difference.

4.3.1 Rat model

The TEER values of the excised rat intestinal tissue, mounted in the Sweetana-Grass diffusion chamber apparatus, showed a decrease of 13.89% during the transport study in the AP - BL direction and a decrease of 22.50% during the transport study in the BL - AP direction over a period of 120 min. These relatively low reductions in TEER values indicate that the viability of the excised rat tissue was maintained throughout the duration of the transport study. The slightly larger percentage TEER decrease in the excised rat intestinal tissue compared to that of the excised pig intestinal tissue (refer to section 4.3.2) could be attributed to the more fragile nature of the thinner rat intestinal tissue.

4.3.1.1 Everted sac technique

The bi-directional transport (i.e. AP - BL and BL - AP) of abacavir was determined across excised rat jejunum fitted on a custom made glass apparatus (based on that of Dixit *et al*., 2012 as described earlier). The average percentage bi-directional transport (Eq. 1) of abacavir across the rat jejunum was plotted as a function of time, and is shown in Figure 4.4.

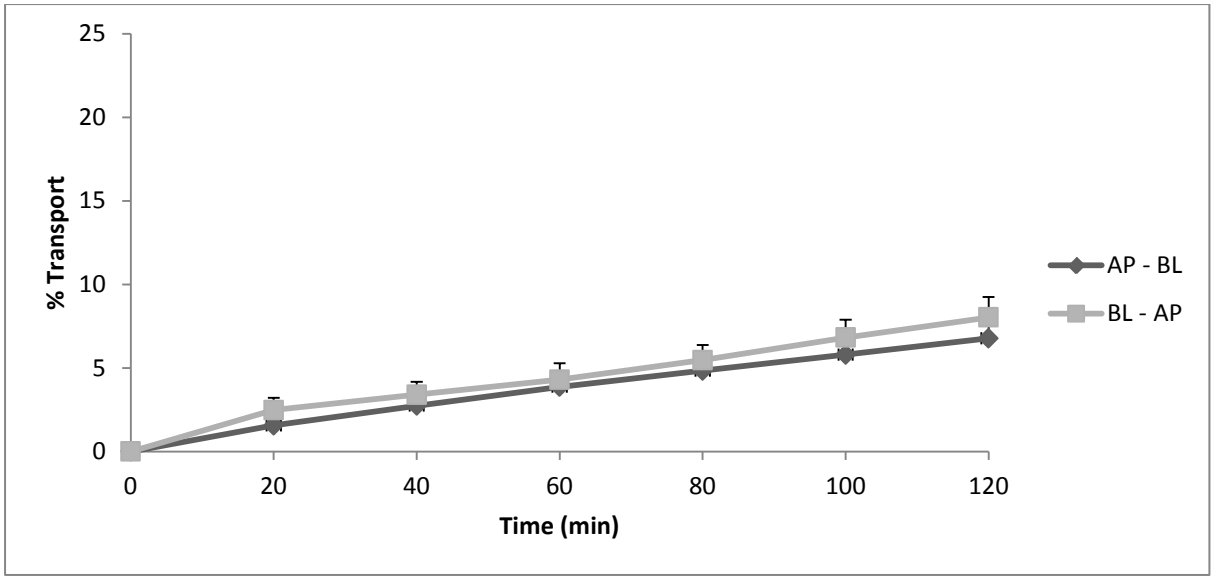

Figure 4.4: Percentage transport (bi-directional) of abacavir across rat jejunum, using the everted sac technique, plotted as a function of time

The total percentage cumulative transport of abacavir (i.e. average of three rats, each repeated in six fold) over a period of 120 min was 6.774% in the AP - BL direction, while it was 8.023% in the BL - AP direction. This higher transport in the BL - AP direction compared to the transport in the AP - BL direction means that active efflux of abacavir occurred. This indicates that excised rat jejunum tissue, mounted on the everted sac apparatus, can be used as a model for investigating transport of compounds that are substrates for active transporters (e.g. P-glycoprotein).

Furthermore, the transport graphs in Figure 4.4 illustrate relatively low SD values (as indicated by the small error bars), which suggest high repeatability and low intra-species variation (i.e. between three rats, each repeated six fold) within this model.

The P_{app} values calculated from the transport curves are shown in Table 4.9 with a mean P_{app} of 6.13 ± 1.13 in the AP - BL direction and 6.68 ± 1.05 in the BL - AP direction.

Table 4.9: Apparent permeability coefficient (P_{app}) values and efflux ratio (ER) values of abacavir transport across excised rat jejunum using the everted sac technique

Repeat	Inter-species	AP - BL (P_{app}) x 10^{-6}	BL - AP (P_{app}) x 10^{-6}	ER
R 1	1	4.71	10.32	2.19
	2	4.81	4.62	0.96
	3	6.65	7.40	1.11
	4	9.13	6.83	0.75
	5	5.36	10.81	2.01
	6	12.03	8.57	0.71
	Average P_{app}	7.12 ± 2.66	8.09 ± 2.11	1.29 ± 0.59
R 2	1	5.53	7.88	1.42
	2	7.75	5.65	0.73
	3	3.23	2.08	0.65
	4	1.78	6.14	3.46
	5	6.73	5.35	0.80
	6	2.24	6.34	2.83
	Average P_{app}	4.54 ± 2.26	5.57 ± 1.75	1.65 ± 1.10
R 3	1	9.42	6.84	0.73
	2	4.83	5.83	1.21
	3	6.22	5.90	0.95
	4	7.42	6.61	0.89
	5	7.178	6.40	0.89
	6	5.28	6.78	1.28
	Average P_{app}	6.72 ± 1.52	6.39 ± 0.40	0.99 ± 0.19
Mean Average		6.13 ± 1.13	6.68 ± 1.05	1.31 ± 0.27

The P_{app} and ER values shown in Table 4.9 are discussed in more detail in section 4.4 where a statistical comparison between the methods is presented.

4.3.1.2 Sweetana-Grass diffusion chamber technique

The bi-directional transport of abacavir was determined across excised rat jejunum mounted in a Sweetana-Grass diffusion apparatus. The average percentage bi-directional transport of abacavir across the rat jejunum was plotted as a function of time, and is shown in Figure 4.5.

The total percentage transport of abacavir (i.e. average of three rats, each repeated in six fold) across the rat jejunum in the AP - BL direction within the Sweetana-Grass diffusion chamber was 4.436% over a period of 120 min, while the average total percentage transport in the BL - AP direction was 6.481%. The SD values in the AP - BL direction was smaller than the SD values in the BL - AP direction (as indicated by the error bars in Figure 4.5).

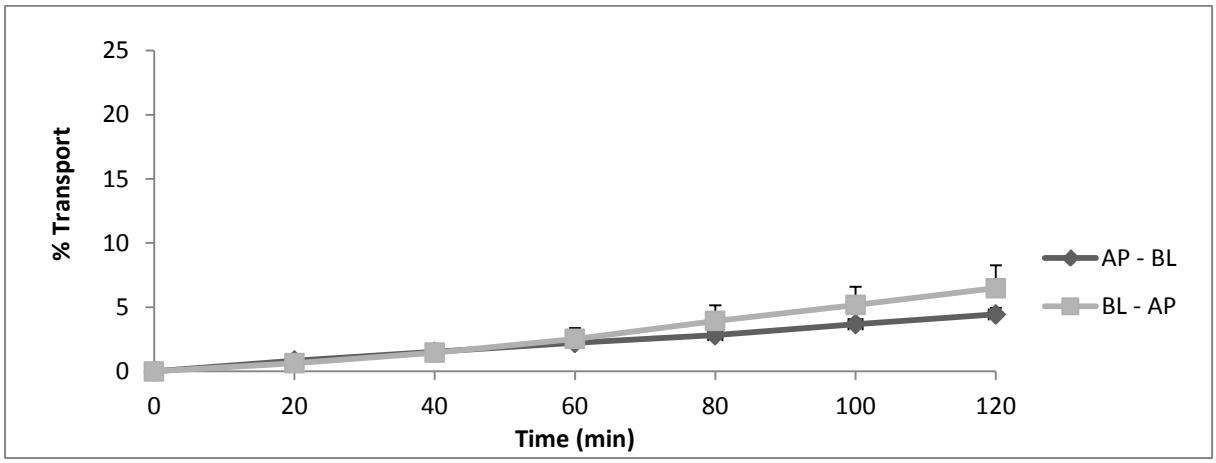

Figure 4.5: Percentage transport (bi-directional) across rat jejunum, using the Sweetana-Grass diffusion technique, plotted as a function of time

The transport of abacavir across excised rat jejunum tissue in Sweetana-Grass diffusion chambers also showed that active efflux transport occurred which is proven by the higher extent of transport in the BL - AP direction compared to that in the AP - BL direction. It is interesting to note that the total percentage abacavir transport across the excised rat intestinal tissue in the diffusion chamber technique was similar to that obtained in the everted sac technique, despite the differences in surface area available for drug transport.

Although the SD values are higher for the transport in the BL - AP direction, they are in general still relatively low, indicating low intra-species variation (i.e. between three rats, each repeated six fold).

The P_{app} values, calculated from the transport curves, are shown in Table 4.10 with a mean P_{app} of 3.39 ± 0.52 in the AP - BL direction and 5.19 ± 1.60 in the BL - AP direction.

Table 4.10: Apparent permeability coefficient (P_{app}) values and efflux ratio (ER) values of abacavir transport across excised rat jejunum using the Sweetana-Grass diffusion technique

Repeat	Inter-species	AP - BL(P_{app}) x 10^{-6}	BL - AP (P_{app}) x 10^{-6}	ER
R 1	**1**	0.883	3.770	4.27
	2	2.912	9.194	3.16
	3	5.290	3.212	0.61
	4	4.052	3.662	0.90
	5	2.340	3.241	1.39
	6	3.371	5.777	1.71
	Average P_{app}	3.14 ± 1.37	4.81 ± 2.14	2.01 ± 1.30
R 2	**1**	3.062	3.372	1.10
	2	2.617	3.678	1.41
	3	2.472	2.805	1.13
	4	3.972	3.969	1.00
	5	2.243	3.375	1.50
	6	3.139	3.457	1.10
	Average P_{app}	2.92 ± 0.57	3.44 ± 0.35	1.21 ± 0.18
R 3	**1**	3.819	6.735	1.76
	2	4.335	9.436	2.18
	3	3.898	4.773	1.22
	4	4.920	5.563	1.13
	5	4.316	9.201	2.13
	6	3.400	8.106	2.38
	Average P_{app}	4.12 ± 0.48	7.30 ± 1.76	1.80 ± 0.48
Mean Average		3.39 ± 0.52	5.19 ± 1.60	1.67 ± 0.34

The P_{app} and ER values shown in Table 4.10 are discussed in more detail in section 4.4 where a statistical comparison between the methods is presented.

4.3.2 Pig model

The TEER values of the excised pig intestinal tissue, mounted in the Sweetana-Grass diffusion chamber apparatus, showed an average decrease of 6.87% during the transport study in the AP - BL direction and 5.53% during the transport study in the BL - AP direction over a period of 120 min. This relatively low reduction in TEER values indicates that the excised pig intestinal tissue remained intact and was viable for the duration of the transport study.

4.3.2.1 Everted sac apparatus

The bi-directional transport of abacavir was determined across excised pig jejunum fitted on a custom made glass apparatus (as described earlier by Dixit *et al.,* 2012:14). The average percentage bi-directional transport of abacavir across pig jejunum was plotted as a function of time, and is shown in Figure 4.6.

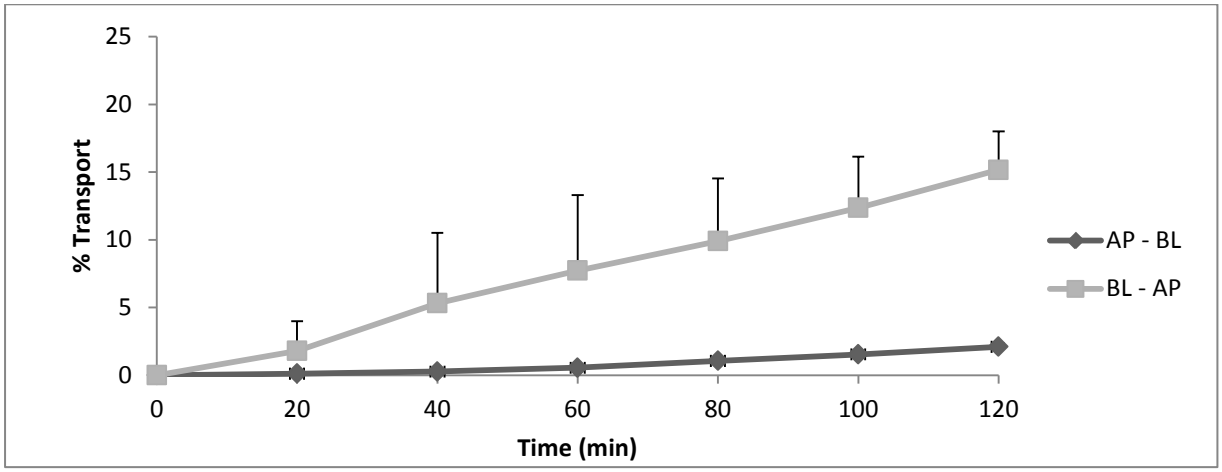

Figure 4.6: Percentage transport (bi-directional) of abacavir across pig jejunum, using the everted sac technique, as a function of time

The total percentage transport of abacavir (i.e. average of three pigs, each repeated in six fold) over a period of 120 min was 2.097%, while it was 15.162% in the BL - AP direction. This much higher transport in the BL - AP direction compared to the transport in the AP - BL direction suggests that active efflux of abacavir occurred and active transporters such as P-glycoproteins (P-gp) were present, and functional, over the entire period of the transport experiment. This attribute suggests that this model may be suitable for investigating transport of compounds which are substrates for active transporters. Transport in the AP -

BL direction displayed a 20 min lag phase in comparison to the BL - AP direction which displayed an immediate onset of transport.

Although the SD values were higher for the transport in the BL - AP direction, they were generally low indicating low intra-species variation.

The P_{app} values calculated from the transport curves are shown in Table 4.11 with a mean P_{app} of 0.05 ± 0.01 in the AP - BL direction and 0.33 ± 0.07 in the BL - AP direction.

Table 4.11: Apparent permeability coefficient (P_{app}) values and efflux ratio (ER) values of abacavir transport across excised pig jejunum using the everted sac technique

Repeat	Inter-species	AP - BL (P_{app}) x 10^{-6}	BL - AP (P_{app}) x 10^{-6}	ER
P1	**1**	0.042	0.299	7.12
	2	0.043	0.462	10.74
	3	0.071	0.423	5.96
	4	0.041	0.481	11.73
	5	0.041	0.267	6.51
	6	0.048	0.390	8.13
	Average P_{app}	0.05 ± 0.01	0.39 ± 0.08	8.37 ± 2.15
P2	**1**	0.029	0.885	30.52
	2	0.061	0.506	8.30
	3	0.044	0.124	2.89
	4	0.038	0.109	2.87
	5	0.028	0.287	10.25
	6	0.029	0.328	11.31
	Average P_{app}	0.04 ± 0.01	0.37 ± 0.27	11.01 ± 9.33
P3	**1**	0.066	0.228	3.45
	2	0.035	0.241	6.89
	3	0.046	0.217	4.72
	4	0.052	0.365	7.02
	5	0.052	0.151	2.90
	6	0.055	0.199	3.62
	Average P_{app}	0.05 ± 0.01	0.23 ± 0.07	4.77 ± 1.64
Mean Average		0.05 ± 0.01	0.33 ± 0.07	8.05 ± 2.56

From Table 4.11, it is clear that the transport of abacavir (as indicated by the P_{app} values) was an order of magnitude smaller than that obtained across the excised rat tissue. The P_{app} and ER values shown in Table 4.11 are discussed in more detail in section 4.4 where a statistical comparison between the methods is presented.

4.3.2.2 Sweetana-Grass diffusion chambers

The bi-directional transport of abacavir was determined across excised pig jejunum mounted in a Sweetana-Grass diffusion apparatus. The average percentage bi-directional transport of abacavir across the pig jejunum was plotted as a function of time, and is shown in Figure 4.7.

The total percentage transport of abacavir (in three pigs, each repeated in six fold) across pig jejunum in the AP - BL direction in Sweetana-Grass diffusion chambers were 6.044% over a period of 120 min, while the mean percentage transport in the BL - AP direction was 12.323%.

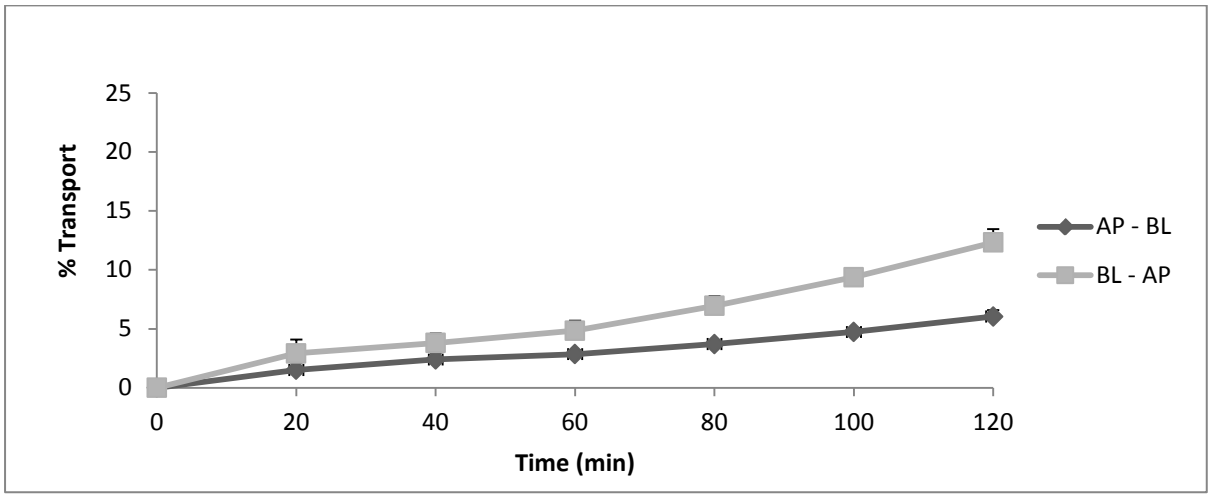

Figure 4.7: Percentage transport (bi-directional) across pig jejunum using the Sweetana-Grass diffusion technique, plotted as a function of time

The transport graphs in Figure 4.7 illustrate exceptionally low SD values (as indicated by the small error bars), which suggest high repeatability and low intra-species variation in this model. The total transport of abacavir in the BL - AP direction was similar in the everted sac and diffusion chamber techniques, while the transport in the AP - BL direction was higher when using the diffusion chamber technique than when using the everted sac technique.

The P_{app} values calculated from the transport curves are shown in Table 4.12 with a mean P_{app} of 4.33 ± 0.16 in the AP - BL direction and 8.87 ± 0.65 in the BL - AP direction.

Table 4.12: Apparent permeability coefficient (P_{app}) values and efflux ratio (ER) values of abacavir transport across excised pig jejunum using the Sweetana-Grass diffusion chamber technique

Repeat	Inter-species	AP - BL (P_{app}) x 10^{-6}	BL - AP (P_{app}) x 10^{-6}	ER
P1	1	3.80	8.82	2.32
	2	4.32	6.60	1.53
	3	3.88	8.20	2.11
	4	4.90	12.96	2.64
	5	4.30	4.43	1.03
	6	3.38	17.16	5.07
	Average P_{app}	4.10 ± 0.48	9.69 ± 4.22	2.45 ± 1.29
P2	1	7.38	11.97	1.62
	2	1.28	6.80	5.30
	3	2.84	7.90	2.78
	4	1.09	6.83	6.27
	5	7.30	13.25	1.81
	6	6.87	6.05	0.88
	Average P_{app}	4.46 ± 2.78	8.80 ± 2.77	3.11 ± 1.99
P3	1	3.10	6.51	2.10
	2	7.71	6.80	0.88
	3	4.25	8.69	2.05
	4	1.30	7.40	5.70
	5	7.07	11.71	1.66
	6	3.07	7.51	2.45
	Average P_{app}	4.42 ± 2.28	8.10 ± 1.75	2.47 ± 1.52
Mean Average		4.33 ± 0.16	8.87 ± 0.65	2.68 ± 0.31

The P_{app} and ER values shown in Table 4.12 are discussed in more detail in section 4.4 where a statistical comparison between the methods is presented.

4.3.3 Caco-2 cell monolayers (Human)

Before the start of the transport study, the TEER measurements of the Caco-2 cell monolayers were higher than 250 Ω with an average percentage decrease in TEER of 19.81% and 11.49% in the AP - BL and BL - AP directions, respectively (Appendix D). According to the literature this is in an acceptable range for cell culture based drug transport studies (Le Ferrec *et al*., 2001:658).

4.3.3.1 Transwell® plates

The bi-directional transport of abacavir was determined across Caco-2 cell monolayers on Transwell® plates. The average percentage bi-directional transport of abacavir across Caco-2 cell monolayers was plotted as a function of time, and is shown in Figure 4.8.

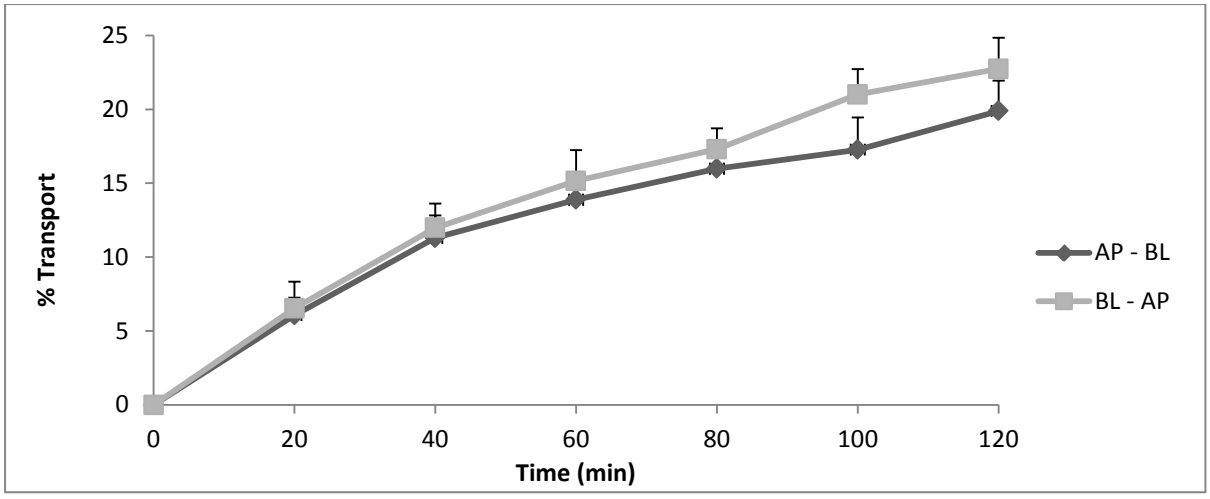

Figure 4.8: Percentage transport (bidirectional) of abacavir across Caco-2 cell monolayers on Transwell® membrane plates plotted as a function of time

The percentage transport, in both directions, was nearly identical for the first 40 min. An initial rapid onset of transport occurred, thereafter (40 to 120 min), the overall percentage transport in the BL - AP direction showed a more rapid increase than in the AP - BL direction. The total percentage transport of abacavir (repeated in six fold) across the Caco-2 cell monolayers in the AP - BL direction in the diffusion chamber was 19.89 % over a period of 120 min, while the mean percentage transport in the BL - AP direction was 22.732%. The SD values in both directions (AP - BL and BL - AP) was small (as indicated by the error bars

in Figure 4.8), which suggest high repeatability and low intra-species variation when using Caco-2 cell monolayers.

The P_{app} values calculated from the transport curves are shown in Table 4.13.

Table 4.13: Apparent permeability coefficient (P_{app}) values and efflux ratio (ER) values of abacavir transport across Caco-2 cell monolayers using the Transwell® plate technique

Repeat	Inter-species	AP - BL (P_{app}) x 10^{-6}	BL - AP (P_{app}) x 10^{-6}	ER
H1	1	6.74	6.25	0.93
	2	4.98	8.15	1.64
	3	6.06	6.28	1.04
	4	5.08	5.99	1.18
	5	5.43	6.59	1.21
	6	4.87	5.91	1.21
Average P_{app}		5.53 ± 0.67	6.53 ± 0.76	1.20 ± 0.22

The P_{app} and ER values shown in Table 4.13 are discussed in more detail in section 4.4 where a statistical comparison between the methods is presented.

4.4 Statistical comparison of transport data

The calculated P_{app} values, described above and summarised in Appendix E, were statistically analysed by means of ANOVA (Univariate Test of Significance) and Tukey HSD tests (Appendix E). From this analysis no normal distribution characteristics of the data could be found. The analysis was then repeated by using non-parametric tests (e.g. Mann-Whitney U test and Kruskal-Wallis ANOVA test). This approach was followed to minimise any discrepancies that may arise due to data not exhibiting normal distribution characteristics. P-values were used to identify any statistically significant differences between the methods and techniques. $P \leq 0.05$ indicates a statistically significant difference between the compared groups.

4.4.1 Comparison of drug transport between species using the Sweetana-Grass diffusion chamber technique

4.4.1.1 Transport across excised rat tissue compared to transport across excised pig tissue

The P_{app} values based on abacavir transport across excised rat and pig intestinal tissue are shown in Figure 4.9.

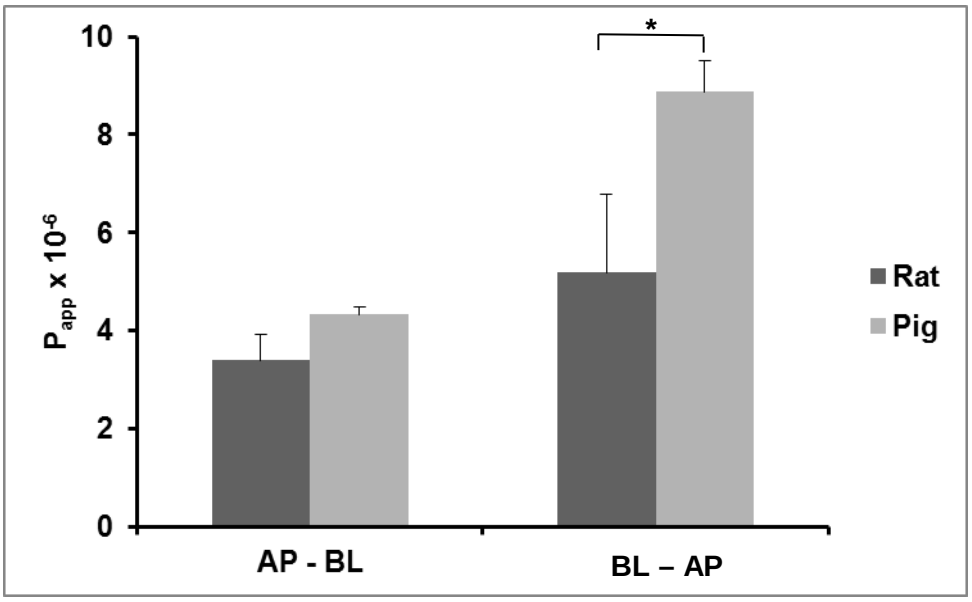

Figure 4.9: Comparison of drug transport between species (i.e. rat and pig) using the Sweetana-Grass diffusion chamber technique (* denotes a statistically significant difference, p = 0.001)

Due to non-normal distribution of the transport data with a non-parametrical nature, the Mann-Whitney U test was applied for statistical analysis. In the AP - BL direction, there was no statistically significant difference (p = 0.367) between the P_{app} values calculated from the transport across excised rat and pig intestinal tissues. This indicates similar transport across excised rat and pig intestinal tissues when using the Sweetana-Grass diffusion technique.

A statistically significant difference was, however, observed in the BL - AP direction (p = 0.001) between the rat and pig model. Further investigation is needed to verify if this may be due to a higher number of efflux transporters present in the pig jejunum comopared to that of the rat jejunum.

From this comparison between the rat and pig model using the Sweetana-Grass diffusion technique, it is apparent that they did not deliver similar results in terms of active efflux

transport and are therefore not comparable as models for predicting efflux of the BCS class II compounds such as abacavir. The models also differ in terms of passive diffusion characteristics of drugs probably due to differences in thickness. This would suggest that the excised pig intestinal model may exhibit lower transport of a drug with consequent underestimation of the bioavailability of the drug.

4.4.1.2 Rat (Sweetana-Grass diffusion chambers) compared to Caco-2 (Transwell® membrane plates)

The P_{app} values based on abacavir transport across the excised rat intestinal tissue and Caco-2 cell monolayers are shown in Figure 4.10.

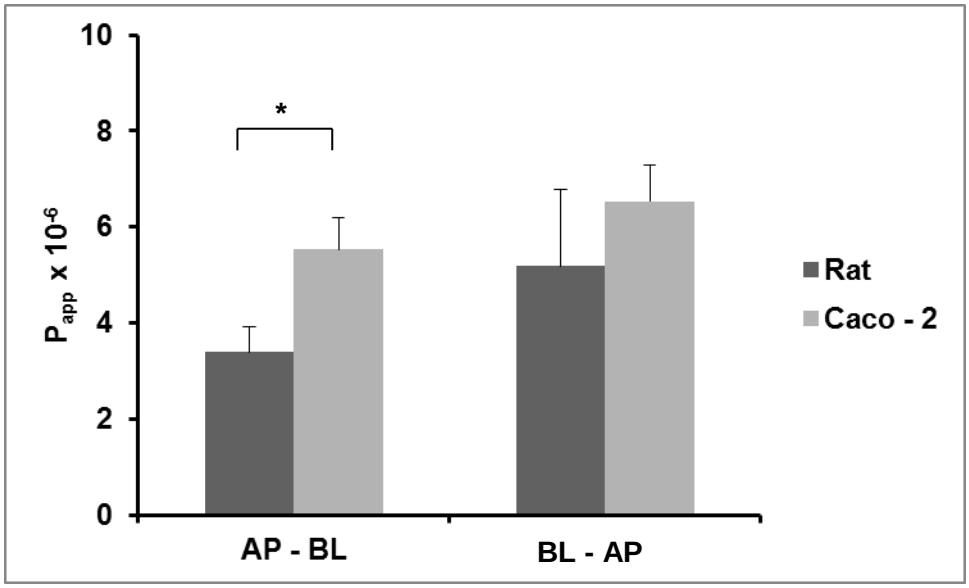

Figure 4.10: Comparison of drug transport between species (i.e. rat and human Caco-2 cells) using Sweetana-Grass diffusion and Transwell® plate techniques (*denotes a statistically significant difference, $p \leq 0.001$)

The statistical comparison of drug transport across rat intestinal tissue (Sweetana-Grass diffusion chamber) and Caco-2 cell monolayers (Transwell® membrane plates) were done with the non-parametric Kruskal-Wallis ANOVA test. In the AP - BL direction, there was a statistically significant difference ($p = 0.0105$) between the P_{app} values calculated for the transport data of abacavir across excised rat tissue and Caco-2 cell monolayers.

However, there was no statistically significant difference ($p = 0.1435$) in drug transport across either rat intestinal tissue or Caco-2 cell monolayers. This indicates that similar drug efflux could be expected across excised rat intestinal tissue (Sweetana-Grass diffusion technique) and Caco-2 cell monolayers (Transwell® plates).

The Caco-2 cell membrane is only a flat monolayer of cells. The rat intestinal tissue is much thicker and this may explain (by Fick's law) why transport across the intestinal tissue occurred to a lesser extent than across the Caco-2 cell monolayer. The absence of a mucus layer on the Caco-2 cell monolayer's AP surface may also have had an influence on the transport. Drugs are known to bind with mucin to form a non-absorbable chelate which cannot be transported across the intestinal wall and diffusion across the mucus layer will have a negative effect on the rate of transport. The presence of a lamina propria and muscularis mucosa, in rat intestinal tissue, may also influence drug transport negatively (Le Ferrec *et al*., 2001:654-655).

4.4.1.3 Pig (Sweetana-Grass diffusion chambers) compared to Caco-2 (Transwell® membrane plates)

The P_{app} values based on abacavir transport across excised pig intestinal tissue and Caco-2 cell monolayers are shown in Figure 4.11.

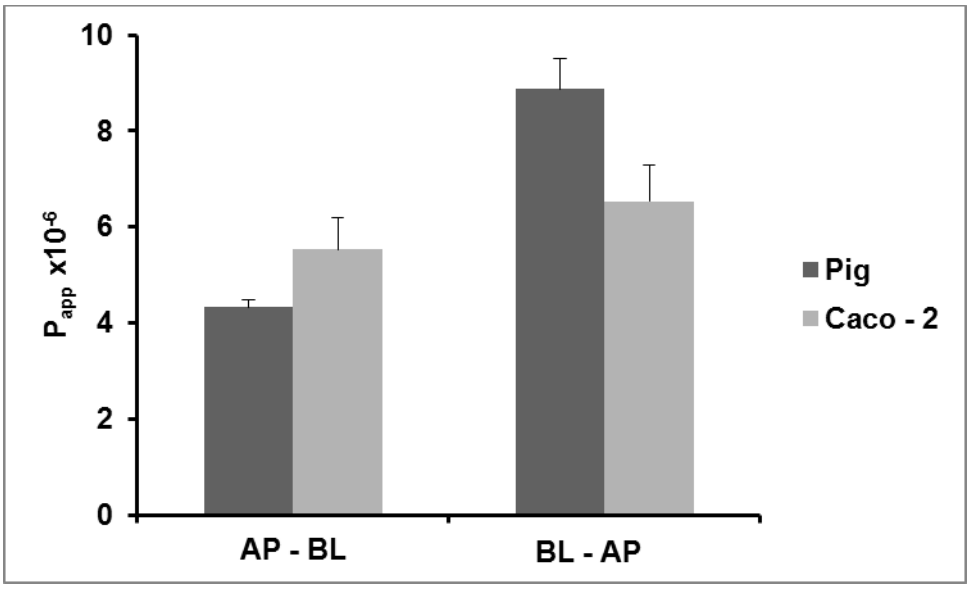

Figure 4.11: Comparison of drug transport between species (i.e. pig and human Caco-2 cells) using Sweetana-Grass diffusion and Transwell® plate techniques, respectively

When comparing drug transport (AP - BL) between pig intestinal tissue (Sweetana-Grass diffusion chambers) and Caco-2 cell monolayers (Transwell® membrane plates) by means of the non-parametric Kruskal-Wallis ANOVA test; it was found that there were no statistically significant difference ($p = 1$) between pig jejunum and Caco-2 cell monolayers. The BL - AP direction also showed no statistically significant difference ($p = 1$) in drug transport.

Although transport was generally higher across the Caco-2 cell monolayers, the comparison between the pig and Caco-2 model rendered similar permeation characteristics (no statistically significantly difference) of drug transport in both directions (AP - BL and BL - AP) and it can be concluded that either model could be used as alternative during drug transport studies.

4.4.2 Comparison of drug transport between species using the everted sac apparatus technique

4.4.2.1 Transport across excised rat tissue compared to transport across excised pig tissue

The P_{app} values based on abacavir transport across excised rat and pig intestinal tissue are shown in Figure 4.12.

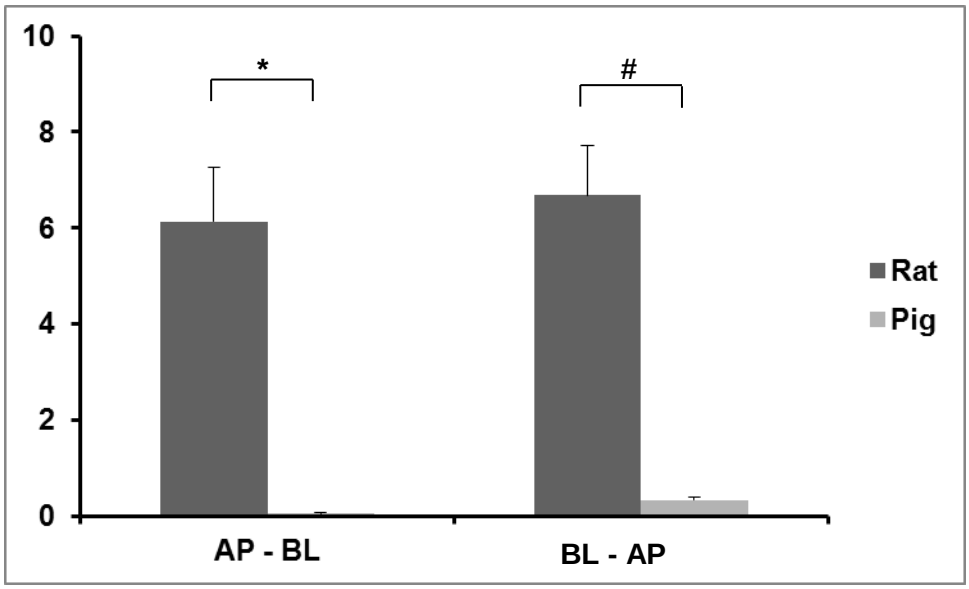

Figure 4.12: Comparison of drug transport between species (i.e. rat and pig) using the Everted sac apparatus technique (the symbols * and # denote a statistically significant difference, $p \leq 0.001$)

Due to non-normal distribution of the transport data with a non-parametrical nature, the Mann-Whitney U test was applied to the data for statistical analysis. In both directions a statistically significant difference ($p \leq 0.001$) was observed between the P_{app} values calculated from the transport data across the excised rat and pig intestinal tissues. This can partially be explained by the fact that the pig intestinal tissue is much thicker than that of the rat (235 µm and 127 µm, respectively). By taking Fick's first law (Equation 4) into

consideration, it is clear that the thickness of the membrane (h) will have a direct influence on the rate of passive diffusion (i.e. the thicker the membrane, the lower the rate of diffusion).

$$\frac{dQ}{dt} = \frac{DAK}{h}(C_{Gi}-C_p) \qquad \text{Eq. 4}$$

Where dQ/dt is the rate of diffusion across a membrane, D the diffusion coefficient, A the surface area of absorption, K the partition coefficient, h the membrane thickness and (C_{GI} - C_P) the drug concentration gradient between the AP and BL chambers.

From this comparison between the rat and pig model using the everted sac technique, it is apparent that they differ in terms of passive diffusion and active efflux characteristics of drugs which can most likely be attributed to differences in membrane thickness. This finding suggests that the excised pig intestinal model may exhibit lower transport of a drug with a consequent underestimation of the bioavailability of the drug.

4.4.2.2 Rat (everted sac apparatus) compared to Caco-2 (Transwell® membrane plates)

The P_{app} values based on abacavir transport across excised rat intestinal tissue and Caco-2 cell monolayers are shown in Figure 4.13.

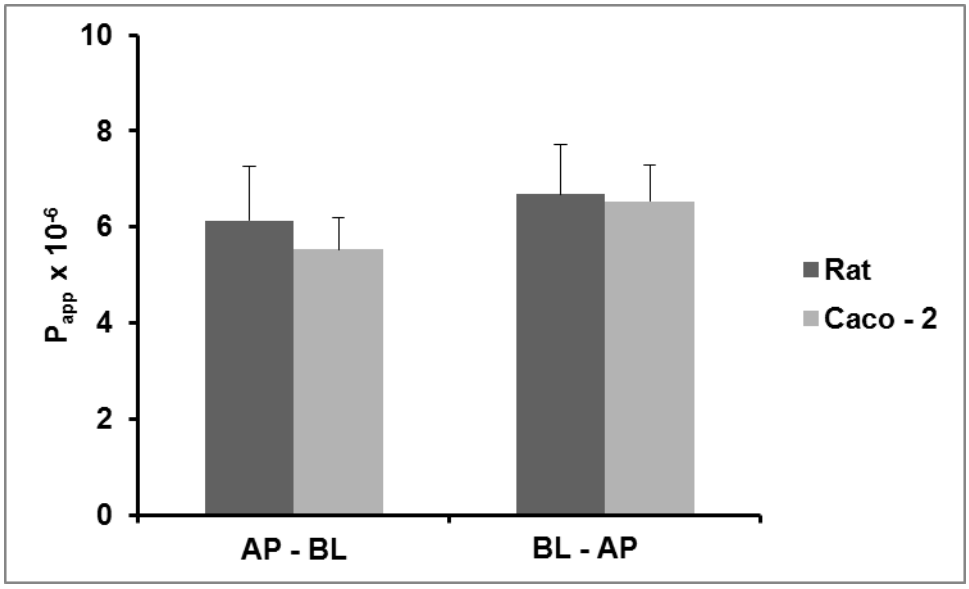

Figure 4.13: Comparison of drug transport between species (i.e. rat and human Caco-2 cells) using the everted sac and Transwell® plate techniques, respectively

Comparing rat intestinal tissue (everted sac apparatus) to Caco-2 cell monolayers (Transwell® membrane plates) by means of a Kruskal-Wallis ANOVA test, showed that there was no statistically significant difference ($p \geq 0.05$) in drug transport between rat jejunum and Caco-2 cell monolayers in the AP - BL direction.

The data of the BL - AP direction of transport also showed no statistically significant difference ($p \geq 0.05$) in drug transport between rat intestinal tissue and Caco-2 cell monolayers.

From this comparison between the rat and Caco-2 model it is clear that they provide similar permeation properties of drug transport in both directions (AP - BL and BL - AP) and that either model can be used as an alternative method for drug transport studies for drugs from the BCS class II, such as abacavir, which are substrates for P-gp.

4.4.2.3 Pig (everted sac apparatus) compared to Caco-2 (Transwell® membrane plates)

The P_{app} values based on abacavir transport across excised pig intestinal tissue and Caco-2 cell monolayers are shown in Figure 4.14.

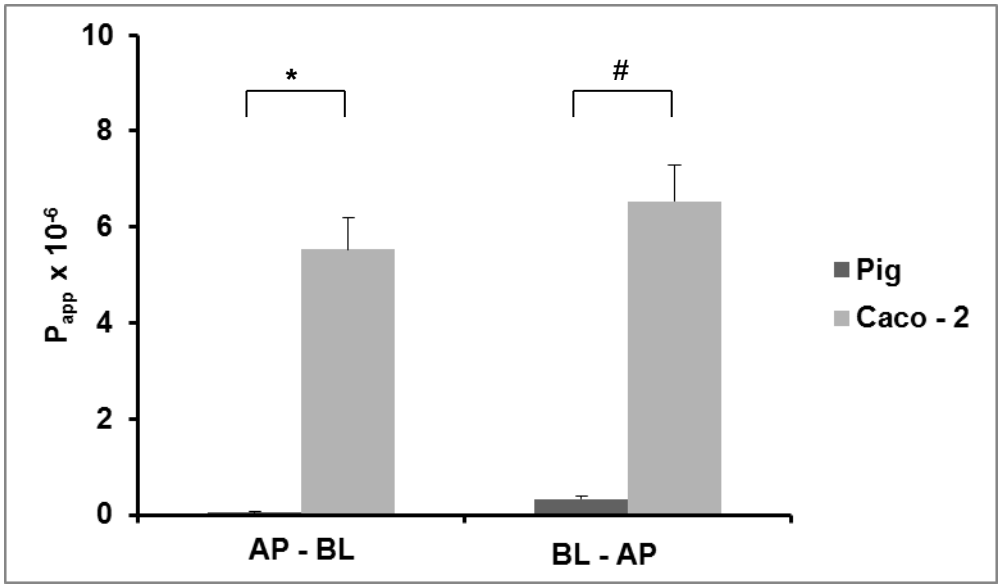

Figure 4.14: Comparison of drug transport between species (i.e. pig and human Caco-2 cells) using the everted sac and Transwell® plate techniques, respectively (the symbols * and # denote a statistically significant difference, $p \leq 0.001$)

The comparison of pig intestinal tissue (everted sac apparatus) and Caco-2 cell monolayers (Transwell® membrane plates) in the AP - BL direction showed that there was a statistically

significant difference ($p \leq 0.001$) in drug transport between pig intestinal tissue and Caco-2 cell monolayers. This could be explained by the fact that the pig intestinal barrier of absorption was a lot thicker than that of the Caco-2 cell monolayers (Fick's law, Eq. 4) with the presence of muscle layers. A second explanation may be the fact that there was an absence of a mucus layer on the AP side of the Caco-2 cell monolayer membrane. Both of these factors may result in a lower rate and extent of drug transport across excised pig intestinal tissue compared to that of the Caco-2 cell monolayer.

When comparing drug transport across pig intestinal tissue and Caco-2 cell monolayers, in the BL - AP direction, a p-value of 0.007 was generated. This can directly be related to the overall difference in transport of the model compound across the excised pig intestinal tissue as compared to the Caco-2 cell monolayers as discussed above.

4.4.3 Comparison between all species, methods and techniques

Due to non-normal distribution of the transport data with a non-parametrical nature, the Mann-Whitney U test was applied in statistical analysis of the two techniques used during the transport studies across rat and pig jejunum.

4.4.3.1 Sweetana-Grass diffusion apparatus compared to the everted sac technique in the rat

Statistical analysis showed that there was a statistically significant difference ($p = 0.002$) between the data generated in the Sweetana-Grass diffusion chambers and the everted sac apparatus when rat intestinal tissue was used to study drug transport in the AP - BL direction. A possible explanation for this occurrence may be attributed to the difference in surface area of intestinal tissue used in each technique. The larger piece of jejunum that was used during the everted sac apparatus technique had a larger surface area available for absorption in comparison to the Sweetana-Grass diffusion chambers. This large surface area will consequently lead to a higher degree of drug transport (Fick's law of diffusion, Eq. 4).

Drug transport in the BL - AP direction showed no statistically significant difference ($p = 0.060$). This indicates that both the everted sac and Sweetana-Grass diffusion technique can be used alternatively when studying the efflux of drugs across excised rat intestinal tissue.

4.4.3.2 Sweetana-Grass diffusion apparatus compared to the everted sac technique in the pig

Drug transport across pig intestinal tissue in the AP - BL as well as the BL - AP direction, between Sweetana-Grass diffusion chambers and the everted sac apparatus, showed a statistically significant difference ($p \leq 0.001$).

This can be explained by the larger surface area (Fick's law of diffusion, Eq. 4), the larger piece of intestinal tissue being used and the higher number of active transporters (P-gp) in the larger pig intestinal tissue in the everted sac techniques as compared to the diffusion chamber technique.

4.5 Statistical comparison of efflux ratios

An efflux ratio takes into consideration the P_{app} values in both AP - BL and BL - AP directions of transport. The ER was calculated using Equation 3 (section 3.6.3).

4.5.1 Comparison between all species, methods and techniques

The ER and SD values of the different species, methods and techniques are given in Table 4.14 and compared in Figure 4.15. These values were used to statistically analyse and compare all the groups.

Table 4.14: ER and SD values of all species, methods and techniques

	Average ER	**SD**
Rat EV	1.31	0.27
Rat SG	1.67	0.34
Pig EV	8.05	2.56
Pig SG	2.68	0.31
Caco-2	1.20	0.22

Where EV denotes Everted sac technique and SG the Sweetana-Grass technique.

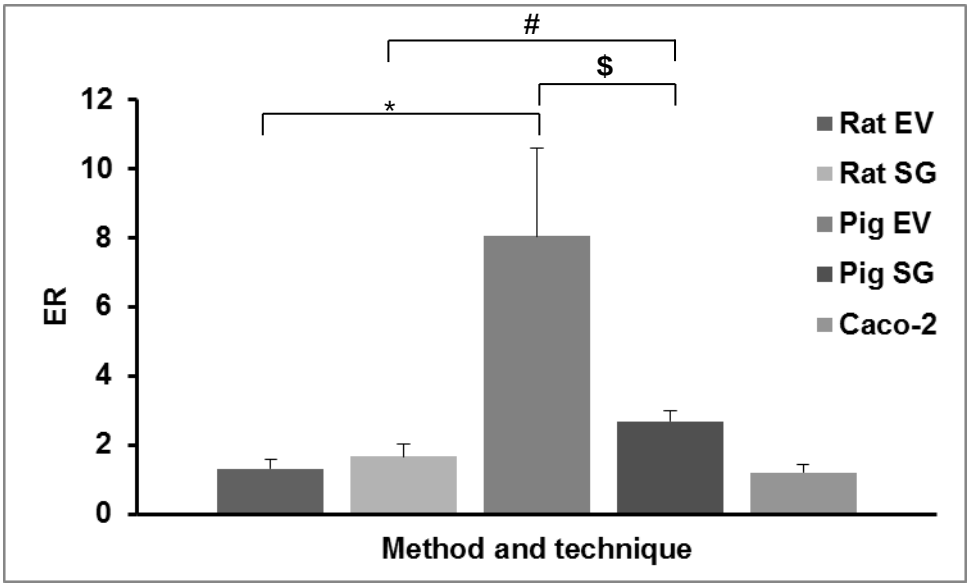

Figure 4.15: Statistical comparison between ER values of all species, methods and techniques. Corresponding symbols indicate groups with a statistically significant difference

Rat intestinal tissue differs statistically significantly from pig intestinal tissue when studies are done using the everted sac technique ($p = 0.025$). A statistically significant difference was also evident when comparing transport in rat and pig intestinal tissue when using Sweetana-Grass diffusion chambers ($p = 0.030$). The difference in morphology (thickness of the intestinal tissue and mucus layer, absorption area and expression of P-gp transporters) may have had a significant influence on the results.

When comparing pig intestinal tissue in Sweetana-Grass diffusion chambers and the everted sac apparatus, a statistically significant difference was evident ($p \leq 0.001$). Again the area available for drug absorption may be the main reason for the observed difference.

There were no statistically significant difference ($p = 0.697$) between the Sweetana-Grass diffusion chambers and the everted sac technique when using rat intestinal tissue.

When rat intestinal tissue was compared to Caco-2 cell monolayers there was no statistically significant difference ($p \geq 0.05$) when either the everted sac apparatus or the Sweetana-Grass diffusion chambers were used. There was also no statistically significant difference between pig intestinal tissue and Caco-2 cell monolayers when either the everted sac apparatus or Sweetana-Grass diffusion chambers ($p = 0.657$ and 0.185 respectively) were used.

4.6 Conclusions

The analytical HPLC method for abacavir proved to be robust and repeatable. The Sweetana-grass diffusion chamber technique rendered drug permeability results comparable to that of the Caco-2 cell line for both the excised rat and pig intestinal tissue methods. The everted sac technique, on the other hand, proved to be relatively predictive with rat intestinal tissue and less predictive with pig intestinal tissue in terms of drug transport across human intestinal epithelial cells. The area of absorption, membrane thickness, presence of a mucus layer and P-gp expression may be some of the major factors that may have caused the differences in drug transport between the different methods and techniques that were used.

CHAPTER 5: FINAL CONCLUSIONS AND FUTURE RECOMMENDATIONS

5.1 Introduction

From the scientific literature it is clear that there is a need for *in vitro* methods to screen the pharmacokinetic properties of NCE's as part of the drug discovery and development process. These methods need to be of a high-throughput, high predictive and cost effective nature. Furthermore, it is important to consider ethical aspects when employing these techniques and the 3R's that was described by Russel and Burch are of high importance (Baumans, 2004:S64 - S66).

This study aimed at comparing the drug permeation properties of three *in vitro* epithelial models including excised intestinal tissues from rats and pigs in two different techniques (i.e. Sweetana Grass diffusion chambers and everted sac glass apparatus) as well as human intestinal epithelial cells (i.e. Caco-2) in Transwell® membrane plates. Although pig intestinal tissue was used as an *in vitro* method in previous studies (Pietzonka *et al.,* 2002:39), relatively little information is available about how it compares with other *in vitro* methods in terms of drug transport. Pig intestinal tissue is an ideal *in vitro* model for screening the pharmacokinetic properties of NCEs since it is freely available as a waste product from abattoirs where pigs are slaughtered on a daily basis for meat production purposes. Although Caco-2 cell monolayers meet the 3R requirements, it is a very expensive and time consuming method. Although the rat is an acceptable model to predict drug pharmacokinetics in humans (Lennernäs, 2014:339), it is associated with ethical issues such as high numbers that need to be specifically bred for experimental purposes.

5.2 Final conclusions

Based on the transport results obtained in this study, it can be concluded that the Sweetana-grass diffusion chamber technique exhibited drug permeability results comparable to that of the Caco-2 cell line for both excised rat and pig intestinal tissue methods. For the everted sac technique, on the other hand, the excised rat intestinal tissue method was more comparable to the Caco-2 cell line method than the excised pig intestinal tissue method. The surface area available for drug absorption, thickness of the membrane, presence of a mucus layer and active efflux transporter expression are some of the factors which may play a role in causing differences in drug transport between the different methods and techniques.

It can therefore be concluded that excised pig intestinal tissue could be used as an *in vitro* method for drug transport studies when mounted in the Sweetana-Grass diffusion chambers. The excised pig intestinal tissue mounted in diffusion chambers provides not only a cost effective approach but also complies with the 3R's concept of ethics and is therefore an acceptable alternative method for screening the pharmacokinetic properties of NCEs.

5.3 Future recommendations

The following studies are recommended for future research as follow-up for this study in order to characterise the excised pig intestinal method even further:

- The transport results obtained from the excised pig intestinal tissue mounted in the diffusion chamber type apparatus should be compared to *in vivo* human pharmacokinetic data to establish the extent of *in vitro-in vivo* correlations.
- Transport studies across excised pig intestinal tissue should be conducted for all four BCS class drugs to prove that the model is applicable to a broader spectrum of drug compounds.
- Region specific transport across the excised pig intestine should be conducted by obtaining tissue from different regions in the gastrointestinal tract (e.g. duodenum, proximal jejunum, mid jejunum, distal jejunum, ileum and colon) and compared to other methods.
- The expression of metabolising enzymes and P-gp transporters in different regions of the pig intestinal tissue should be investigated.
- The design of a better standardised everted sac technique for *in vitro* NCE screening, which correlates well with human cell culture as well as *in vivo* data, is required.

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APPENDIX A

CONGRESS PROCEEDINGS

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

Comparison of drug permeability in rat, pig and human *in vitro* models

Ruan Joubert[1], Dewald Steyn[1], Jan Steenekamp[2], Josias Hamman[2]

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Purpose: High throughput, but less predictive *in vitro* models are preferred for primary screening purposes. The aim of this study is to compare three different *in vitro* models, namely the excised rat intestinal tissue, the excised pig intestinal tissue and the Caco-2 human cell culture in terms of drug permeability. Furthermore, the Sweetana-Grass Ussing type diffusion and everted sac techniques were used for both of the excised animal tissue models.

Methods: The transport of abacavir was determined in two directions (i.e. apical-tobasolateral and basolateral-to-apical directions) in all three *in vitro* models and in both techniques. The test solution was applied to the donor side and samples (200 µl) were drawn from the acceptor side at 20 min intervals for a period of 2 h. The concentration of abacavir in the samples was measured by means of a validated high performance liquid chromatography (HPLC) method. The transepithelial electrical resistance (TEER) was measured before and after the transport experiments in all models to determine if membrane integrity was maintained. The models were compared in terms of apparent permeability coefficient (Papp) values and efflux ratio (ER) values.

Results: All three the *in vitro* models, in both the techniques employed, showed higher transport of abacavir in the BL-AP direction than in the AP-BL direction. This indicates that all three *in vitro* models contained intact active efflux transporters over the entire study period. The excised rat intestinal model showed similar drug permeability characteristics in both techniques compared to that of Caco-2 cell monolayers. On the other hand, the excised pig intestinal model only showed similar drug permeability characteristics in the Sweetana-Grass diffusion apparatus compared to that of the Caco-2 cell monolayers, but not in the

everted sac technique. This phenomenon can possibly be explained by the relatively large size of the pig tissue used in the everted sac technique where the role of physiological and other factors take effect. These factors may include the thickness of the membrane and mucus layer as well as variables such as diet, age, gender and size of the pigs obtained from the abattoir that cannot be controlled.

Conclusions: The Sweetana-grass Ussing type diffusion chamber technique rendered drug permeability results comparable to that of the Caco-2 cell line for both the excised rat and pig intestinal tissue models. The everted sac technique, on the other hand, proved to be relatively predictive with rat intestinal tissue and less predictive with pig intestinal tissue in terms of drug transport across human intestinal epithelial cells.

35TH CONFERENCE OF THE ACADEMY OF PHARMACEUTICAL SCIENCES

INTEGRATING EDUCATION, RESEARCH & PRACTICE

CERTIFICATE OF ATTENDANCE

This is to certify that

Mr Ruan Joubert

has attended the 35th Conference of the Academy of Pharmaceutical Sciences held from 12 – 14 September at the Summerstrand Hotel in Port Elizabeth

Proudly hosted by the Department of Pharmacy at the Nelson Mandela Metropolitan University

Prof Gareth Kilian
Conference Convenor

Nelson Mandela Metropolitan University
for tomorrow

APPENDIX B

ETHICAL APPROVAL

NORTH-WEST UNIVERSITY
YUNIBESITI YA BOKONE-BOPHIRIMA
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Ethics Committee
Tel +27 18 299 4852
Email Ethics@nwu.ac.za

20 February 2013

ETHICS APPROVAL OF PROJECT

The North-West University Ethics Committee (NWU-EC) hereby approves your project as indicated below. This implies that the NWU-EC grants its permission that, provided the special conditions specified below are met and pending any other authorisation that may be necessary, the project may be initiated, using the ethics number below.

Project title: Correlation of drug permeability and metabolism between rat intestinal tissue, pig (porcine intestinal tissue) and human in vitro intestinal epithelial models.

Project Leader: Dr D Steyn

Ethics number: N W U - 0 0 0 3 0 - 1 3 - A 5

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Approval date: 2013/05/03 **Expiry date**: 2018/05/29

Special conditions of the approval (if any): None

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- *The project leader (principle investigator) must report in the prescribed format to the NWU-EC:*
  - *annually (or as otherwise requested) on the progress of the project,*
  - *without any delay in case of any adverse event (or any matter that interrupts sound ethical principles) during the course of the project.*
- *The approval applies strictly to the protocol as stipulated in the application form. Would any changes to the protocol be deemed necessary during the course of the project, the project leader must apply for approval of these changes at the NWU-EC. Would there be deviated from the project protocol without the necessary approval of such changes, the ethics approval is immediately and automatically forfeited.*
- *The date of approval indicates the first date that the project may be started. Would the project have to continue after the expiry date, a new application must be made to the NWU-EC and new approval received before or on the expiry date.*
- *In the interest of ethical responsibility the NWU-EC retains the right to:*
  - *request access to any information or data at any time during the course or after completion of the project;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the project are revealed or suspected,*
    - *it becomes apparent that any relevant information was withheld from the NWU-EC or that information has been false or misrepresented,*
    - *the required annual report and reporting of adverse events was not done timely and accurately,*
    - *new institutional rules, national legislation or international conventions deem it necessary.*

The Ethics Committee would like to remain at your service as scientist and researcher, and wishes you well with your project. Please do not hesitate to contact the Ethics Committee for any further enquiries or requests for assistance.

Yours sincerely

Prof Amanda Lourens
(chair NWU Ethics Committee)

APPENDIX C

CERTIFICATE OF ANALYSIS (ABACAVIR) AND CERTIFICATE OF LABORATORY ANIMAL TRAINING COURSE

SRI SAI NIKITHA PHARMA PVT. LTD.

Office : 425/3RT, S.R.Nagar, Hydgrabad - 500 038. A.P. India.
Tel : 0091 40 23801672 / 73, Fax : 0091 40 23707143
E-mail : brcreddy_08@yahoo.co.in

CERTIFICATE OF ANALYSIS

Product Name	ABACAVIR SULPHATE	Page No	1 of 2
Batch Number	CAB0770039	A.R.NO	07CB00436
Mfg.Date	July 2007	Date of Release	20 August 07
Quantity	1.00KG	Retest Date	June 2009

S.NO	TEST	SPECIFICATIONS	RESULT
1.	Description	A white to off - White crystalline powder	An off -White crystalline powder
2	Solubility	Soluble in water, slightly soluble in Methanol	Soluble in water, slightly soluble in Methanol
3.	Identification: A) By IR	The IR absorption spectrum of sample recorded as KBr pellet should exhibit maxima only at the same wave numbers as that of similarly recorded spectrum of the Abacavir sulfate working standard.	The IR absorption spectrum of sample recorded as KBr pellet exhibits maxima only at the same wave numbers as that of similarly recorde spectrum of the Abacavir sulfate working standard.
	B)By HPLC	The retention time of the Abacavir peak in the chromatogram of the sample solution should correspond to that of the standard solution, as obtained in the Assey.	The retention time of the Abacavir peak in the chromatogram of the sample solution corresponds to that of the standard solution as obtained in the assay.
	C) Test for Sulfate	A copious dense white precipitate should form; indicating the presence of sulfate ions.	A copious dense white precipitate formed; indicating the presence of Sulfate ions.
4.	Water (% w/w, by KF)	Not more than 1.5	0.38
5.	Specific Optical Rotation: $[\alpha]^{25}_{D}$: (c=0.5 in Methanol, on anhydrous basis)	Between -32.0° and -38.0°	-36.96°
6.	Residue on ignition (determined on 1.0 g, % w/w)	Not more than 0.5	0.08
7.	Heavy Metals (ppm)	Not more than 20	Less than 20
	PREPARED BY	CHECKED BY	APPROVED BY
Signature			
Date	20/08/07	20/08/2007	20.08.07.

REPRESENTED BY:
D B Fine Chemicals (Pty) Limited
PO Box 786
RIVONIA 2128
Johannesburg
South Africa

10 YEARS
1995 2005

SRI SAI NIKITHA PHARMA PVT. LTD.

Office : 425/3RT, S.R.Nagar, Hyderabad - 500 038. A.P. India.
Tel : 0091 40 23801672 / 73; Fax : 0091 40 23707143
E-mail : brreddy_08@yahoo.co.in

CERTIFICATE OF ANALYSIS

Product Name	: ABACAVIR SULPHATE	Page No.	: 2 of 2
Batch Number	: SAB/07/0083	A.R.NO.	: 07CB00436
M/fg Date	: July 2007	Date of Release	: 30 August '07
Quantity	: 1.00KG	Retest Date	: June 2009

S.NO	TEST	SPECIFICATIONS	RESULT
8.	Sulfate Content (By Titrimetry, % w/w)	Between 13.6 and 15.0	14.55
9.	Abacavir Enantiomer Content (By HPLC, % w/w)	Not more than 0.2	Below limit of Quantification
10.	Related compounds (By HPLC, % w/w):		
	Deoxycloprogyl Abacavir	Not more than 0.1	0.04
	Dihydro Abacavir	Not more than 0.1	Not Detected
	Abacavir Pentafimate	Not more than 0.1	Not Detected
	O-Pyrimidinyl Abacavir	Not More than 0.15	0.057
	Individual unknown related compound	Not more than 0.10	Not Detected
	Total Related compounds	Not more than 0.5	0.10
11.	Assay (By HPLC, %w/w, as $(C_{14}H_{18}N_6O)_2.H_2SO_4$, on anhydrous basis)	Not Less than 98.0 and not more than 102.0	100.56
12.	Residual solvents (By GC, µg/g)		
	Ethanol	Not more than 500	Not Detected
	Isopropyl Alcohol	Not more than 5000	10.98
	Ethyl acetate	Not more than 500	Not detected
	tert-Butylmethylether	Not more than 200	Not Detected
13.	Organic Volatile impurities (GC, µg/g)	Should meet the requirements of USP/ ICH	Meets the requirements of USP/ ICH
14.	Particle size (By Malvern Particle size Analyzer, µm):		
	D_v (v, 0.1)	Between 10 and 20	15.521
	D_v (v, 0.5)	Between 60 and 90	77.998
	D_v (v, 0.9)	Between 150 and 200	212.335
15.	Bulk density (g/ml):		
	Unsifted	Between 0.30 and 0.50	0.477
	Sifted	Between 0.50 and 0.65	0.626
	PREPARED BY	CHECKED BY	APPROVED BY
	Signature		
	Date	20/08/2007	20/08/2007

REPRESENTED BY:
D B Fine Chemicals (Pty) Ltd
P.O. Box 786
RIVONIA 2128
Johannesburg
South Africa

10 YEARS 1995 2005

07/09 2007 10:16 FAX 0118070953 DB FINE CHEMICALS 004

WHO COLLABORATING CENTRE FOR CHEMICAL REFERENCE SUBSTANCES

CENTRE COLLABORATEUR OMS DES SUBSTANCES CHIMIQUES DE RÉFÉRENCE

WHO Collaborating Centre for Chemical Reference Substances
Apoteket AB
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SE-14175 KUNGENS KURVA
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http://www.apl.apoteket.se/who

CERTIFICATE

Proposed International Chemical Reference Substance

ABACAVIR SULFATE

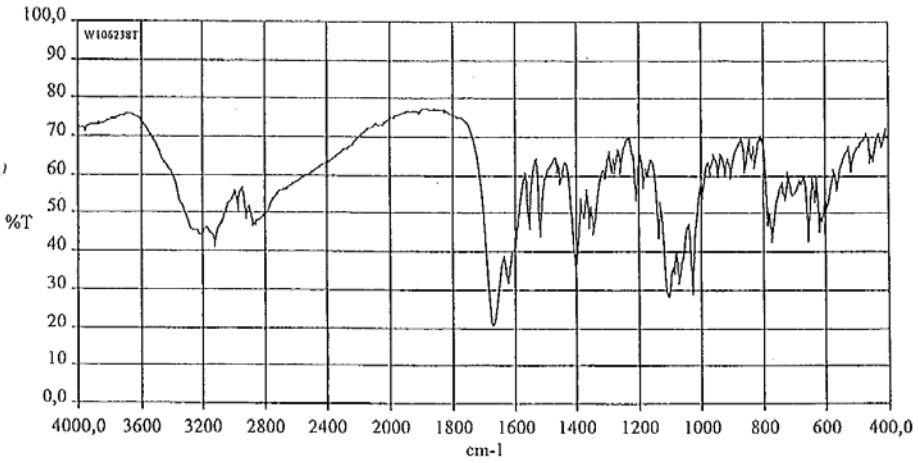
Control No 106238

Intended use

The International Chemical Reference Substance for abacavir sulfate is intended to be used in the infrared absorption spectrophotometric and thin-layer chromatographic tests for identity according to the monograph for Abacavir sulfate in *The International Pharmacopoeia*.

Analytical data

Infrared absorption spectrophotometry (IR): A spectrum, 1.3 mg of abacavir sulfate in 300 mg of potassium bromide, is given in figure W106238T.



30/10/07
RECEIVED

Ultraviolet spectrophotometric absorption data: $A_{1cm}^{1\%} = 416$ at 297 nm, calculated with reference to the dried substance. Abacavir sulfate was dissolved in 0.1 M hydrochloric acid.

High performance liquid chromatography (HPLC): The purity was estimated by peak area normalization to 99.8% at 254 nm.

Thin-layer chromatography (TLC): The purity was estimated to 100.0% at 254 nm, when 100 µg were applied.

Thermogravimetric analysis (TG): When heated to 105 °C a loss of < 0.1% (w/w) was observed.

Storage

Abacavir sulfate should be kept in a tightly closed container, protected from light.

Reference: This certificate is extracted from the report, which is the basis for the adoption of this International Chemical Reference Substance by the WHO Expert Committee on Specifications for Pharmaceutical Preparations.

May 12, 2006
Kungens Kurva

Victoria Hjortsberg, Director

I YUNIBESITI YA BOKONE-BOPHIRIMA
NORTH WEST UNIVERSITY
NOORDWES UNIVERSITEIT

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Tel +27 18 299 2268
Fax +27 18 299 2291
E-mail Erna.Swanepoel@nwu.ac.za

POTCHEFSTROOM CAMPUS

ABBREVIATED CERTIFICATE OF ANALYSIS

PRODUCT: Abacavir sulphate
BATCH NO.: CAB0770029
CONTAINER: Clear glass polytop

COMPANY: Innovasionfund
FORM: Raw Material
STRENGTH: Abacavir sulphate

TEST	SPECIFICATION	RESULTS
IDENTIFICATION (IR):	The infrared absorption spectrum is concordant with the spectrum obtained from abacavir sulfate RS or with the reference spectrum of abacavir sulfate.	Complies
IDENTIFICATION (Optical Rotation):	- 53° to - 57°	- 54°
IDENTIFICATION (Sulphate):		Complies
ASSAY: (Titr)		
ACTIVE INGREDIENT		
Abacavir sulphate	99.0% - 101.0%	99.9% "as is"

QC Release: [signature]

Date: 06 November 2007

ORIGINAL

NORTH-WEST UNIVERSITY ®
YUNIBESITI YA BOKONE-BOPHIRIMA
NOORDWES-UNIVERSITEIT

Vivarium

Hiemee word verklaar dat

RUAN JOUBERT

'n kursus in

PROEFDIERNAVORSING

bygewoon het

16 Februarie 2013

Hoof: Vivarium
en Aanbieder

Aanbieder

Aanbieder

APPENDIX D

TEER MEASUREMENTS AND TRANSPORT DATA

Table D 1: TEER results for pig intestinal tissue

	TEER (kΩ)											
	1		2		3		4		5		6	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
	27	23	26.5	25	33.5	33	24	23	24.4	17.6	32.3	32
	26.8	23	28.4	28	32.4	31	16	15	27	20.6	31	30
	25.4	21.9	26.9	25.3	34.5	32.4	24.7	24.4	28.6	21.2	33.3	33
	25.7	23.2	27	25.5	31.5	27.5	25.4	20	24	17.5	31	30
	25	22.3	26	26	31.6	31	24.5	23	27	20.5	33.5	32.4
	24.9	23.8	26.5	25.4	33.6	28	26	25.4	26	17	31.7	30.3
Ave.	25.8	22.87	26.88	25.87	32.85	30.48	23.43	21.8	26.17	19.07	32.13	31.28
Ave. loss	2.93		1.02		2.37		1.63		7.1		0.85	
% loss	11.37		3.78		7.20		6.97		27.13		2.65	

Table D 2: TEER results for rat intestinal tissue

	TEER (kΩ)											
	1		2		3		4		5		6	
	Before	After	Before	After	Before	After	Before	After	Before	After	Before	After
	30.7	26.3	33.2	28.4	30.8	23.6	11.1	13.5	15.3	10.3	13	14.9
	31.1	27	30	27	31.7	25.5	11.5	13.1	15.8	11.1	15.8	11.4
	31.9	27.2	31.6	27.3	29.9	27.8	11.9	10.4	12.3	14.1	15.6	14.4
	29	23.4	31.5	27.2	29.3	26.6	12.1	13.7	14.8	10.4	15.5	14.7
	31.3	26.4	30.6	26.7	30.7	29.2	14.5	13.3	11.1	11	12.5	14.2
	31.5	23.4	30.2	27.2	30.6	28.2	11.7	13.4	11.9	10.5	15	10.8
Ave.	30.92	25.62	31.18	27.3	30.5	26.82	12.13	12.9	13.53	11.23	14.57	13.4
Ave. loss	5.3		3.88		3.68		-0.77		2.3		1.17	
% loss	17.14		12.45		12.08		-6.32		16.99		8.01	

Table D 3: TEER results for Caco-2 cell monolayers

TEER (Ω)				
	1		2	
	Before	**After**	**Before**	**After**
	486	508	355	290
	501	503	401	180
	431	272	432	474
	519	473	362	392
	411	421	380	474
	458	161	320	455
Ave.	467.67	389.67	375	377.5
Ave. loss	78		-2.5	
% loss	16.68		-0.67	

Table D 4: Mean % transport of abacavir across rat jejunum (AP – BL) by means of the everted sac apparatus

Mean % Transport AP - BL (Rat EV)						
Time	**Rat 1**	**Rat 2**	**Rat 3**	**Mean %**	**SD**	**Ave $P_{app}(10^{-6})$**
0	0	0	0	0	0	6.13 ± 1.13
20	1.563	1.108	2.019	1.563	0.322	
40	3.200	1.976	3.051	2.742	0.472	
60	4.299	2.702	4.623	3.874	0.727	
80	5.716	3.234	5.558	4.836	0.983	
100	6.738	4.155	6.495	5.796	1.008	
120	7.664	5.182	7.475	6.774	0.977	

Table D 5: Mean % transport of abacavir across rat jejunum (BL - AP) by means of the everted sac apparatus

Mean % Transport BL - AP (Rat EV)						
Time	**Rat 4**	**Rat 5**	**Rat 6**	**Mean %**	**SD**	**Ave $P_{app}(10^{-6})$**
0	0	0	0	0	0	6.68 ± 1.05
20	3.6333	1.6159	2.3491	2.4803	0.296	
40	4.5449	2.3707	3.2632	3.397	0.4103	
60	5.5735	2.8407	4.2695	4.301	0.4676	
80	6.6688	4.1337	5.2805	5.4707	0.4245	
100	8.2649	5.2518	6.4711	6.8182	0.5867	
120	9.7979	6.3513	7.3175	8.023	0.3682	

Table D 6: Mean % transport of abacavir across rat jejunum (AP – BL) by means of the Sweetana-Grass diffusion chambers

Mean % Transport AP - BL (Rat SG)						
Time	**Rat 1**	**Rat 2**	**Rat 3**	**Mean**	**SD**	**Ave P_{app} ($x10^{-6}$)**
0	0	0	0	0	0	3.39 ± 0.52
20	1.193	0.661	0.653	0.836	0.219	
40	1.789	1.243	1.523	1.518	0.193	
60	2.412	1.867	2.364	2.214	0.213	
80	2.584	2.519	3.335	2.813	0.321	
100	3.647	3.123	4.254	3.675	0.400	
120	4.361	3.750	5.198	4.436	0.514	

Table D 7: Mean % transport of abacavir across rat jejunum (BL - AP) by means of the Sweetana-Grass diffusion chambers

Mean % Transport BL - AP (Rat SG)						
Time	**Rat 4**	**Rat 5**	**Rat 6**	**Mean**	**SD**	**Ave P_{app} ($x10^{-6}$)**
0	0	0	0	0	0	5.19 ± 1.60
20	0.409	0.371	1.114	0.632	0.296	
40	0.978	1.008	2.388	1.458	0.570	
60	1.938	1.768	3.885	2.530	0.832	
80	3.345	2.601	5.845	3.930	1.202	
100	4.657	3.498	7.380	5.178	1.409	
120	5.967	4.248	9.229	6.481	1.789	

Table D 8: Mean % transport of abacavir across pig jejunum (AP – BL) by means of everted sac apparatus

Mean % Transport AP - BL (Pig - EV)						
Time	**Pig 1**	**Pig 2**	**Pig 3**	**Mean**	**SD**	**Ave P_{app} (x 10^{-6})**
0	0	0	0	0	0	0.05 ± 0.01
20	0.262	0.004	0.078	0.115	0.094	
40	0.327	0.127	0.348	0.268	0.087	
60	0.576	0.417	0.663	0.552	0.088	
80	1.231	0.810	1.142	1.061	0.157	
100	1.685	1.231	1.692	1.536	0.187	
120	2.290	1.690	2.311	2.097	0.249	

Table D 9: Mean % transport of abacavir across pig jejunum (BL - AP) by means of everted sac apparatus

Mean % Transport BL - AP (Pig EV)						
Time	**Pig 4**	**Pig 5**	**Pig 6**	**Mean**	**SD**	**Ave P_{app} (x 10^{-6})**
0	0	0	0	0	0	0.33 ± 0.07
20	5.392	0	0	1.797	2.201	
40	13.704	2.236	0	5.314	5.199	
60	16.580	5.151	1.446	7.725	5.578	
80	17.061	8.399	4.244	9.901	4.624	
100	17.679	12.437	6.981	12.366	3.783	
120	18.422	16.402	10.66	15.162	2.847	

Table D 10: Mean % transport of abacavir across pig jejunum (AP – BL) by means of the Sweetana-Grass diffusion chambers

Mean % Transport AP - BL (Pig SG)						
Time	**Pig 1**	**Pig 2**	**Pig 3**	**Mean**	**SD**	**Ave P_{app} (x 10^{-6})**
0	0	0	0	0	0	4.33 ± 0.16
20	0.620	2.333	1.607	1.520	0.608	
40	1.497	3.048	2.658	2.401	0.571	
60	2.335	3.236	2.996	2.856	0.330	
80	3.303	4.104	3.719	3.709	0.283	
100	4.222	5.160	4.820	4.734	0.336	
120	5.167	6.657	6.308	6.044	0.551	

Table D 11: Mean % transport of abacavir across pig jejunum (BL - AP) by means of the Sweetana-Grass diffusion apparatus

Mean % Transport BL - AP (Pig SG)						
Time	**Pig 4**	**Pig 5**	**Pig 6**	**Mean**	**SD**	**Ave P_{app} (x 10^{-6})**
0	0	0	0	0	0	8.87 ± 0.65
20	4.032	1.020	3.716	2.923	1.171	
40	4.594	2.519	4.311	3.808	0.796	
60	5.790	3.490	5.264	4.848	0.852	
80	8.083	5.737	7.014	6.945	0.831	
100	10.019	8.550	9.566	9.378	0.532	
120	14.171	11.447	11.352	12.323	1.132	

Table D 12: Mean % transport of abacavir across Caco-2 cell monolayers (AP - BL) by means of Transwell membrane plates

Caco-2 (AP - BL)	% Transport						Average	SD	Slope	P_{app} ($x 10^{-6}$)
Well	**1**	**2**	**3**	**4**	**5**	**6**	**%**	**%**	**0.1549**	**5.53 ± 0.67**
Time										
0	0	0	0	0	0	0	0	0		
20	6.7369	5.4017	3.9228	6.6415	4.3754	9.3884	6.0778	1.8125		
40	12.6610	13.6150	11.7880	8.6238	10.1530	10.9415	11.2970	1.6357		
60	17.3450	14.6280	13.6610	12.9660	10.3530	14.3794	13.8890	2.0872		
80	18.3670	15.5740	17.0910	14.0340	14.9340	15.9258	15.9880	1.4131		
100	20.4970	15.9600	17.7640	15.1330	17.7300	16.5199	17.2670	1.7183		
120	24.1660	18.3360	20.7270	19.1080	17.9220	19.0807	19.8900	2.1031		

Table D 13: Mean % transport of abacavir across Caco-2 cell monolayers (BL – AP) by means of Transwell membrane plates

Caco-2 (BL - AP)	% Transport						Average	SD	Slope	P_{app} ($x 10^{-6}$)
Well	**1**	**2**	**3**	**4**	**5**	**6**	**%**	**%**	**0.1829**	**6.53 ± 0.76**
Time										
0	0	0	0	0	0	0	0	0		
20	6.5250	4.9025	8.2423	5.1963	7.3383	7.00173	6.5343	1.1716		
40	13.4840	14.2450	10.0920	11.3160	10.4190	12.3649	11.9870	1.5267		
60	15.1470	17.8340	15.7790	13.3880	14.0100	14.7869	15.1570	1.4220		
80	16.5840	20.3550	16.8110	16.7640	17.2200	16.0661	17.3000	1.4083		
100	20.3940	25.4290	20.5530	19.2940	21.5670	18.7712	21.0010	2.1750		
120	22.3950	26.9150	22.4170	20.1100	22.7150	21.8400	22.7320	2.0567		

Table D 14: Mean P_{app}'s and ER's of all the methods and techniques investigated

Repeat (inter)	Repeat (intra)	Dif/Eve/Caco2	AP-BL P_{app} x10^{-6}	BL-AP P_{app} x10^{-6}	ER (BL-AP/AP-BL)
R 1	1	D	0.883	3.770	4.27
	2	D	2.912	9.194	3.16
	3	D	5.290	3.212	0.61
	4	D	4.052	3.662	0.90
	5	D	2.340	3.241	1.39
	6	D	3.371	5.777	1.71
R 2	1	D	3.062	3.372	1.10
	2	D	2.617	3.678	1.41
	3	D	2.472	2.805	1.13
	4	D	3.972	3.969	1.00
	5	D	2.243	3.375	1.50
	6	D	3.139	3.457	1.10
R 3	1	D	3.819	6.735	1.76
	2	D	4.335	9.436	2.18
	3	D	3.898	4.773	1.22
	4	D	4.920	5.563	1.13
	5	D	4.316	9.201	2.13
	6	D	3.400	8.106	2.38
R 1	1	E	4.71	10.32	2.19
	2	E	4.81	4.62	0.96
	3	E	6.65	7.40	1.11
	4	E	9.13	6.83	0.75
	5	E	5.36	10.81	2.01
	6	E	12.03	8.57	0.71

R 2	1	E	5.53	7.88	1.42
	2	E	7.75	5.65	0.73
	3	E	3.23	2.08	0.65
	4	E	1.78	6.14	3.46
	5	E	6.73	5.35	0.80
	6	E	2.24	6.34	2.83
R 3	1	E	9.42	6.84	0.73
	2	E	4.83	5.83	1.21
	3	E	6.22	5.90	0.95
	4	E	7.42	6.61	0.89
	5	E	7.178	6.40	0.89
	6	E	5.28	6.78	1.28
P1	1	D	3.80	8.82	2.32
	2	D	4.32	6.60	1.53
	3	D	3.88	8.20	2.11
	4	D	4.90	12.96	2.64
	5	D	4.30	4.43	1.03
	6	D	3.38	17.16	5.07
P2	1	D	7.38	11.97	1.62
	2	D	1.28	6.80	5.30
	3	D	2.84	7.90	2.78
	4	D	1.09	6.83	6.27
	5	D	7.30	13.25	1.81
	6	D	6.87	6.05	0.88
P3	1	D	3.10	6.51	2.10
	2	D	7.71	6.80	0.88
	3	D	4.25	8.69	2.05

	4	D	1.30	7.40	5.70
	5	D	7.07	11.71	1.66
	6	D	3.07	7.51	2.45
P1	1	E	0.042	0.299	7.12
	2	E	0.043	0.462	10.74
	3	E	0.071	0.423	5.96
	4	E	0.041	0.481	11.73
	5	E	0.041	0.267	6.51
	6	E	0.048	0.390	8.13
P2	1	E	0.029	0.885	30.52
	2	E	0.061	0.506	8.30
	3	E	0.044	0.124	2.89
	4	E	0.038	0.109	2.87
	5	E	0.028	0.287	10.25
	6	E	0.029	0.328	11.31
P3	1	E	0.066	0.228	3.45
	2	E	0.035	0.241	6.89
	3	E	0.046	0.217	4.72
	4	E	0.052	0.365	7.02
	5	E	0.052	0.151	2.90
	6	E	0.055	0.199	3.62
H1	1	C	6.74	6.25	0.93
	2	C	4.98	8.15	1.64
	3	C	6.06	6.28	1.04
	4	C	5.08	5.99	1.18
	5	C	5.43	6.59	1.21
	6	C	4.87	5.91	1.21

APPENDIX E

STATISTICAL ANALYSIS

Table E 1: Univariate test of significance for P_{app} comparing human and pig species (AP - BL)

Effect	SS	Degr. of Freedom	MS	F	p
Direction=AP-BL Univariate Tests of Significance for Papp (Statistiek_finale_data) Sigma-restricted parameterization Effective hypothesis decomposition Include condition: Species='Human' or species='Pig'					
Intercept	332.0664	1	332.0664	172.0907	0.000000
Method	204.0831	2	102.0416	52.8822	0.000000
Error	75.2544	39	1.9296		

Table E 2: Univariate test of significance for P_{app} comparing human and pig species (BL - AP)

Effect	SS	Degr. of Freedom	MS	F	p
Direction=BL-AP Univariate Tests of Significance for Papp (Statistiek_finale_data) Sigma-restricted parameterization Effective hypothesis decomposition Include condition: Species='Human' or species='Pig'					
Intercept	811.7087	1	811.7087	164.7369	0.000000
Method	664.6364	2	332.3182	67.4442	0.000000
Error	192.1649	39	4.9273		

Table E 3: Tukey HSD test comparing human and pig species using P_{app} values (AP - BL)

Cell No.	Method	{1} 4.0295	{2} .05722	{3} 5.5174
Direction=AP-BL Tukey HSD test; variable Papp (Statistiek_finale_data) Approximate Probabilities for Post Hoc Tests Error: Between MS = 1.9296, df = 39.000 Include condition: Species='Human' or species='Pig'				
1	D		0.000123	0.071919
2	E	0.000123		0.000123
3	C	0.071919	0.000123	

Table E 4: Tukey HSD test comparing human and pig species using P_{app} values (BL - AP)

Cell No.	Method	{1} 8.4584	{2} .03814	{3} 6.5192
	Direction=BL-AP Tukey HSD test; variable Papp (Statistiek_finale_data) Approximate Probabilities for Post Hoc Tests Error: Between MS = 4.9273, df = 39.000 Include condition: Species='Human' or species='Pig'			
1	D		0.000123	0.166116
2	E	0.000123		0.000123
3	C	0.166116	0.000123	

Table E 5: Univariate test of significance for ER comparing human and pig species and techniques

Effect	SS	Degr. of Freedom	MS	F	p
	Univariate Tests of Significance for ER (Statistiek_finale_data) Sigma-restricted parameterization Effective hypothesis decomposition Include condition: Species='Human' or species='Pig'				
Intercept	82.75211	1	82.75211	58.76712	0.000000
Method	33.64740	2	16.82370	11.94750	0.000090
Error	54.91731	39	1.40814		

Table E 6: Tukey HSD test for ER comparing human and pig species and techniques

Cell No.	Method	{1} 2.7367	{2} .85611	{3} 1.2017
	Tukey HSD test; variable ER (Statistiek_finale_data) Approximate Probabilities for Post Hoc Tests Error: Between MS = 1.4081, df = 39.000 Include condition: Species='Human' or species='Pig'			
1	D		0.000192	0.024341
2	E	0.000192		0.811411
3	C	0.024341	0.811411	

Table E 7: Kruskal-Wallis ANOVA for P_{app} comparing human and pig species (AP - BL)

Depend.: Papp	Code	Valid N	Sum of Ranks	Mean Rank
	Direction=AP-BL Kruskal-Wallis ANOVA by Ranks; Papp (Statistiek_finale_data) Independent (grouping) variable: Method Kruskal-Wallis test: H (2, N= 42) =31.06238 p =.0000 Include condition: Species='Human' or species='Pig'			
D	1	18	524.0000	29.11111
E	2	18	171.0000	9.50000
C	3	6	208.0000	34.66667

Table E 8: Kruskal-Wallis ANOVA for P_{app} comparing human and pig species and techniques (BL - AP)

	Direction=BL-AP Kruskal-Wallis ANOVA by Ranks; Papp (Statistiek_finale_data) Independent (grouping) variable: Method Kruskal-Wallis test: H (2, N= 42) =30.79070 p =.0000 Include condition: Species='Human' or species='Pig'			
Depend.: Papp	Code	Valid N	Sum of Ranks	Mean Rank
D	1	18	570.0000	31.66667
E	2	18	171.0000	9.50000
C	3	6	162.0000	27.00000

Table E 9: Kruskal-Wallis ANOVA for P_{app} comparing human and pig species and techniques (AP - BL)

	Direction=AP-BL Multiple Comparisons p values (2-tailed); Papp (Statistiek_finale_data) Independent (grouping) variable: Method Kruskal-Wallis test: H (2, N= 42) =31.06238 p =.0000 Include condition: Species='Human' or species='Pig'		
Depend.: Papp	D R:29.111	E R:9.5000	C R:34.667
D		0.000005	1.000000
E	0.000005		0.000041
C	1.000000	0.000041	

Table E 10: Kruskal-Wallis ANOVA for P_{app} comparing human and pig species and techniques (BL - AP)

	Direction=BL-AP Multiple Comparisons p values (2-tailed); Papp (Statistiek_finale_data) Independent (grouping) variable: Method Kruskal-Wallis test: H (2, N= 42) =30.79070 p =.0000 Include condition: Species='Human' or species='Pig'		
Depend.: Papp	D R:31.667	E R:9.5000	C R:27.000
D		0.000000	1.000000
E	0.000000		0.007433
C	1.000000	0.007433	

Table E 11: Kruskal-Wallis ANOVA for ER comparing human and pig species and techniques

	Kruskal-Wallis ANOVA by Ranks; ER (Statistiek_finale_data) Independent (grouping) variable: Method Kruskal-Wallis test: H (2, N= 42) =19.31929 p =.0001 Include condition: Species='Human' or species='Pig'			
Depend.: ER	Code	Valid N	Sum of Ranks	Mean Rank
D	1	18	553.0000	30.72222
E	2	18	230.5000	12.80556
C	3	6	119.5000	19.91667

Table E 12: Multiple comparisons (Kruskal-Wallis ANOVA) using P_{app} values to compare human and pig species and techniques

	Multiple Comparisons p values (2-tailed); ER (Statistiek_finale_data) Independent (grouping) variable: Method Kruskal-Wallis test: H (2, N= 42) =19.31929 p =.0001 Include condition: Species='Human' or species='Pig'		
Depend.: ER	D R:30.722	E R:12.806	C R:19.917
D		0.000035	0.185092
E	0.000035		0.656504
C	0.185092	0.656504	

Table E 12: Univariate test of significance for P_{app} comparing human and rat species (AP - BL)

	Direction=AP-BL Univariate Tests of Significance for Papp (Statistiek_finale_data) Sigma-restricted parameterization Effective hypothesis decomposition Include condition: species='Human' or species='Rat'				
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	749.3371	1	749.3371	212.8539	0.000000
Method	50.9755	2	25.4877	7.2400	0.002119
Error	137.2968	39	3.5204		

Table E 13: Univariate test of significance for P_{app} comparing human and rat species (BL - AP)

	Direction=BL-AP Univariate Tests of Significance for Papp (Statistiek_finale_data) Sigma-restricted parameterization Effective hypothesis decomposition Include condition: species='Human' or species='Rat'				
Effect	SS	Degr. of Freedom	MS	F	p
Intercept	1144.879	1	1144.879	263.7965	0.000000
Method	14.646	2	7.323	1.6873	0.198244
Error	169.260	39	4.340		

Table E 14: Tukey HSD test comparing human and rat species using P_{app} values (AP - BL)

Cell No.	Method	{1} 3.3348	{2} 5.5751	{3} 5.5174
	Direction=AP-BL Tukey HSD test; variable Papp (Statistiek_finale_data Approximate Probabilities for Post Hoc Tests Error: Between MS = 3.5204, df = 39.000 Include condition: species='Human' or species='Rat'			
1	D		0.002718	0.046659
2	E	0.002718		0.997733
3	C	0.046659	0.997733	

Table E 15: Tukey HSD test comparing human and rat species using P_{app} values (BL - AP)

Cell No.	Method	{1} 5.1087	{2} 6.2053	{3} 6.5192
	Direction=BL-AP Tukey HSD test; variable Papp (Statistiek_finale_data Approximate Probabilities for Post Hoc Tests Error: Between MS = 4.3400, df = 39.000 Include condition: species='Human' or species='Rat'			
1	D		0.266673	0.332674
2	E	0.266673		0.945394
3	C	0.332674	0.945394	

Table E 16: Univariate test of significance for ER comparing human and rat species and techniques

Effect	SS	Degr. of Freedom	MS	F	p
	Direction=AP-BL Univariate Tests of Significance for ER (Statistiek_finale_data) Sigma-restricted parameterization Effective hypothesis decomposition Include condition: species='Human' or species='Rat'				
Intercept	70.20733	1	70.20733	54.99273	0.000000
Method	1.06992	2	0.53496	0.41903	0.660611
Error	49.78996	39	1.27667		

Table E 17: Tukey HSD test for ER comparing human and rat species and techniques

Cell No.	Method	{1} 1.6861	{2} 1.5283	{3} 1.2017
	Tukey HSD test; variable ER (Statistiek_finale_data) Approximate Probabilities for Post Hoc Tests Error: Between MS = 1.2767, df = 39.000 Include condition: species='Human' or species='Rat'			
1	D		0.908091	0.637682
2	E	0.908091		0.813826
3	C	0.637682	0.813826	

Table E 18: Kruskal-Wallis ANOVA for P_{app} comparing human and rat species (AP - BL)

	Direction=AP-BL Kruskal-Wallis ANOVA by Ranks; Papp (Statistiek_finale_data) Independent (grouping) variable: Method Kruskal-Wallis test: H (2, N= 42) =14.48210 p =.0007 Include condition: species='Human' or species='Rat'			
Depend.: Papp	Code	Valid N	Sum of Ranks	Mean Rank
D	1	18	239.0000	13.27778
E	2	18	483.0000	26.83333
C	3	6	181.0000	30.16667

Table E 19: Kruskal-Wallis ANOVA for P_{app} comparing human and rat species and techniques (BL - AP)

	Direction=BL-AP Kruskal-Wallis ANOVA by Ranks; Papp (Statistiek_finale_data) Independent (grouping) variable: Method Kruskal-Wallis test: H (2, N= 42) =5.283130 p =.0713 Include condition: species='Human' or species='Rat'			
Depend.: Papp	Code	Valid N	Sum of Ranks	Mean Rank
D	1	18	301.0000	16.72222
E	2	18	433.0000	24.05556
C	3	6	169.0000	28.16667

Table E 20: Multiple comparison (Kruskal-Wallis ANOVA) for P_{app} comparing human and rat species and techniques (AP - BL)

	Direction=AP-BL Multiple Comparisons p values (2-tailed); Papp (Stati Independent (grouping) variable: Method Kruskal-Wallis test: H (2, N= 42) =14.48210 p =.00(Include condition: species='Human' or species='Rat'		
Depend.: Papp	D R:13.278	E R:26.833	C R:30.167
D		0.002750	0.010488
E	0.002750		1.000000
C	0.010488	1.000000	

Table E 21: Kruskal-Wallis ANOVA for P_{app} comparing human and rat species and techniques (BL - AP)

	Direction=BL-AP Multiple Comparisons p values (2-tailed); Papp (Stati Independent (grouping) variable: Method Kruskal-Wallis test: H (2, N= 42) =5.283130 p =.071 Include condition: species='Human' or species='Rat'		
Depend.: Papp	D R:16.722	E R:24.056	C R:28.167
D		0.218772	0.143468
E	0.218772		1.000000
C	0.143468	1.000000	

Table E 22: Kruskal-Wallis ANOVA for ER comparing human and rat species and techniques

	Kruskal-Wallis ANOVA by Ranks; ER (Statistiek_finale_data) Independent (grouping) variable: Method Kruskal-Wallis test: H (2, N= 42) =3.001829 p =.2229 Include condition: species='Human' or species='Rat'			
Depend.: ER	Code	Valid N	Sum of Ranks	Mean Rank
D	1	18	454.0000	25.22222
E	2	18	328.5000	18.25000
C	3	6	120.5000	20.08333

Table E 23: Multiple comparisons (Kruskal-Wallis ANOVA) using ER values to compare human and rat species and techniques

	Multiple Comparisons p values (2-tailed); ER (Statistiek_finale_data) Independent (grouping) variable: Method Kruskal-Wallis test: H (2, N= 42) =3.001829 p =.2229 Include condition: species='Human' or species='Rat'		
Depend.: ER	D R:25.222	E R:18.250	C R:20.083
D		0.264584	1.000000
E	0.264584		1.000000
C	1.000000	1.000000	

Table E 24: Comparing rat and pig species (Sweerana-Grass diffusion chambers) by means of a Mann-Whitney U test

variable	Method=D Mann-Whitney U Test (w/ continuity correction) (Statistiek_finale_data) By variable Species Marked tests are significant at p <.05000 Exclude condition: method='C' Rank Sum Rat	Rank Sum Pig	U	Z	p-value	Z adjusted	p-value	Valid N Rat	Valid N Pig	2*1sided exact p
Papp	1058.000	1570.000	392.0000	-2.87752	0.004008	-2.87752	0.004008	36	36	0.003596
ER	264.000	402.000	93.0000	-2.16724	0.030217	-2.16738	0.030206	18	18	0.028958

Table E 25: Comparing rat and pig species (everted sac apparatus) by means of a Mann-Whitney U test

variable	Method=E Mann-Whitney U Test (w/ continuity correction) (Statistiek_finale_data) By variable Species Marked tests are significant at p <.05000 Exclude condition: method='C' Rank Sum Rat	Rank Sum Pig	U	Z	p-value	Z adjusted	p-value	Valid N Rat	Valid N Pig	2*1sided exact p
Papp	1962.000	666.0000	0.00000	7.292333	0.000000	7.292333	0.000000	36	36	0.000000
ER	404.500	261.5000	90.50000	2.246341	0.024683	2.246919	0.024646	18	18	0.022359

Table E 26: Comparing rat and pig species and techniques by means of a Univariate test of significance

Effect	All Groups Univariate Tests of Significance, Effect Sizes, and Powers for Papp (Statistiek_finale_data Over-parameterized model Type III decomposition Include condition: species='Rat' or species='Pig' SS	Degr. of Freedom	MS	F	p	Partial eta-squared
Intercept	2421.709	1	2421.709	262.8655	0.000000	0.657385
Species	131.350	1	131.350	14.2574	0.000237	0.094259
Repeat_inter(Species)	64.659	4	16.165	1.7546	0.141590	0.048733
Method	184.515	1	184.515	20.0283	0.000016	0.127546
Error	1262.144	137	9.213			

Table E 27: Comparing rat and pig species and techniques (AP – BL) by means of a Univariate test of significance using P_{app} values

Effect	Direction=AP-BL Univariate Tests of Significance, Effect Sizes, and Powers for Papp (Statistiek_finale_da Over-parameterized model Type III decomposition Include condition: species='Rat' or species='Pig'					
	SS	Degr. of Freedom	MS	F	p	Partial eta-squared
Intercept	760.1148	1	760.1148	136.9940	0.000000	0.678208
Species	104.6836	1	104.6836	18.8669	0.000050	0.224963
Repeat_inter(Species)	20.1957	4	5.0489	0.9100	0.463522	0.053028
Method	13.4996	1	13.4996	2.4330	0.123660	0.036080
Error	360.6542	65	5.5485			

Table E 28: Comparing rat and pig species and techniques (BL - AP) by means of a Univariate test of significance using P_{app} values

Effect	Direction=BL-AP Univariate Tests of Significance, Effect Sizes, and Powers for Papp (Statistiek_finale_dat Over-parameterized model Type III decomposition Include condition: species='Rat' or species='Pig'					
	SS	Degr. of Freedom	MS	F	p	Partial eta-squared
Intercept	1766.058	1	1766.058	161.1769	0.000000	0.712614
Species	35.719	1	35.719	3.2598	0.075628	0.047756
Repeat_inter(Species)	49.864	4	12.466	1.1377	0.346683	0.065431
Method	241.367	1	241.367	22.0280	0.000014	0.253114
Error	712.222	65	10.957			

Table E 29: Comparing everted sac apparatus and Sweetana-Grass diffusion chamber technique when investigating drug transport across pig intestinal tissue

Variable	Direction=BL-AP T-tests; Grouping: Method (Statistiek_finale_data) Group 1: D Group 2: E Include condition: Species='Pig'								
	Mean D	Mean E	t-value	df	p	Valid N D	Valid N E	Std.Dev. D	Std.Dev. E
Papp	8.458448	0.038142	10.72236	34	0.000000	18	18	3.331650	0.027026

Table E 30: Comparing everted sac apparatus and Sweetana-Grass diffusion chamber technique when investigating drug transport across rat intestinal tissue

Variable	All Groups T-tests; Grouping: Method (Statistiek_finale_data) Group 1: D Group 2: E Include condition: Species='Rat' Mean D	Mean E	t-value	df	p	Valid N D	Valid N E	Std.Dev. D	Std.Dev. E
Papp	4.221757	5.890188	-3.24883	70	0.001782	36	36	1.993582	2.349468

Table E 31: Comparing everted sac apparatus and Sweetana-Grass diffusion chamber technique when investigating drug transport across rat intestinal tissue (AP – BL)

Variable	Direction=AP-BL T-tests; Grouping: Method (Statistiek_finale_data) Group 1: D Group 2: E Include condition: Species='Rat' Mean D	Mean E	t-value	df	p	Valid N D	Valid N E	Std.Dev. D	Std.Dev. E
Papp	3.334826	5.575116	-3.37782	34	0.001844	18	18	1.070227	2.602402

Table E 32: Comparing everted sac apparatus and Sweetana-Grass diffusion chamber technique when investigating drug transport across rat intestinal tissue (BL - AP)

Variable	Direction=BL-AP T-tests; Grouping: Method (Statistiek_finale_data) Group 1: D Group 2: E Include condition: Species='Rat' Mean D	Mean E	t-value	df	p	Valid N D	Valid N E	Std.Dev. D	Std.Dev. E
Papp	5.108687	6.205259	-1.48970	34	0.145523	18	18	2.317607	2.093322

APPENDIX F

Language editing certificate

Gill Smithies

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Work Certificate

To	Ruan Joubert
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Date	20/11/2014
Subject	Masters: Chapters 1and 2, by Ruan Joubert
Ref	GS/RJ /01

I, Gill Smithies, certify that I have proofed and language edited:

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