Effect of the transport medium composition on *in vitro* drug permeation across excised pig intestinal tissue

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Dissertation submitted in fulfilment of the requirements for the degree *Master of Science* in Pharmaceutics at the Potchefstroom Campus of the North-West University

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November 2016

Acknowledgements

- God, for granting me this opportunity to complete this study. Without Him, it would not have been possible.
- My wife, Sanet, thank you for your support, love and encouragement. You were always there for me. Love you!
- Dr. Dewald Steyn, thank you for being my supervisor during this study. Your advice and friendship is appreciated. Thank you for always making time to read and re-read my work!
- Prof. Sias Hamman, thank you for the advice and encouragement given. Your friendship and support is greatly appreciated.
- Liezl Badenhorst, my lab partner. Thank you for your help in the lab! Without it this study would have been difficult to complete. Your friendship and support is much appreciated.
- Prof Jan du Preez, thank you for all the help, advice and assistance with the HPLC analysis.
- Prof Jan Steenekamp, thank you for professional advice and guidance.
- Potch abattoir for providing the pig's intestines.
- To my friends and colleagues at the university. Thank you for the conversations, coffee breaks and shared humor. You made this a very enjoyable two years!
- My parents and my parents-in-law. Thank you for always being just a phone call away! Your support and love is irreplaceable.
- The North-West University for funding this study.

Abstract

A crucial step in the process of drug research and development is the investigation of permeation characteristics of new chemical entities across biological membranes to give an indication of their pharmacokinetic properties. Permeation studies are necessary to ensure that drug candidates have acceptable permeability properties before proceeding to more expensive clinical trials. Various techniques are currently employed by researchers to determine the pharmacokinetic properties of pharmaceutical compounds. *In vitro* techniques are commonly used as screening tools to quickly evaluate the ADME-Tox properties of pharmacologically active compounds.

Ex vivo methods, a subdivision of *in vitro* techniques, include the evaluation of drug transport across excised segments of intestinal tissue. Excised pig intestinal tissue models are commonly used in *ex vivo* pharmacokinetic studies. Due to physiological, biochemical and anatomical similarities to humans, the pig model is considered to be sufficiently accurate to be used to predict the pharmacologically active compound's absorption and efflux rates in humans.

Due to the difficulty associated with obtaining aspirated intestinal fluid, simple aqueous buffers are often employed in permeation and solubility studies. These simple buffer systems are, however, not necessarily adequate to predict intestinal solubility or transport. This is due to the effect of pH, stomach content and to a certain extent the ionisation of the drug. Transport medium components used in *in vitro* permeation tests may significantly alter the effect of P-gp on the transport of compounds across the intestinal epithelium. This may be attributed to the fact that the transport media may alter the "tightness" of the intercellular juctions as indicated by the trans-epithelial electrical resistance (TEER) values. The media may either inhibit or stimulate P-gp proteins to such an extent that the rate of P-gp mediated efflux is modulated. Due to these factors, the results obtained from the bi-directional transport studies may differ significantly from that which is encountered in a live animal. To preserve the predictive value of screening tests, it is important to know what influence the transport medium composition may have on the activity of the efflux transporter proteins, tight junctions and solubility of the model drugs

The aim of this study was to compare different types of transport media (which included Phosphate buffer, Krebs Ringer Bicarbonate buffer and simulated intestinal fluids in the fed and fasted states) on the bi-directional transport of four model compounds from the different classes of the Biopharmaceutical Classification System (BCS) in the Sweetana-Grass diffusion chamber apparatus across excised pig intestinal tissue.

Abacavir (BCS class 1) exhibited lower P_{app} values in the simulated intestinal fluid (SIF) than in the buffer solutions. Lamivudine (BCS class 3) exhibited markedly lower P_{app} values in the Phosphate buffer than in any of the other media. Dapsone (BCS class 2) showed marked drug efflux, which to the knowledge of this author, has not previously been experimentally proven. Furosemide (BCS class 4) exhibited higher transport in the Phosphate buffer, with pronounced efflux in the FeSSIF. The different behavior of the drugs in the respective media may be explained by the differences in the physico-chemical properties of the different model compounds, the interaction of the compounds with the transport media and the effect of the transport medium on the pig's intestinal tissue.

Key words: *in vitro* model, Sweetana-Grass diffusion chamber apparatus, simulated intestinal fluid, drug transport studies, abacavir, furosemide, lamivudine, dapsone.

Uittreksel:

'n Belangrike stap tydens geneesmiddelnavorsing en -ontwikkeling is die bepaling van die permeasie eienskappe van nuwe chemiese verbindings oor biologiese membrane om inligting te verskaf rakende die farmakokinetiese eienskappe van die verbindings. Permeasie studies word benodig om te verseker dat nuwe chemiese verbindings oor aanvaarbare permeasie eienskappe beskik voordat duur kliniese toetse onderneem word. *In vitro* tegnieke word algemeen gebruik om die ADME-Toks eienskappe van farmakologies aktiewe verbindings te bepaal.

Ex vivo metodes, 'n onderafdeling van *in vitro* tegnieke, sluit die bepaling van geneesmiddeltransport oor verwyderde dermweefsel in. Vark-intestinumweefsel word algemeen gebruik tydens *ex vivo* studies omdat die fisiologiese, biochemiese en anatomiese eienskappe van die vark baie oreenstem met die van die mens. As gevolg van hierdie verwantskappe is dit moontlik om akkurate absorbsie- en effluksvoorspellings te maak aangaande die nuwe farmakologies aktiewe verbindings in die mens.

Ge-aspireerde intestinale vloeistowwe is oor die algemeen moeilik verkrygbaar en daarom word eenvoudige waterige buffers gereeld gebruik tydens permeasie- en oplosbaarheids-studies. Hierde eenvoudige buffers is nie noodwendig voldoende om die invloed van pH, maaginhoud en mate van ionisasie van die chemise verbindings op intestinale permeasie of -oplosbaarheid akkuraat te voorspel nie. Die samestelling van die transportmedium kan moontlik die fisiologie van die intestinale weefsel beïnvloed wat daartoe kan lei dat intestinale transporters, soos P-gp, se effekte op intestinale permeasie moontlik kan verander. Hierdie verandering kan toegeskryf word aan die feit dat transportmedia die intersellulêre hegtings kan beïnvloed en daardeur die TEER waardes kan verander. Die media kan ook P-gp transporter proteïene inhibeer of stimuleer, wat daartoe kan lei dat die mate van P-gp gemedieërde effluks *in vitro* merkbaar kan verskil van die mate van effluks *in vivo*. Die voorspellingswaarde van die siftingstoets kan slegs bewaar word indien die invloed van die transportmedium op die faktore wat permeasie beïnvloed bekend .is. Dit sluit in die effek van die transportmedium of die intersellulêre hegtings, transporterproteïene en die oplosbaarheid van die geneesmiddel.

Die doel van die studie was om die invloed van verskillende tipes transportmedia (fosfaatbuffer, Krebs-Ringer Bikarbonaat buffer, gesimuleerde intestinale vloeistowwe in die vastende en nie-vastende toestand) op die bi-direksionele transport van vier

modelverbindings uit elkeen van die klasse van die “Biopharmaceutical Classification System” (BCS) te toets. Die toetse is uitgevoer op intestinale varkweefsel in ‘n Sweetana-Grass diffussie-apparraat.

Abacavir (Klas 1) het laer P_{app} waardes in die SIF getoon as in die bufferoplossings, terwyl lamivudine (Klas 3) aansienlik laer P_{app} waardes in die fosfaatbuffer getoon het as in enige van die ander buffers. Dapsoon (Klas 2) het geneesmiddel-effluks getoon, wat tot die kennis van die outeur nog nie vantevore eksperimenteel bewys is nie. Furosemied (klas 4) het beter transport getoon in die fosfaatbuffer met merkbare effluks in FeSSIF media. Die verskillende gedrag van die geneesmiddels in die onderskeie media kan verklaar word deur verskille in die verbindings se fisies-chemiese eienskappe, interaksies tussen die verbindings met spesifieke komponente in die transportmedia en die effek van die transportmedia op die intestinale weefsel van die vark.

Sleutelwoorde: *In vitro* modelle, Sweetana-Grass diffusie-apparaat, gesimuleerde intestinale vloeistof, geneesmiddel-permeasiestudies, abacavir, furosemied, lamivudien, dapsoon.

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List of abbreviations

- % RSD Percentage relative standard deviation
- 3 R's Reduce, Replace, Refine
- ABA Abacavir
- ABC ATP-binding cassette
- ANOVA Analysis of variance
- AP – BL Apical to basolateral
- AP Apical
- ATP Adenosine triPhosphate
- BCS Biopharmaceutics Classification System
- BL – AP Basolateral to apical
- BL Basolateral
- Caco-2 Human Caucasian colon adenocarcinoma
- CYP 3A4 Cytochrome P450 3A4
- Daps Dapsone
- ECACC European Collection of Cell Cultures
- EDTA Ethylene tetra-Diamine Tetra-Acetic acid
- ER Efflux ratio
- F Ratio
- FaSSIF Fasting State Simulated Intestinal Fluid
- FeSSIF Fed State Simulated Intestinal Fluid

- Furos Furosemide
- HBSS Hank’s Balanced Salt Solution
- HCl Hydrochloric acid
- HIF Human Intestinal Fluid
- HPLC High performance liquid chromatography
- ICH International Conference of Harmonisation
- KRB Krebs-Ringer bicarbonate
- LAM Lamivudine
- LOD Limit of detection
- LOQ Limit of quantitation
- MDCK II Madin-Darby canine kidney II
- NRTI Nucleoside reverse transcriptase inhibitor
- P_{app} Apparent permeability coefficient
- PBS Phosphate buffered saline
- P-gp P-glycoprotein
- Phos Phosphate buffer
- R2 Linear regression coefficient
- RSD Relative standard deviation
- SD Standard deviation
- SGF Simulated Gastric Fluid
- SIF Simulated intestinal fluid
- TEER Transepithelial electrical resistance

- USP United States Pharmacopoeia

Chapter 1: Background and justification

1.1 Oral drug delivery

The oral route is the most commonly used and preferred route of administration over intravenous administration due to its convenience, acceptability and safety (Jibodh *et al.,* 2013). Successful delivery of an orally administered drug necessitates identification of a formulation that will provide the required pharmacokinetic profile (Mrsny, 2012). The bioavailability of a drug is a function of many contributing factors that impact on the solubility and permeability of the drug molecules after administration. Drug dissolution and absorption processes are for example dependent on the physicochemical properties of the drug and physiological factors such as pre-systemic metabolism (Jambhekar & Breen, 2013).

1.2 Screening of new chemical entities

A crucial step in the process of drug research and development is to investigate the permeation characteristics of new chemical entities across biological membranes as an indication of their pharmacokinetic properties. Permeation studies are necessary to ensure that promising drug candidates have acceptable pharmacokinetic profiles before proceeding to more expensive clinical trials (Panchagnula & Thomas, 2000). The development of new low cost, high accuracy and high throughput pharmacokinetic screening models with high predictive value are becoming a priority for the pharmaceutical industry and researchers alike. These models should be robust and generate repeatable data that are predictive of the *in vivo* situation, while also taking into consideration all the factors that may influence absorption, distribution, metabolism, excretion and toxicity (ADME-Tox) of the drug under investigation (Balimane & Chong, 2005).

1.3 Models to predict pharmacokinetic properties

Various techniques are currently employed by researchers to measure the pharmacokinetic properties of pharmaceutical compounds. The models used for intestinal permeation studies during the pre-clinical research stages can be divided into the following categories (Alqatani *et al.,* 2013):

- *In vitro* models (e.g. cell cultures such as Caucasian colon adenocarcinoma (Caco-2) and Martin-Darby canine kidney (MDCK) cell lines;
- *In vivo* models (e.g. whole live animals such as rats);
- *Ex vivo* models (e.g. excised animal tissues in Sweetana-Grass diffusion chambers and/or everted intestinal sacs);
- *In situ* perfusion models (e.g. Segments of intestine as part of live animals).

In vitro techniques are commonly used as screening tools to quickly evaluate the ADME-Tox properties of pharmacologically active compounds. A large number of lead compounds can be screened in quick succession by means of high throughput robotic systems. It is also considerably less costly than *in vivo* and *in situ* techniques. Furthermore, *in vitro* techniques have less ethical implications to consider and these models can be manipulated to closely mimic conditions encountered in the human intestinal mucosa. These models can also be used to select lead compounds with satisfactory ADME-Tox properties for further development and clinical evaluation. In some cases the data generated by these models is sufficiently predictive to approve generic products for marketing purposes by the appropriate authorities. A major drawback with all *in vitro* models is the fact that it is impossible to account for complex physiological factors such as blood flow, nerve supply, disease state, hepatic and renal dysfunctions as well as age (Sarmento *et al.,* 2012).

In vivo bioavailability studies in animals have several advantages over other models, mainly due to the presence of blood circulation and intact intestinal membranes. However, disadvantages of *in vivo models* include a time-consuming aspect, the high costs associated with this model and results that may be variable due to interspecies differences in the expression of active transporters and metabolic enzymes (Alqatani *et al.,* 2013).

Ex vivo methods include the measurement of drug transport across an excised segment of intestinal tissue mounted on a Sweetana-Grass diffusion chamber or as everted sacs. Excised pig intestinal tissue models are commonly used in *ex vivo* biopharmaceutical studies. Because of physiological, biochemical and anatomical similarities to humans, the pig model is considered to be sufficiently accurate to predict the pharmacologically active compound's absorption and efflux rates in humans (Sjogren *et al.,* 2014). This method can be used to measure passive and active carrier mediated transport across epithelial tissue in the apical to basolateral direction and also in the basolateral to apical direction (Alqatani *et al.,* 2013).

Due to an increasing emphasis on animal wellbeing and research ethics, the 3R concept (i.e. replace, reduce and refine) has been introduced as a guideline to encourage responsible

use of animals in research. The term “replace” refers to substitution of living animals with alternative models such as *in vitro* techniques. “Reduce” refers to the development of more sophisticated methods that would render accurate data while reducing the amount of animals required for experimental purposes. “Refine” refers to the development of techniques to reduce the pain and distress animals experience during experimentation (Zurlow *et al.,* 1996).

1.4 Active efflux of drug molecules

A wide variety of different active transporters can cause the efflux of drugs across epithelial membranes, including the ATP-binding cassette transporters (ABC) and multidrug resistance associated proteins (MRP). Efflux refers to the counter transport of drug molecules from the basolateral side to the apical side of a physiological membrane, which means drug molecules are pumped back from the epithelium into the gastrointestinal lumen (Kis *et al.,* 2010; Deferme, Annaert & Augustijns, 2010). Efflux transporters such as P-glycoprotein (P-gp) have a significant effect on the bioavailability of many compounds (Balimane *et al.,* 2006).

1.5 The effect of transport media on drug bioavailability

Drug solubility in intestinal fluid is often the rate limiting step in the absorption process. It is therefore important to identify possible solubility issues during the pre-clinical trials of a new drug compound. Due to the high demand for effective new drugs, high throughput solubility testing in a simple aqueous buffer system is usually employed. These simple buffer systems are, however, not necessarily adequate to predict intestinal solubility due to the effect of pH, stomach content and to a certain extent the ionisation of the drug (Augustijns *et al.,* 2014).

The type of transport medium used in *in vitro* screening tests can significantly alter the effect of P-gp on the transport of compounds across the intestinal epithelium. This may be attributed to the fact that the transport media may either inhibit or stimulate P-gp proteins to such an extent that the rate of P-gp mediated efflux may differ significantly from that which is encountered in a live animal. To preserve the predictive value of screening tests, it is important to know what influence the transport medium may have on the activity of the efflux transporter proteins (Balimane *et al.,* 2006).

The composition of the medium from which a drug is absorbed is therefore an important factor to consider when investigating drug bioavailability. Currently, no standard system has been developed in this regard and this lack of data warrants urgent research in this field of

study (Custodio, Wu & Benet, 2008). To effectively mimic the effect of intestinal fluids on the absorption of a drug, it is necessary to use a transport medium that has similar physico-chemical attributes as human intestinal fluid. Simulated intestinal fluid (SIF) has previously been used in transport studies in the Caco-2 cell monolayer model, but this method is plagued by membrane integrity issues due to the damaging nature of this transport medium. Some studies have been undertaken to improve the composition of SIF, but currently there is not sufficient data available to compile a standardised method of preparation (Markopoulos *et al.,* 2014).

1.6 Representative drug compounds for *in vitro* transport studies

Due to varying physico-chemical properties of drug compounds, the Biopharmaceutics Classification System (BCS) has been developed, which classifies drugs based on their solubility and membrane permeability properties. To represent the complete spectrum of drug properties, one compound from each of the four classes of the BCS was used in this study. The following compounds have been selected from a comprehensive list that was previously published (Wu & Benet, 2005).

- Class 1 (high solubility and high permeability): Abacavir
- Class 2 (low solubility, high permeability): Dapsone
- Class 3: (high solubility, low permeability): Lamivudine
- Class 4: (low solubility, low permeability): Furosemide

1.6.1 Abacavir

Abacavir is a nucleoside reverse transcriptase inhibitor (NRTI) used for the treatment of human immunodeficiency virus type 1 infection (HIV-1) with a reported bioavailability of 83% after oral administration. It is reported that 2% of the drug is excreted unchanged through the urinary tract, whilst the metabolic inactivation takes place in the liver, through the alcohol dehydrogenase and glucoronyltransferase enzymes. Abacavir is not significantly metabolised by Cytochrome (CYP) P450 enzymes (Drugbank, 2014). Abacavir inhibits P-gp efflux to a limited extent (Storch *et al.,* 2007).

1.6.2 Dapsone

Dapsone is an antifolate drug that is used in the treatment of leprosy. It is strongly bound to plasma protein (70%-90%) and extensively metabolised by liver enzymes, mainly CYP 2E1 and excreted via the kidneys. Bioavailability after oral dosage is between 70 and 80% (Drugbank, 2015).

1.6.3 Lamivudine

Lamivudine is a NRTI used for the treatment of HIV-1 infection. Lamivudine is loosely bound to protein (<36%), with only a small fraction being metabolised. Bioavailability is 86% after oral dosage in healthy individuals. The largest fraction is excreted unchanged by the renal pathway (Drugbank, 2015).

1.6.4 Furosemide

Furosemide is a diuretic that is used in the treatment of congestive heart failure and hypertension. It is strongly bound to plasma protein (95%) with only a small fraction metabolised by the liver. The bulk of the dose is excreted unchanged in the urine (Drugbank, 2015). In healthy persons, furosemide has a bioavailability of 50-70% in tablet formulations (Bragatto, 2011).

1.2 Problem statement

Several *in vitro* and *ex vivo* models exist to evaluate drug permeability across intestinal epithelium, however, many of these models use Phosphate type buffers and saline as transport medium, which may not necessarily mimic *in vivo* conditions optimally and can therefore have an effect on drug solubility and membrane permeability. It is important to investigate the effect that transport medium composition may have on the *in vitro* permeability of compounds and on drug solubility and membrane integrity. Results from this comparative study can be used to make recommendations regarding the type of transport medium that should be used in future *in vitro* permeability studies to produce more repeatable and reliable data.

1.3 General aim

The aim of this study is to compare different types of transport media (which include Phosphate buffer, Krebs Ringer Bicarbonate buffer, simulated intestinal fluids in the fed and fasted states) on the bi-directional transport of four model compounds from the different classes of the BCS in the Sweetana-Grass diffusion apparatus across excised pig intestinal tissue.

To reach this aim, the following objectives were set:

- To select model drugs from each class of the BCS (abacavir, lamivudine, dapsone and furosemide).
- To select four different transport media in which bi-directional transport of the selected drugs will be performed (Krebs Ringer Bicarbonate buffer, Phosphate buffer, fed and fasting state simulated intestinal fluid).
- To compare the bi-directional transport of each model drug in the different transport media.
- To develop and validate a high performance liquid chromatography (HPLC) method for the selected drugs.

1.4 Dissertation layout

The introductory chapter (Chapter 1) is followed by a thorough review of relevant literature (Chapter 2) regarding oral drug delivery and the models that are used to measure drug permeation across the intestinal epithelium. In Chapter 3 the experimental design, data collection and statistical processing of the data are described. The results and discussions are outlined in Chapter 4, which were obtained from the experiments executed in this study. The final conclusions and future recommendations are made in Chapter 5.

Chapter 2: Literature review on intestinal drug absorption evaluation

2.1 Introduction

The oral route is the most commonly used route of administration for medicine. This route of administration is preferred in most instances due to the convenience of administration, acceptability by patients and safety. Systemic effects are elicited after oral administration due to absorption of the drug through the intestinal tissue and the subsequent distribution of the drug throughout the body to the desired target tissues (Alqatani *et al.,* 2013).

After oral ingestion, the drug has to be released from the dosage form via a process that includes dosage form disintegration and dissolution of the active ingredient. After completion of the dissolution process, the drug is absorbed across the intestinal wall via a process termed permeation. The absorption process, and ultimately drug bioavailability, is dictated by a multitude of factors which can be divided into two groups, namely physicochemical and physiological factors. Passive diffusion is directly related to the concentration of the drug in the gastro-intestinal tract (GIT) fluids as well as the degree of ionization. Some dosage form related parameters such as disintegration and dissolution of the drug can be tested by using simple *in vitro* tests. These tests, however, do not take into account the *in vivo* physiological conditions that are presented to the dosage form during oral administration (Kostewicz *et al.,* 2014).

There is thus a pressing need to develop *in vitro* models that mimic physiological conditions to aid in the accurate prediction of intestinal drug permeation and subsequent bioavailability. Current *in vitro* systems are attempting to include physiological factors such as GIT motility, pH differences in the GIT, digestion processes such as enzyme secretion, food effects and intestinal fluid composition (Kostewicz *et al.,* 2014). However, further refinement of these advanced *in vitro* systems will enable researchers to predict the performance of new drug molecules with a greater degree of accuracy (Alqatani *et al.,* 2013).

2.2 Anatomy of the gastrointestinal tract

The GIT can be described as a tube lined with a single layer of epithelial cells and can be divided into different ‘compartments’, each with its own function. These compartments include the oesophagus, stomach, small and large intestine and rectum. The small intestine is further divided into the duodenum, jejunum and ileum (DeSesso & Jacobson, 2001). An illustration of the human GIT is depicted in Figure 2.1.

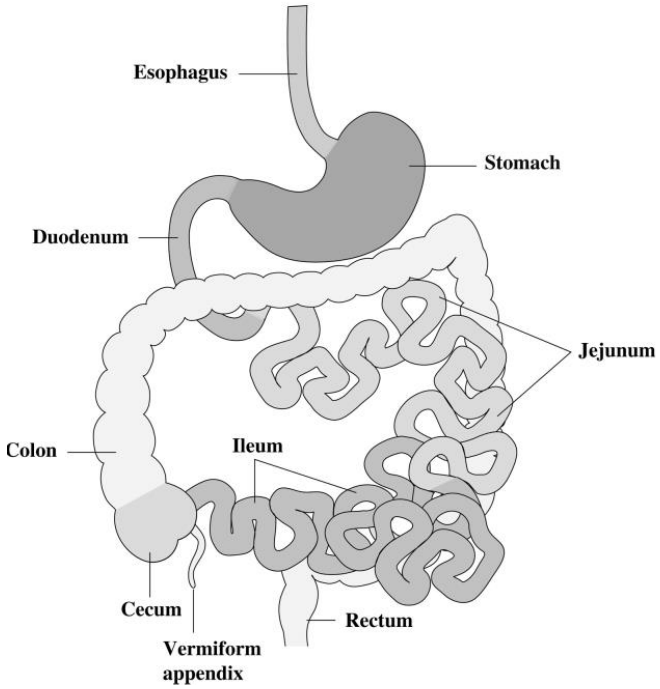


Figure 2.1: Schematic illustration of the human gastro-intestinal tract (DeSesso & Jacobson, 2001)

When a cross-section of the jejunum is examined, five main layers can be identified, namely the serosa, outer and inner smooth muscle layers, muscularis externa, the sub-mucosa and mucosa, as shown in Figure 2.2.

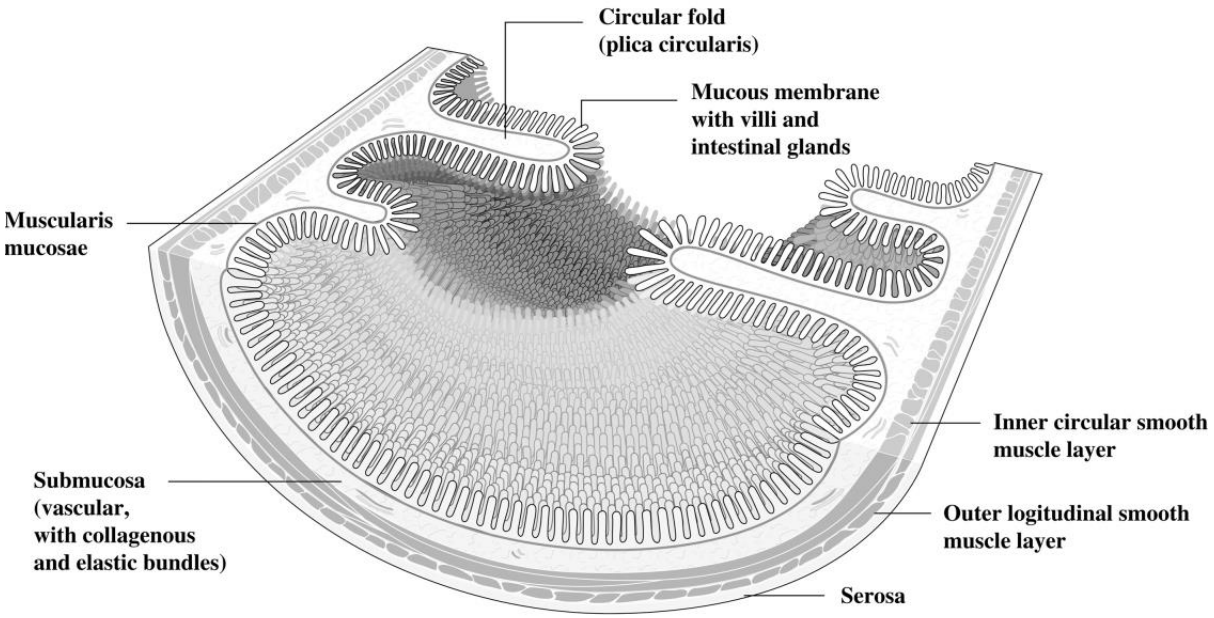


Figure 2.2: Schematic illustration of a cross section of the human jejunum (DeSesso & Jacobson, 2001)

The outermost layer, the serosa, consists of connective tissue which is responsible for providing attachment points to keep the GIT in place. The two smooth muscle layers are responsible for peristalsis, the rhythmic contractions that move intestinal content through the GIT. Just underneath the smooth muscle layers is the sub-mucosa, which is a highly innervated plexus with many blood vessels running through it. The muscularis mucosa is a thin layer of smooth muscle, which is situated just below the sub-mucosa. The function of the sub-mucosa is thought to be related to the movement of the villi. This movement agitates the intestinal content, which reduces the thickness of the unstirred water layer (DeSesso & Jacobson, 2001).

The lamina propria, a thin layer of loose connective tissue and the epithelium is the innermost layer of the intestine. The epithelium consists of intestinal folds and villi, which increases the surface area of the intestine and promote absorption. The epithelium is mainly comprised of enterocytes with the apical side of the cell covered in microvilli, which further increases the surface area available for absorption. A schematic illustration of a cross section of a villus is shown in Figure 2.3.

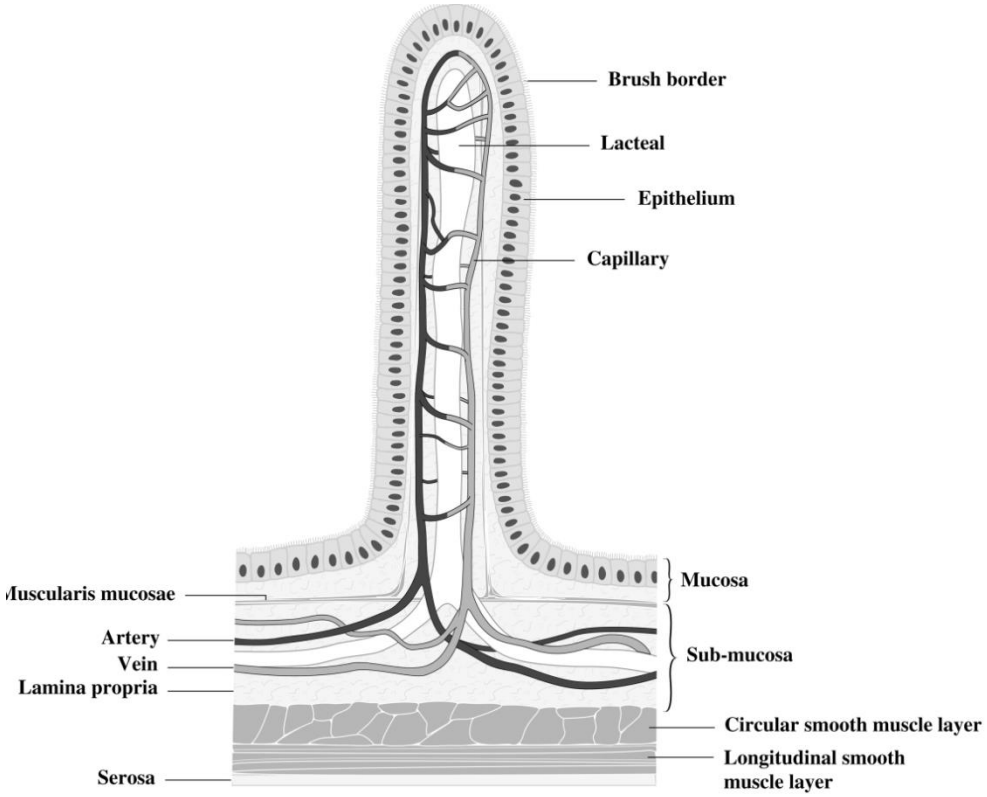


Figure 2.3: Schematic illustration of a cross section of an intestinal villus with underlying tissue layers and blood supply (DeSesso & Jacobson, 2001)

Alongside the absorptive cells (or enterocytes), there are also parietal cells in the stomach that are responsible for the secretion of hydrochloric acid and also goblet cells, which are responsible for intestinal mucous secretion. These secretions are controlled by both neural and hormonal systems. When the acidic stomach content reaches the intestine, it is neutralized by the secretion of alkaline fluids from the pancreas and intestinal wall (Kararli, 1995).

Lymphoid tissue is also found in the intestinal epithelium, which is arranged in groups called Peyer's patches. These patches differentiate into M-cells, capable of transporting substances in the form of particles across the intestinal tissue as part of an immunological response. Due to this particle sampling process, Peyer's patches have altered permeability compared to the surrounding intestinal tissue (Daugherty & Mrsny, 1999).

2.3 Comparison of the pig and human gastrointestinal tract

The pig is considered as a translational model for medical research. This stems from the fact that there are anatomical and physiological similarities between humans and pigs. These similarities are especially valuable when GIT tissues are used for *in vitro* studies.

Pigs and humans have similar (omnivorous) diets (Westerhout *et al.,* 2014) and both species are monogastric (Sjogren *et al.,* 2014). Gastric secretions in both species are dependent on mutual factors, such as hormonal influences and neural impulses from the vagus nerve. This similarity, along with the fact that pigs are roughly the same mass as humans, makes the pig model a useful alternative for *in vitro* studies where correlation with human data is investigated (Sjogren *et al.,* 2014).

Table 2.1: Comparason of anatomical and physiological parameters of human and pig intestinal tracts (adapted from Fagerholm & Lennernäs, 1995; Varum *et al.,* 2010; Sjogren *et al.,* 2014; Hatton *et al.,* 2015)

Parameter	**Human**	**Pig**
Transit time: Stomach	30 min – 3 h	1,5 - 6 hs
Transit time: Small intestine	3 - 4 h	3 - 4 h
Transit time: Large intestine	8 - 18 hours	24 - 48 hours
Length	Small intestine: 7 m Large intestine: 1.5 m	Small intestine: 4.7 - 20 m Large intestine: 3 m
pH	Stomach: 1 - 6 Small intestine: 5 - 7 Large intestine: 5.5 - 8	Stomach: 1.2 - 4.4 Small intestine: 4.7 - 6.1 Large intestine: 6.8 - 7.1
Jejunal mucus layer thickness	83 - 188 µm	28.9 ± 13.7 µm

2.4 Absorption mechanisms, efflux and metabolism

After a drug has been administered orally, it needs to be absorbed into systemic circulation in order to elicit a pharmacological effect. The drug molecules need to cross the intestinal epithelium and reach the systemic circulation and sites of action in an unchanged, therapeutically active form. This can be accomplished by means of different absorption mechanisms as schematically illustrated in Figure 2.4.

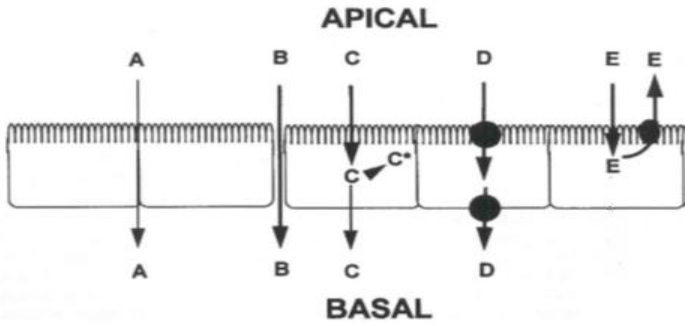


Figure 2.4: Schematic illustration of different mechanisms of intestinal absorption. A and B) paracellular transport via intercellular spaces and through the tight junctions, C) transcellular diffusion with intercellular metabolism illustrated, D) transporter mediated absorption and E) transporter mediated efflux (Versantvoor *et al.,* 2000)

2.4.1 Paracellular transport

Paracellular transport can be defined as transport of molecules via the aqueous "channels" between adjoining epithelial cells. For molecules to pass through this pathway, they should not have a molecular weight larger than 200 Da and occupy a space larger than 11 Å (Ashford, 2013). The tight junctions act as gates, restricting the movement of molecules through the paracellular pathway and thereby limiting the absorption of compounds from the intestine by means of this mechanism (Kawauchiya *et al.,* 2011; Yu *et al.,* 2013). The "tightness" of the junctions may be determined by measuring the trans-epithelial electrical resistance (TEER) across the relevant epithelial membrane. The difference between "tight and leaky" epithelia may be illustrated by comparing the TEER values of different tissue types. Intestinal tissue usually has a TEER value below 200 $\Omega.cm^2$ whilst bladder tissue has a TEER value above 100 000 $\Omega.cm^2$. High TEER values indicate very tight intercellular junctions and a corresponding decrease in paracellular transport is normally observed for tissues with this characteristic (Anderson & Van Itallie, 2009).

2.4.2 Transcellular transport

Transcellular transport encompasses both passive diffusion through a cell membrane as well as active transporter mediated uptake. This is thought to be the main absorption pathway for most drugs after oral administration (Van de Waterbeemd & Testa, 2009). This transport route is mainly responsible for transporting lipophilic molecules with a molecular weight below 500 Da across the intestinal epithelium. Molecules larger than 500 Da are usually not transported across the intestinal epithelium by means of passive diffusion (Lipinski 2004).

2.4.3 Passive diffusion

Passive transcellular diffusion is defined as the movement of molecules from a high concentration (i.e. the lumen of the intestine) to a low concentration (i.e. the basolateral side of the intestinal membrane). Small lipophilic molecules, which occupy a space of less than 100 Å, are usually absorbed via this transport mechanism (Van de Waterbeemd & Testa, 2009). This is the most important mechanism of drug absorption, it is not energy dependent, and is prevalent across the large surface area of the small intestinal epithelium (Lennernas, 1997).

2.4.4 Active transport

Active transcellular transport, or carrier mediated absorption is defined as the energy–dependent transport of a molecule across the intestinal epithelium. This mechanism of transport is relevant to any drug molecule that is a substrate of the active transporter Active transcellular transport is a saturable process, which requires energy acquired from ATP hydrolysis and the direction of transport is against the concentration gradient (Ashford, 2013).

A summary of the location of the most relevant active drug transporters in the intestinal epithelial cells are given in Figure 2.5.

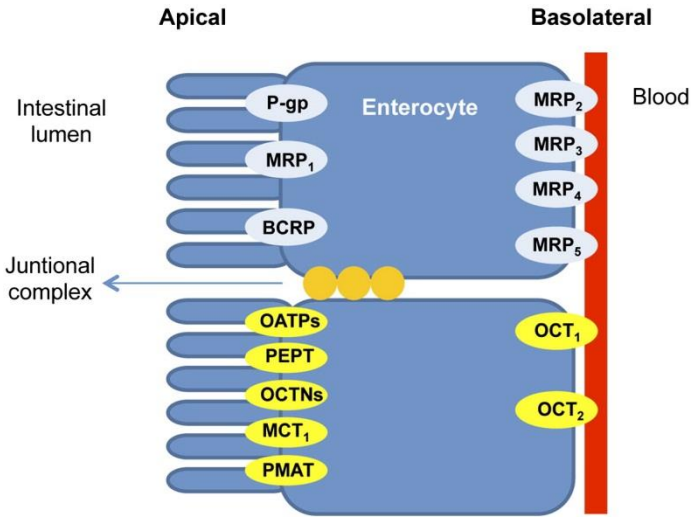


Figure 2.5: Schematic illustration of active drug transporters and their location on the intestinal epithelial cell. Uptake transporters are illustrated in yellow, while efflux transporters are illustrated in white. Multidrug resistance protein (MDR1, P-glycoprotein), multidrug resistance associated protein (MRP), breast cancer resistance protein (BCRP), monocarboxylate transporter protein (MCT), peptide transporter protein (PEPT), organic anion transporting polypeptide (OATP), organic cation transporter (OCT), carnitine/organic cation transporter (OCTN), and plasma membrane monoamine transporter (PMAT) (Estudante *et al.,* 2013)

2.4.5 Transporter mediated efflux

In an effort to protect the body from foreign compounds, transporters known as efflux pumps actively transport compounds from the epithelial cell back to the lumen of the intestine (Pelkonen, Boobis & Gundert-Remy, 2001). Efflux mechanisms will affect drug bioavailability to varying extents depending on the degree of substrate specificity. A wide variety of transporters exhibit drug efflux characteristics, including the ATP-binding cassette transporters (ABC) and solute carrier uptake transporters (SLC). The best-studied efflux transporter is the P-glycoprotein efflux pump (P-gp) which forms part of the ATP-binding cassette (ABC) transporter group. (Pelkonen, Boobis & Gundert-Remy, 2001). Other transporters that are reported to contribute to drug efflux are breast cancer resistance protein (BCRP) and multidrug resistance protein type 2 (MDR-2). These transporters are expressed more extensively in the human jejunum than P-gp (Kis *et al.,* 2010; Deferme, Annaert & Augustijns, 2010). Intestinal metabolism and efflux work in combination to form a co-ordinated barrier to drug absorption which decreases the bioavailability of most drugs (Suzuki & Sugiyama, 2000).

2.4.6 Intestinal drug metabolism

Drug bioavailability is defined as the rate and amount of drug that reaches the systemic circulation unchanged (Van de Waterbeemd & Testa, 2009). The majority of drugs, however, are metabolised in the intestinal wall and liver by a large variety of enzymes, especially enzymes belonging to the Cytochrome P450 (CYP-450) family. These metabolizing enzymes, which are expressed in the intestinal wall, are responsible for pre-systemic metabolism and subsequently a reduction in drug bioavailability (Pelkonen, Boobis & Gundert-Remy, 2001). Metabolism neutralises the potency of most drugs, except in the case of pro-drugs (Silverman, 2004), and adds chemical groups to the molecule to increase hydrophilicity, which in turn facilitates urinary excretion from the body (Silverman, 2004; Hughes, 2014).

2.5 Factors that can influence drug absorption

2.5.1 Physicochemical factors

The physicochemical properties of the model compound play a significant role in the “drug-ability” of the compound. It became apparent that most successful compounds shared certain physicochemical traits, and that focusing on this during drug development, the failure rate of lead compounds may be reduced (Keller, Pichota & Yin, 2016).

2.5.1.1 Rule of five

One of the most often used methods of predicting the *in vivo* performance of new drug molecules is the "Rule of five” (Ro5) as proposed by Lipinski, which states that for a molecule to be able to dissolve in the GIT fluids and to achieve acceptable membrane permeability, it must conform to the following parameters:

- Molecular weight < 500 Da
- Log P < 5
- Hydrogen bond acceptors < 10
- Hydrogen bond donors < 5

Should one or more of the parameters not be complied to, the molecule can be expected to show poor solubility and/or permeability (Lipinski *et al.,* 2001).

2.5.1.2 Beyond the Rule of five

The Ro5 criteria cannot, however, discriminate between drug molecules and non-drug molecules and is only applicable to about half of the drug molecules in use today. The other half consists of large molecules with more hydrogen acceptors and donors (Mattson *et al.,* 2016). This has led to the development of evaluation techniques that lie "beyond the Rule of five" (bRo5) (Benet *et al.,* 2016). A summary of the physicochemical properties of molecules that comply with the Ro5 and those that fall outside the Ro5 (i.e. bRo5 and eRo5) is given in Figure 2.6.

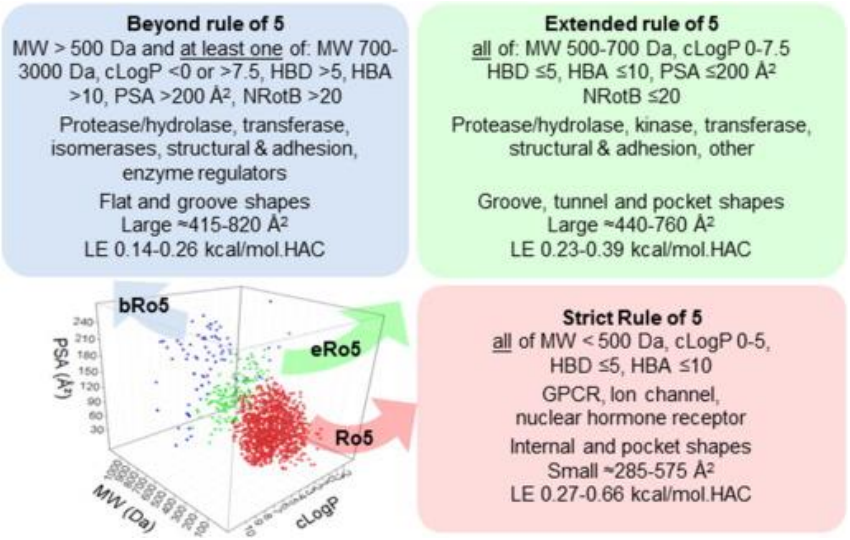


Figure 2.6: A summary of the properties of molecules classified in and beyond the Rule of 5 (Mattson *et al.,* 2016).

2.5.1.3 Rule of unity

The "Rule of unity" (RoU) stemmed from the marked increase in the discovery of lead compounds that exhibited properties beyond the Rule of five. Several mathematical models have been proposed in order to predict the oral absorption of pharmaceutical compounds. The RoU was derived from taking several mathematical equations and deriving a single variable from them. The RoU equation is represented by $\Pi = K_{ow}/O_{lumen}$, with Π being the single absorption parameter, K_{ow} the octanol/water partition coefficient and O_{lumen} the luminal oversaturation number. Due to the equation taking into account both permeability and solubility, the absorption of any passively absorbed molecule can be predicted (Shangvi *et al.,* 2003.) The predictive superiority of the RoU over the Ro5 has been proven experimentally (Yalkowsky *et al.,* 2006).

2.5.2 Physiological factors

After oral ingestion, various physiological factors act as barriers to absorption. These factors tend to limit the bioavailability of compounds .These include, blood supply to the intestine, the contents of the intestine, disease state, the presence of mucus and various other factors (Versantvoort, Rompelberg & Sips, 2000). Some of these factors are discussed in more detail in the following section.

2.5.2.1 Mucus

Mucus, a mixture of mucin and water, is secreted by goblet cells and present a physical barrier to the absorption of most drugs across the intestinal epithelium (Sigurdsson, Kirch & Lehr, 2013). The presence of mucus may hinder drug absorption by one of two mechanisms or a combination of both, namely size exclusion and/or chemical exclusion. Most drug molecules are extremely small compared to the size of the "pores" in mucus, so size exclusion can be ruled out. This is not true for peptide- and protein-based drugs. The molecules are so large that absorption is limited by the inability of the drug molecule to cross the mucus layer and intestinal wall (Choonara *et al.,* 2014; Netsomboon & Bernkop-Schnurch, 2016). Some parts of the mucin backbone is "protein-like" in structure, lipophilic drugs will readily bind to it, thereby reducing the amount available for absorption (Sigurdsson, Kirch & Lehr, 2013; Haegesaether *et al.,* 2013).

Muco-adhesion also plays an important role in the absorption of drugs. If a compound becomes attached to the mucus, the contact time of the drug is lengthened and close contact between the membrane and the molecule is established. This may increase the bioavailability of the drug. However, muco-adhesion only serves to increase bioavailability if extended release dosage forms are used. Formulations are being developed to maximize this phenomenon (Haegesaether, Hiorth & Sande, 2009). This is experimentally proven by the higher bioavailability of valsartan when formulated as a muco-adhesive pellet (Cao *et al.,* 2012). Sloughing of the mucus, on the other hand, may reduce the amount of drug close to the intestinal wall, reducing absorption. This is due to the rapid removal of unattached mucus in the intestinal tract (Netsomboon & Bernkop-Schnurch, 2016).

2.5.2.2 pH

Molecules exhibit different bioavailability at different pH. This is directly linked to the degree of ionisation of the molecule. A molecule is more likely to be in solution if it is ionised, but can only cross a biological membrane if it is un-ionised. The pH varies along the GIT, ranging from pH = 2.4 in the duodenum to pH = 8 in the distal ileum. The abovementioned

variability in pH have a profound effect on the peak anatomical area of absorption for different drugs. (Amidon *et al.,* 1995*;* Van de Waterbeemd & Testa 2009).

2.5.2.3 Gastric emptying

The rate of gastric emptying has been shown to influence both the peak plasma concentration and rate of onset of an orally administered compound with the jejunum as main absorption area. When the gastric emptying rate is reduced, the peak plasma concentration will be higher, and the onset of action slower (Heading *et al.,* 1973). The presence of food also influence the gastric emptying rate, with the fasting rate being much slower than the emptying rate when food is present in the stomach (Dressman *et al.,* 1997).

2.5.2.4 Intestinal transporters and metabolism.

Various energy dependant transporters regulate the active uptake and efflux of molecules in the GIT. These include transporters such as peptide transporter (PepT1), organic anionic anion transporter polypeptide (OATP) and various multidrug resistance transporters, of which P-gp is well known. Substrates of the efflux transporters are pumped out of the intestinal wall and into the lumen of the intestine, reducing the bioavailability of the compound. In conjunction to the above-mentioned transporters, metabolising enzymes such as Cytochrome P450 (CYP3A family) are present within the intestinal wall, further reducing the bioavailability of the compound (Suzuki & Sugiyama, 2000; Pelkonen, Boobis & Gundert-Remy, 2001).

2.6 Drug permeability and solubility

2. 6.1 The Biopharmaceutical Classification System

In 1995, a drug classification system was proposed that grouped drugs into four classes based on their aqueous solubility and membrane permeability characteristics. The system, known as the Biopharmaceutical Classification System (BCS), is schematically depicted in Figure 2.7 (Amidon *et al.,* 1995; Wu & Benet 2005).

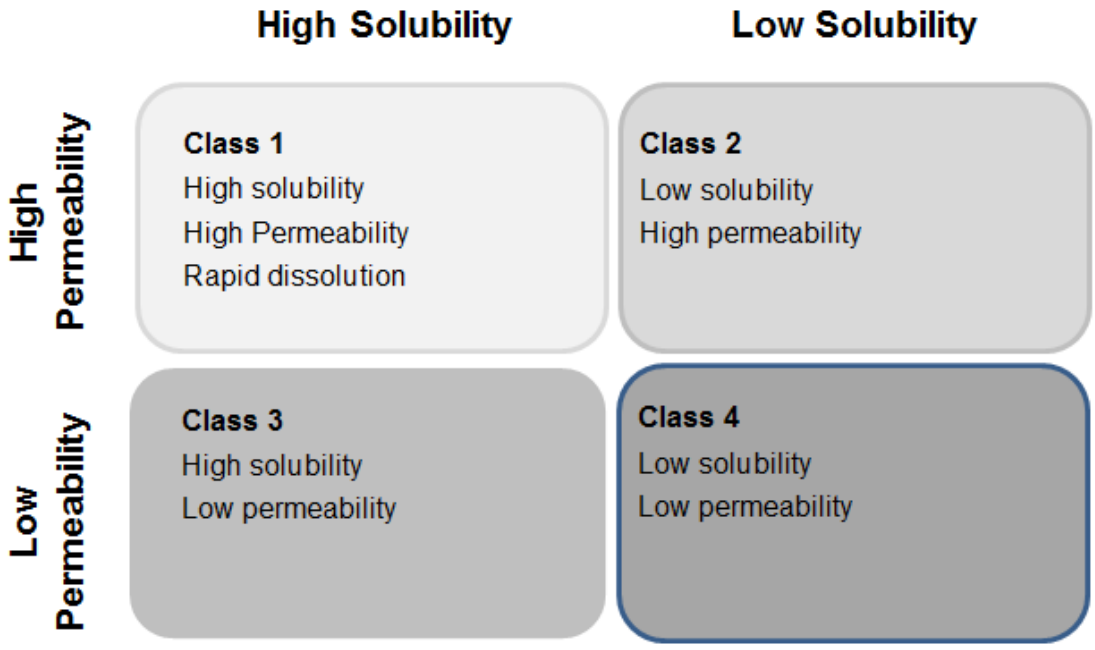


Figure 2.7: Illustration of the characteristics of the four classes of the Biopharmaceutical Classification System (Benet, 2009).

Class 1 drugs of the BCS are highly soluble and permeate easily across the intestinal wall due to its low molecular weight and non-polar properties. Due to their high degree of solubility, class 1 drugs usually exhibit rapid dissolution and subsequent absorption together with a relatively quick onset of therapeutic action (Benet, 2009; Estudante *et al.,* 2013).

Class 2 drugs of the BCS are known to exhibit low solubility but a high degree of membrane permeability. This is due to the lipophilic nature of these molecules which negate the effect of intestinal uptake transporters because of the rapid partitioning of the drug into the membrane (Estudante *et al.,* 2013).

Class 3 drugs of the BCS have good solubility and low permeability characteristics, which originates from the hydrophilic nature of these molecules, while class 4 drugs exhibit poor solubility as well as permeability. In the intestinal environment, class 4 drugs may achieve sufficient solubility due to the presence of surfactants (e.g. bile salts) present in the GIT. This allows some class 4 drugs to exhibit class 3-type behaviour in *in vivo* conditions. The uptake of class 3 and 4 drugs is transporter dependent (Estudante *et al.,* 2013).

2.6.2 The Biopharmaceutical Drug Disposition Classification system

The Biopharmaceutical Drug Disposition Classification System (BDDCS) was developed based on the BCS, but also takes into account drug metabolism. An illustration of the BDDCS is summarized in Figure 2.8**.**

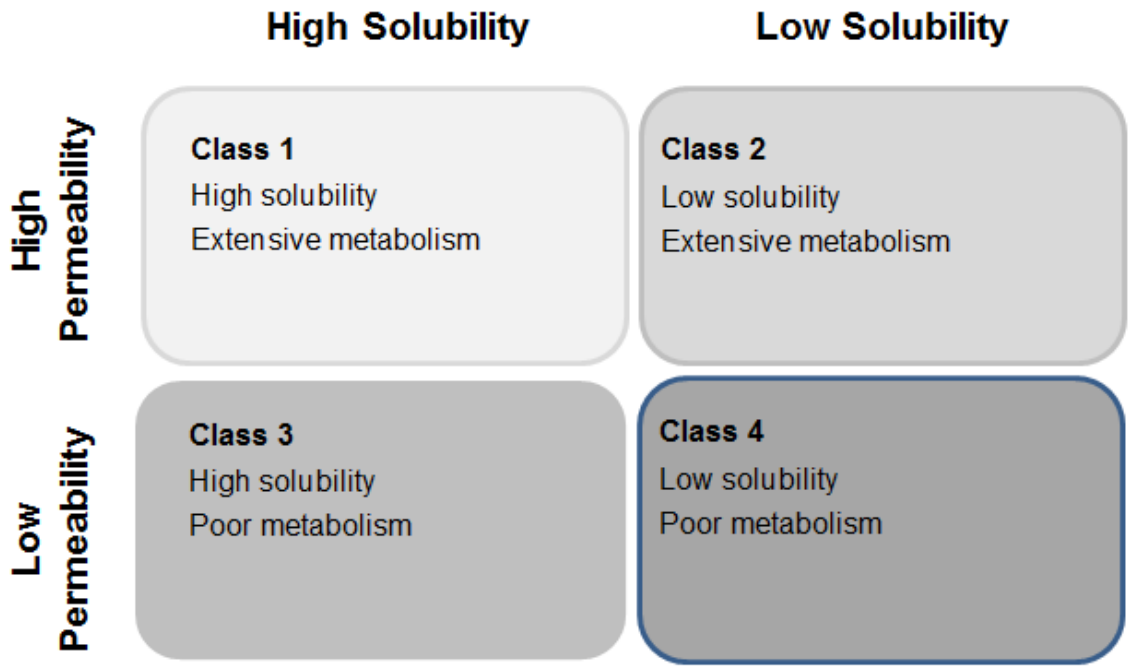


Figure 2.8: Illustration of the four Biopharmaceutical Drug Disposition Classification System classes (Wu & Benet, 2005; Benet, 2009)

Class 1 and 2 drugs of the BDDCS experience pronounced metabolism due to the large amount of drug moving into the systemic circulation, which is exposed to metabolic enzymes. Class 3 and 4 drugs, although they might be substrates for these enzymes, experience less metabolism due to poor drug permeability (Benet 2009).

This BDDCS system makes it possible to predict potential interactions that different drugs may have with intestinal transporters as illustrated in Figure 2.9.

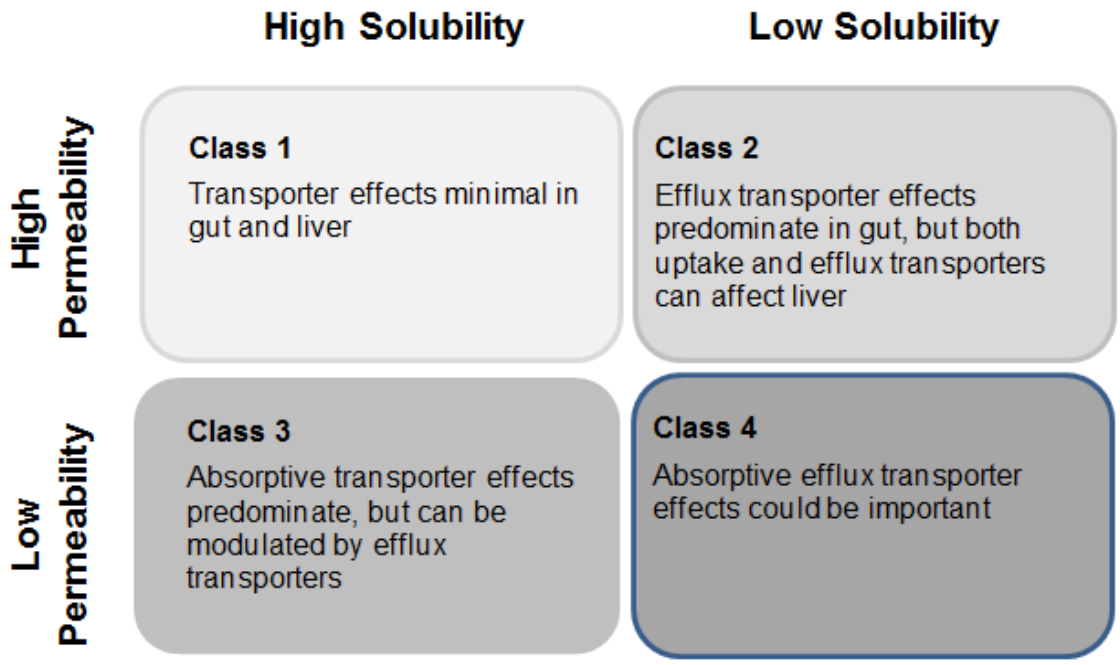


Figure 2.9: Intestinal transporter effects as predicted by the Biopharmaceutics Drug Disposition Classification System (Benet, 2009)

Class 1 drugs of the BDDCS, although they might be substrates for intestinal transporters, do not experience significant transport alterations due to transporter related effects. The solubility and permeability of these compounds are so high that passive diffusion is considered to be the primary means of drug transport for these compounds (Estudante *et al.,* 2013).

The uptake of class 2 drugs of the BDDCS may be affected by transporter related efflux. Class 2 drugs are lipophilic in nature and are able to readily cross intestinal tissue, but are not able to saturate efflux transporters due to their low solubility characteristics. This may cause pronounced drug efflux into the intestinal lumen, which may significantly impede the bioavailability of these compounds. Class 3 and 4 drugs are influenced by both uptake and efflux transporters (Estudante *et al.,* 2013).

2.7 *In vitro* pharmacokinetic screening models

Various *in vitro* techniques exist to predict the *in vivo* pharmacokinetic behaviour of drug molecules in the GIT. The techniques are broadly divided into two groups, where the one group addresses the physiological factors such as gut motility, pH changes in the GIT and gastric lipolysis on the solubility of compounds and the other addresses the transport of compounds in solution across a membrane (Kostewicz *et al.,* 2014). The advantages and

limitations of these two groups are listed in Table 2.2 (Fotaki *et al.,* 2009, Deferme *et al.,* 2010; Klein, 2010; Kostewicz *et al.,* 2014).

One of the biggest challenges is to combine the abovementioned systems into one. Some work has already been done on the development of such a system namely the “Gut-in-a-lab” system. However, it is important to note that all artificial systems are a simplification of a complex biological entity and all the variables cannot be taken into consideration (Thomas *et al.,* 2012; Kostewics *et al.,* 2014).

Table 2.2: Advantages and limitations of different *in vitro* models

Motility and digestion models			**Absorption models**		
	Advantages	**Limitations**		**Advantages**	**Limitations**
Basket and paddle dissolution methods	Large medium volume simulates sink conditions	Volume is not the only factor in sink conditions Stirring may produce coning	Cellular techniques	Good screening model Medium throughput Unending supply of membrane	Inter-laboratory variability due to do different culture techniques used Mucus layer lacking in most monocultures
Reciprocating cylinder method	Assays on a wide range of dosage forms No hydrodynamic dead zone (coning)	New method: Useful parameter combinations needs development	Excised tissue techniques	Directional transport Retains intestinal architecture Could be used to differentiate between intestinal regions	Limited tissue supply Limited time viable Stirring conditions not optimal
Flow-through cell	Simulate retention of solid particles in the stomach Used in conjunction with simulated intestinal fluid (SIF)	Superiority over other dissolution techniques needs to be proved.	Artificial membranes	High throughput Simple membrane preparation Long viability time	No active transport or efflux

2.7.1 Classification of models

Various approaches are employed by researchers to investigate the pharmacokinetic properties of novel pharmaceutical compounds. The models used for intestinal permeation studies during the pre-clinical research stages can be divided into the following categories (Alqatani *et al.,* 2013):

- *In vivo* models (e.g. whole live animals such as rats);
- *In situ* perfusion models (e.g. Segments of intestine as part of live animals);
- *In silico* models (e.g. computer-based simulations);
- *Ex vivo* models (e.g. excised animal tissues in Sweetana-Grass diffusion chambers and/or everted intestinal sacs);
- *In vitro* models (e.g. cell cultures such as Caucasian colon adenocarcinoma (Caco-2) and Martin-Darby canine kidney (MDCK) cell lines.

2.7.1.1 *In vivo*

In vivo bioavailability studies in animals have several advantages over *in vitro* models mainly due to the interaction between complex physiological parameters within the intact organism, which include blood circulation, nerve supply and viable tissues such as intestinal membranes. The disadvantages of *in vivo models* include time-consuming studies, high cost and the results can be highly variable due to interspecies variation in drug metabolism and expression of active transporters such as P-gp (Hämäläinen & Frostell-Karlsson, 2004; Alqatani *et al.,* 2013).

2.7.1.2 *In situ*

In Situ perfusion models are used to study drug absorption and metabolism on isolated segments of tissue acquired from that are still part of various living, but anaesthetised animal sources, e.g. pig or rat intestine. The difference in concentration between the inlet and outlet samples drug solution is measured to determine the rate and extent of drug absorption. This model is a valuable tool in ADME pharmacokinetic studies due to the fact that the organ of choice is still attached to a living organism. Disadvantages associated with this method include the effects of the anaesthesia on drug absorption and the labour intensive preparation of the test animals (Alqatani *et al.,* 2013).

2.7.1.3 *In silico*

The term "*in silico*" encompasses all computer-based models used to predict drug-likeness and pharmacokinetic and/or pharmacological interactions. These models are derived from a combination of data acquired from a large number of pharmacokinetic and other studies to build a database which mathematically combine these variables in order to predict drug characteristics. This technique is popular due to its high throughput potential and cost-effectiveness (Spalding *et al.,* 2000).

2.3.1.4 *Ex vivo*

Ex vivo methods include the measurement of drug transport across an excised segment of intestinal tissue mounted in an Ussing type diffusion chamber or as everted sacs. Excised pig intestinal tissue models are commonly used in *ex vivo* biopharmaceutical studies. Because of physiological, biochemical and anatomical similarities to humans, the pig model is considered to be sufficiently accurate to predict the pharmacologically active compound's absorption and efflux rates in humans (Sjogren *et al.,* 2014). This method can be used to measure passive and active carrier mediated transport across epithelial tissue in the apical to basolateral direction and also in the basolateral to apical direction (Alqatani *et al.,* 2013).

2.7.1.5 *In vitro*

In vitro methods refer to studies done in specialised equipment (outside living organisms) designed to stimulate one or more of the variables influencing *in vivo* conditions. Some equipment makes use of mechanical stirrers to simulate gastric motility, whilst others employ cultured cell lines to act as a permeation membrane. Some of the more complex experimental setups attempt to mimic the entire GIT (Kostewicz *et al.,* 2014; Bedunau *et al., 2013*).

A common *in vitro* cell culture model used to predict drug absorption in humans after oral administration is the Caco-2 cell line. This human intestinal epithelial cell line culture model is often employed to investigate drug permeability and is known to have sufficient P-glycoprotein (P-gp) efflux pump expression and tight cellular junctions, which limits the extent of paracellular transport (Crowe & Wright, 2012).

2.7.2 Cell culture models

Cell cultures offer a broad range of different *in vitro* models and techniques to mimic *in vivo* conditions during drug absorption. It is important, however, to keep in mind that there is no "one size fits all" technique to use for the determination/prediction of the absorption of all

drug molecules. Each cell line is specifically suited for a specific type of experiment, for example, the Caco-2 cell line is better suited to investigate intestinal drug transport (permeation) than the Madin Darby Canine Kidney (MDCK) cell line due to the fact that Caco-2 is of human origin (Wrzesinski & Fey, 2015).

There are also different types of cell sources namely primary cells (i.e. cells isolated from a specific organ or tissue type), cells that are derived from tumors, genetically engineered cell lines and stem cells (Elsheikh *et al.,* 2014; Wrzesinski & Fey, 2015). Stem cells and primary cells have one major disadvantage over tumor and engineered cell lines, they expire within a finite time. Primary cells have the added disadvantage of not being able to be expanded in continuous culture (Elsheikh *et al.,* 2014; Wrzesinski & Fey, 2015).

Although tumor cells offer an unlimited supply of cells, it is imperative to take note that the cells are genetically flawed; it may therefore not be an accurate representation of normal cells (Wrzesinski & Fey, 2015). These cells also exhibit properties of the organ in which the cells originated. Caco-2 cells, the most used cell line in intestinal permeation studies is a prime example because they form tight junctions and express transporters that more closely represent the colon than the small intestine (Araujo & Sarmento, 2013). Caco-2 cells also do not excrete mucus, which is normally present in the gastro-intestinal tract (Gamboa & Leong, 2013; Antunes *et al.,* 2013).

Cell manipulation is needed to create sub-cultures to stop cells dying off due to contact inhibition. This is usually done by trypsinisation, which accelerates cell growth, but causes the cells to lose some functionality (Elsheikh *et al.,* 2014; Wrzesinski & Fey, 2015). Despite these drawbacks, cell culture models have shown relatively good *in vitro-in vivo correlation (*IVIC) and can be used to relatively successfully predict drug transport in humans (Lennernas *et al.,* 1995; Balimane, Chong & Morrison, 2000).

2.7.2.1 Tight junctions

The paracellular pathway can be defined as the aqueous space or “channel” between epithelial cells, allowing for small hydrophilic molecules to cross the epithelium between cells. The tight junctions act as gates, restricting the movement of these molecules, and thereby severely limiting the absorption of xenobiotics via the paracellular pathway (Kawauchiya *et al.,* 2011; Yu, Wang *et al.,* 2013). Proteins such as claudins and occludin contribute to the barrier properties of the tight junctions. These proteins may be influenced by trace element supplementation (Wang *et al.,* 2013; Valenzano *et al.,* 2015).

The Caco-2 cell culture model cannot accurately predict the paracellular permeability of drugs (Balimane, Chong & Morrison, 2000). This is due to the fact that Caco-2 monolayers form very tight intercellular junctions similar to those found in the colon, the anatomical site of its origin (Balimane, Chong & Morrison, 2000; Rocha, Velez & Devesa, 2012; Araujo & Sarmento, 2013). It is also important to consider that Caco-2 cell tight junctions become tighter with time in culture, with the 4-day old culture showing similar paracellular permeability to some animal colon models (Lozoya-Agullo *et al.,* 2015).

2.7.2.2 Transporter expression and mucosal metabolism

Efflux, pre-systemic metabolism and active transporter-mediated uptake are factors that largely influence drug absorption. The key to developing an accurate model lies in the ability to predict the interaction between these processes (Pelkonen, Boobis & Gundert-Remy, 2001). Although Caco-2 cells originated in the colon, transporter expression differs markedly from all human gastro-intestinal regions with some transporters showing more than a five-fold difference. The greatest correlation is with the human small intestine, which this cell model emulates. It is also important to take into account that transporter expression varies with different culturing techniques, time spent in culture and the source of the cells (Englund *et al.,* 2006).

2.7.2.3 Loss of cellular function

Trypsinisation, or the breaking of intercellular connections with trypsin or ethyline diamine tetra-acetic acid (EDTA) in order to re-culture the cells, stimulates the cells into high-growth mode in order to repair the “damage” done. This may lead to loss of certain cellular functions (Elsheikh *et al.,* 2014; Wrzesinski & Fey, 2015). In classical 2D cell culturing techniques, the cells are “forced” to grow on a flat surface that do not represent an accurate framework of the *in vivo* situation (Pereira *et al.,* 2015). One of the drawbacks of 2D cell culture techniques is that the cells “crowd” or agglomerate, and depending on the age of the culture, may lead to morphological changes that influence cellular function (Wang *et al.,* 2016).

These factors influence the predictability of cellular models and can be mitigated by using 3D cell culture modeling techniques that simulate the *in vivo* environment much more accurately and usually do not make use of trypsinisation (Pereira *et al.,* 2015; Wrzesinski & Fey, 2015).

2.7.3 Excised tissue models

Excised tissues mounted between the half-cells of an Ussing type diffusion apparatus is considered to be an acceptable model to use to study drug permeation across the intestinal epithelium. Human intestinal tissue obtained after bariatric surgery is the ideal, but due to its limited availability, animal tissues are more often used for these types of studies. Animal intestinal tissues may be obtained from an abattoir where animals are routinely slaughtered for meat production purposes (Westerhout *et al.,* 2014; Alqatani *et al.,* 2013). This complies with the 3R concept in which researchers attempt to replace laboratory animals with alternative models, refine the experimental methods and to reduce the number of animals sacrificed for research (Zurlo, Rudacille & Goldberg, 1996). Implementation of the 3R concept makes the research more ethically acceptable and cost-effective (Pelegatti, 2012).

2.7.4 Interspecies variation

Various animal models (such as pigs, rats and dogs) have been used to predict *in vivo* drug behaviour in humans. However, the internal structures of the intestine, villi expression, size and shape as well as intestinal length and the" tightness" of the tight junctions differ between the various animal species and humans. A combination of these factors is responsible for the sometimes poor correlation of data between animals and humans in terms of drug pharmacokinetic behaviour (Hatton *et al.,* 2015; Sjogren *et al.,* 2014).

Metabolic variation between humans and animals may also lead to poor data correlation. For example, human and landrace pigs show similar expression of several CYP-450 isozymes, including 2C9 and 2D6, while the minipig show no expression of the abovementioned isozymes and the functions of isozyme 2D6 are fulfilled by isozyme 2B (Dalgaard, 2015, Sjogren *et al.,* 2014).

2.8 Factors that influence *in vitro* screening models

2.8.1 Dissolution medium

2.8.1.1 Solubility in different media

As stated in the introduction, bioavailability can be linked to permeation of the compound across the intestinal membrane and the solubility of the compound in the GIT fluids. Simple aqueous solubility tests are often employed, but are not always adequate in determining *in vivo* solubility. This is due to the ability of bile components to improve the solubility of lipophilic compounds (Augustijns *et al.,* 2014). This phenomenon may lead to the under-

prediction of the intestinal performance of lipophilic compounds and attempts have been made to conduct solubility tests in aspirated human intestinal fluids (HIF). This, however, is not an ideal solvent system for high throughput screening as it requires the constant use of volunteers that need to subject themselves to medical procedures (Augustijns *et al.,* 2014).

2.8.1.2 Simulated intestinal fluids (SIF)

The difficulties experienced with the limited supply of HIF have led to the development of media containing lecithin and bile components in the form of taurocholate. Although these media do not exactly replicate the composition of HIF, they do reflect the effect of HIF on the solubility of lipophilic compounds (Augustijns *et al.,* 2014). In an attempt to develop simulated gastric fluid (SGF) of the stomach and (SIF), various compositions have been suggested to improve the biorelevancy of these media (Schwebel *et al.,* 2011). This has led to various levels of simulation of the intestinal fluid, with each attempt encompassing an increase in the accuracy of the simulation of the luminal conditions (Markopoulos *et al.,* 2014). The different levels of intestinal fluid simulation are illustrated in Figure 2.10.

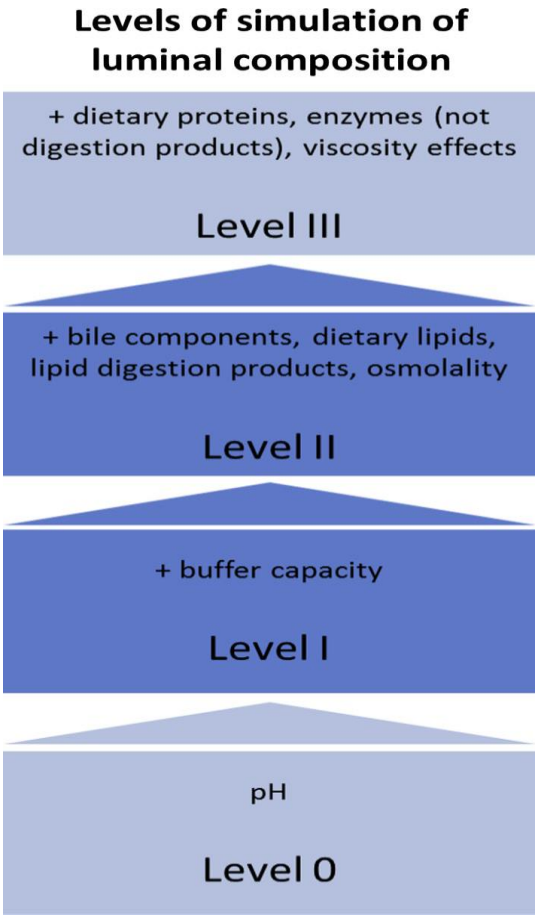


Figure 2.10: Schematic illustration of the different levels of intestinal fluid simulation (Markopoulos *et al.,* 2015).

Each of these simulation levels lends it towards a specific application with level zero being suitable for dissolution testing. Level 1 media is suitable for the pH effect that food may have on very soluble drugs, since these drugs do not require the addition of bile components. Level 2 lends itself to testing the luminal performance of poorly soluble drugs, as the addition of bile and fatty acid components may aid in the solubilisation of lipophilic compounds. Level 3 media are still under development as some incompatibilities exist between the components. It is thought to be useful in the evaluation of extended release lipid-based dosage forms (Markopoulos *et al.,* 2015).

2.8.1.3 *In vitro* transport studies using biorelevant media

Drug transport studies involving excised animal tissues require the use of physiological buffers such as Krebs-Ringer bicarbonate (KRB) buffer, Phosphate-buffered saline (PBS) or Hank's Balanced Salt Solution (HBSS). During these *in vitro* transport studies, oxygen is bubbled through the medium of choice to keep the tissue oxygenated and to prolong tissue viability (Alqatani, Mahomed & Kaddoumi, 2013).

However, these simple experimental setups with physiological buffers, do not address other variables such as the influence of bile components or the presence of lipids in the intestinal fluids and therefore transport media with a more complex composition is required to achieve more reliable/repeatable *in vitro* transport results. These conditions may lead to the formation of micelles that aid in the absorption of lipophilic compounds. This is especially relevant to poorly soluble BCS class 2 and 4 compounds (Markopoulos *et al.,* 2014; Benet, 2009).

As mentioned before, to address these shortcomings, studies were done using aspirated HIF. Due to the difficulty associated with obtaining HIF, attempts have been made to simulate the composition of HIF, which resulted in the production of SIF to represent both the fed and fasted states of the GIT (Stappaerts *et al.,* 2014). Both types of media have been tested in Caco-2 cell based experiments, but incompatibilities between the cell monolayer and the media were identified (Westerhout *et al.,* 2014; Wuyts *et al.,* 2015. Some success has been reported with modified SIF in the Caco-2 cell model, with an increase in the Phosphatidylcholine (lechetin) concentration and a corresponding reduction in the tauracholine concentration (Markopoulos *et al.,* 2014).

2.8.2 Excised animal tissue techniques

The Ussing chamber model was first described in 1951. It was developed in order to better understand the transport mechanisms of ions across biological membranes. The first study was done using frog's skin mounted between the two halves of an Ussing chamber while measuring the potential difference across the skin which gave an indication of the extent of ion transport. The Ussing chamber diffusion method was further developed and validated for a variety of tissues, including intestinal mucosa (Luo *et al.,* 2013).

The Sweetana-Grass diffusion chamber system was developed as an improved system based on the initial design of the original Ussing chamber design apparatus. In this system, a segment of tissue is mounted between two chambers with one of the chambers containing a medium with the drug/compound of interest (donor chamber), whilst the other contain medium without any compound/drug added (acceptor chamber). Medical oxygen is bubbled through both of the chambers to ensure oxygenation of the tissue and agitation of the contents of the chamber. Samples are withdrawn from the acceptor chamber and analysed to determine the amount of drug/compound that has permeated across the tissue segment (Bohets *et al.,* 2001). Pig intestinal tissue is readily available from abattoirs and due to the fact that it is very similar in structure to the human intestine, makes it a very popular choice

for *ex vivo* permeation studies in Sweetana-Grass diffusion chamber apparatus (Pietzonka *et al.,* 2001).

The big disadvantage of this method is the limited time of tissue viability after the death of the animal which is typically in the region of a three hour period (Alqatani, Mahomed & Kaddoumi, 2013). Some studies, however has shown that tissue can be compromised in as soon as 20 minutes after slaughter (Pietzonka *et al.,* 2001).

2.9 Conclusion

From the literature, it is apparent that better *in vitro* models are necessary to better predict the pharmacokinetic behaviour of new drugs *in vivo*. These improved methods need to mimic *in vivo* parameters more realistically and they must be time and cost-effective as well as ethically acceptable. One of the shortcomings of conventional *in vitro* techniques is the use of non-biorelevant buffers such as Phosphate buffer saline. Various studies have been done to investigate what the influence of alteration in medium composition may have on drug solubility and/or transport of test compounds over a cell-based monolayer such as Caco-2 monolayers.

In vitro models are developed to ensure the implementation of the 3R ethics concept in research, but if that data cannot predict the *in vivo* situation effectively, it will not be useful. It is clear, however, that further studies on the effect media composition has to be done when used to investigate the transport of drugs across animal tissue models.

CHAPTER 3: Materials and Methods

3.1 Introduction

A commonly used technique to study drug permeation is the *in vitro* use of excised animal tissues. This includes the removal and isolation of the relevant tissue, removing any excess tissue, mounting it between the half-cells of a diffusion chamber apparatus and measuring the drug transport across the tissue by drawing samples from the acceptor chamber. Other *in vitro* techniques may also be used such as the everted sac technique, where a segment of intestinal tissue is everted, mounted and submerged in a suitable medium, with a drug-containing fluid circulating through the intestine. One of the advantages of these *in vitro* permeability techniques is that the studies take place in a controlled environment, allowing no external factors to influence the outcome (Balimane *et al.,* 2000; Westerhout *et al.,* 2014). Various animal species have been used as models for providing tissues to be used in *in vitro* transport studies, which include pigs, dogs, monkeys and rats (Kararli, 1995; Dalgaard, 2014; Jung & Maybach, 2015).

For this study, the Sweetana-Grass diffusion chamber technique was used to determine the bi-directional transport of selected drugs (i.e. abacavir, lamivudine, dapsone and furosemide) across excised pig intestinal tissue. This was done in four different transport media, namely Kreb's Ringer bicarbonate (KRB) buffer, Phosphate buffer (Phos), fed state simulated intestinal fluid (FeSSIF) and fasting state simulated intestinal fluid (FaSSIF). The transport and efflux of the selected drugs in the selected transport media were compared by calculating and statistically comparing the apparent permeability coefficient (P_{app}) and efflux ratio (ER) values.

3.2 Materials

Abacavir (batch number: CAD0770029, DB Fine Chemicals, South Africa), lamivudine (batch number: RM144AE, Kirsch Pharma, South Africa), dapsone (batch number: 2954, DB Fine Chemicals, South Africa) and furosemide (batch number: M110401, Warren Chem Specialities, South Africa) were kindly donated by the staff of the NWU (Proff: W. Liebenberg, J. du Preez and J. Steenekamp).

Krebs Ringer bicarbonate buffer (product number: K4002) and sodium taurocholate hydrate (product number: 86339) were purchased from Sigma Aldrich (Johannesburg, South Africa). Sodium dihydrogen Phosphate (CAS number: 10049-21-5), sodium hydrogen carbonate (CAS number: 144-55-8), sodium hydroxide (CAS number: 1310-73-2), potassium dihydrogen orthoPhosphate (CAS number: 7778-77-0), sodium chloride (CAS number: 7647-09-9) and dipotassium hydrogen orthoPhosphate (CAS number: 7758-11-4) were

purchased from Associated Chemicals Enterpises (ACE) (Johannesburg, South Africa. Lecithin was purchased from Alfa Aesar (Karlsruhe, Germany).

3.3 *In vitro* transport studies across excised pig intestinal tissues

3.3.1 Transport media preparation

3.3.1.1 Krebs Ringer bicarbonate buffer

The contents of one container of KRB containing 9.5 g of powder for reconstitution were dissolved in 800 ml deionized water in a 1 liter volumetric flask. The container of KRB was rinsed twice with water, which was also added to the volumetric flask to ensure that all the powder was removed from the container. Sodium bicarbonate (1.26 g) was added to the solution. This solution was stirred for 5 min to ensure that all the powder was dissolved. Water was then added to make up the required volume of 1 liter. This solution was thoroughly stirred for 5 min to ensure dissolution of all the powder particles. The pH of the solution was measured, and if necessary, the pH was adjusted by adding drops of a 1 M sodium hydroxide or hydrochloric acid solution until a pH of 7.4 was reached.

3.3.1.2 Phosphate buffer

Dipotassium hydrogen Phosphate (3.448 g) and potassium dihydrogen Phosphate (10.91 g) were weighed off and dissolved in 900 ml deionized water in a 1 liter volumetric flask. The solution was stirred for 5 min to ensure that all the powder particles were dissolved. Water was added to 1 liter and the pH was then adjusted to 7.4 by adding drops of a 1 M sodium hydroxide or hydrochloric acid solution.

3.3.1.3 Simulated intestinal fluids

The quantities of the ingredients used to prepare FaSSIF and FeSSIF are outlined in Table 3.1.

Table 3.1 Composition of simulated intestinal fluids (for the preparation of 1 l simulated intestinal fluid)

Component	**FaSSIF**	**FeSSIF**
Sodium taurocholate	3 mmol/l (1.89 g)	15 mmol/l (8.09 g)
Phosphatidylcholine as lechitin	0.75 mmol/l (0.48 g)	3.75 mmol/l (2.41 g)
NaH_2PO_4	28.66 mmol/l (3.44 g)	0
Acetic acid (glacial)	0	144 mmol/l (9.1 ml)
NaCl	106 mmol/l (6.19 g)	173 mmol/l (10.11 g)
NaOH q.s. (quantis sufficit)	pH 7.4	pH 7.4

The required quantity of each compound was weighed off and transferred to a 1 liter volumetric flask. A volume of 900 ml of deionized water was added and the mixture was stirred thoroughly. The pH was adjusted to 7.4 with NaOH and water was added to make up to a final volume of 1 l.

3.3.2 Preparation of drug test solutions

A quantity of 80 mg of each drug (i.e. abacavir, lamivudine, dapsone, and furosemide) was dissolved in 1 l of the various transport media on a magnetic stirrer at 37°C. Each solution was freshly prepared on the day of the transport study. The solutions were placed in an ultrasonic bath and sonicated for 2 min. The test solutions were then transferred and kept in a heated water bath at 37°C and each solution was sonicated again for 1 min directly before use in the transport study.

3.3.3 Tissue preparation

Pig intestinal tissue was collected from the local abattoir in Potchefstroom directly after the removal of the intestine from the slaughtered animal. A piece of the proximal jejunum of approximately 40 cm in length was excised from the gastro-intestinal tract of the pig (± 30 cm from the pyloric sphincter). The excised tissue was then rinsed with ice-cold KRB, placed in KRB and transported on ice to the laboratory.

In the laboratory the segment of excised jejunum was pulled over a glass tube and the serosa was removed by blunt dissection as illustrated in Figure 3.1.

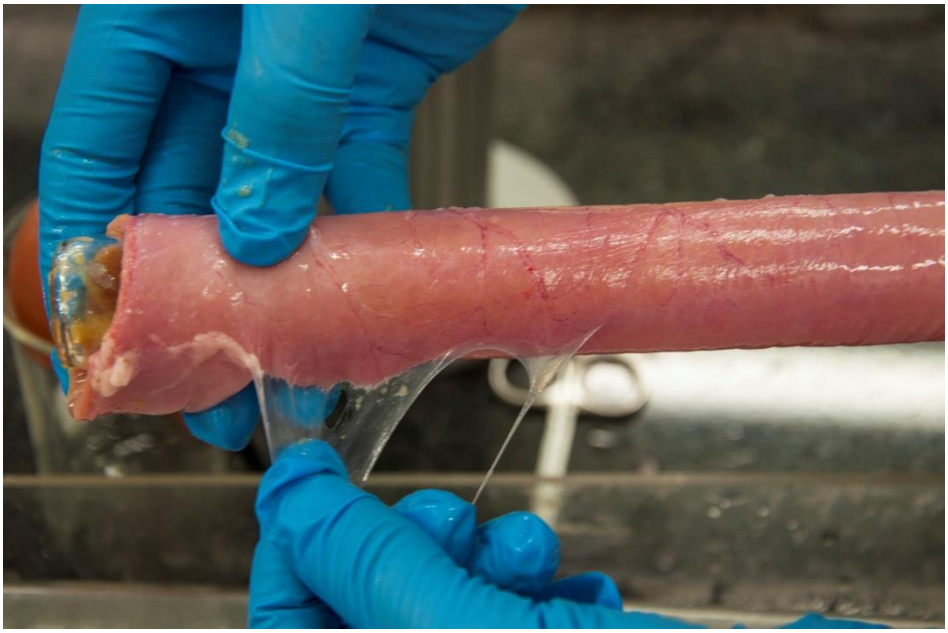


Figure 3.1: Photograph illustrating a segment of excised pig jejunum on a glass tube with removal of the serosa

Once the serosa was removed, the intestinal tissue was cut along the mesenteric border and rinsed off the glass tube onto filter paper. The tissue was spread open as a flat piece of tissue without any visible folds. Smaller pieces, ± 2 cm in width, were then cut with care taken to ensure that segments containing Peyer's patches (illustrated in Figure 3.2) were not used (Daugherty & Mrsny, 1999). Care was also taken to ensure that the tissue remained moist throughout the procedure by wetting it with ice-cold KRB.

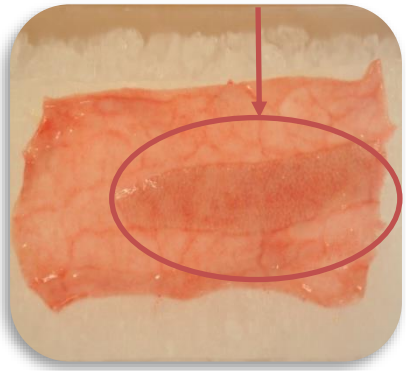
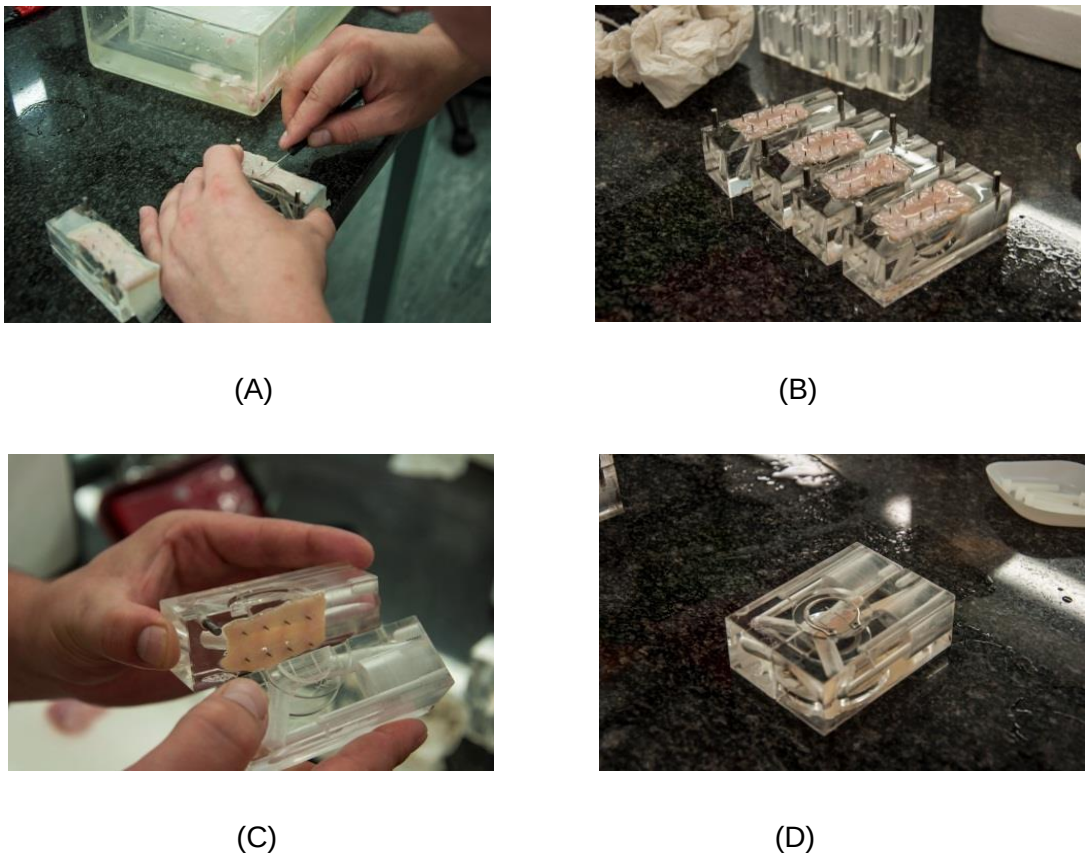


Figure 3.2: Photograph illustrating a piece of excised pig intestine with a Peyer's patch visible (shown by the arrow and oval)

The excised tissue pieces were mounted onto the pins of the diffusion apparatus so that the apical side was facing downward (Figure 3.3 A and B). The two half-cells were then joined tightly to form a diffusion cell/chamber (Figure 3.3 C and D), with the two half-cells kept in

place by a metal circlip. The diffusion cells were placed on the pre-heated manifold (Figure 3.4). The half-cells were filled with 7 ml of pre-heated (37°C) KRB in each half-cell. The carbogen gas supply line was connected to each half-cell to circulate bubbles through the medium inside the cells causing a stirring effect. Electrodes were inserted to measure the transepithelial electrical resistance (TEER) across the half-cells to give an indication of the integrity of the intestinal tissue over the duration of the study. A 20-min window for environmental equilibration was allowed before commencing with the permeation experimental procedures. The total time from collection of the tissue until equilibration commenced did not exceed 60 min.



(A) (B)

(C) (D)

Figure 3.3: Photographs illustrating: (A) Mounting a tissue segment onto the metal pins of the half-cell, (B) mounted tissue segments with filter paper removed, (C) joining the half-cells to complete the diffusion chamber and (D) assembled diffusion chamber ready for insertion into heating block clamp

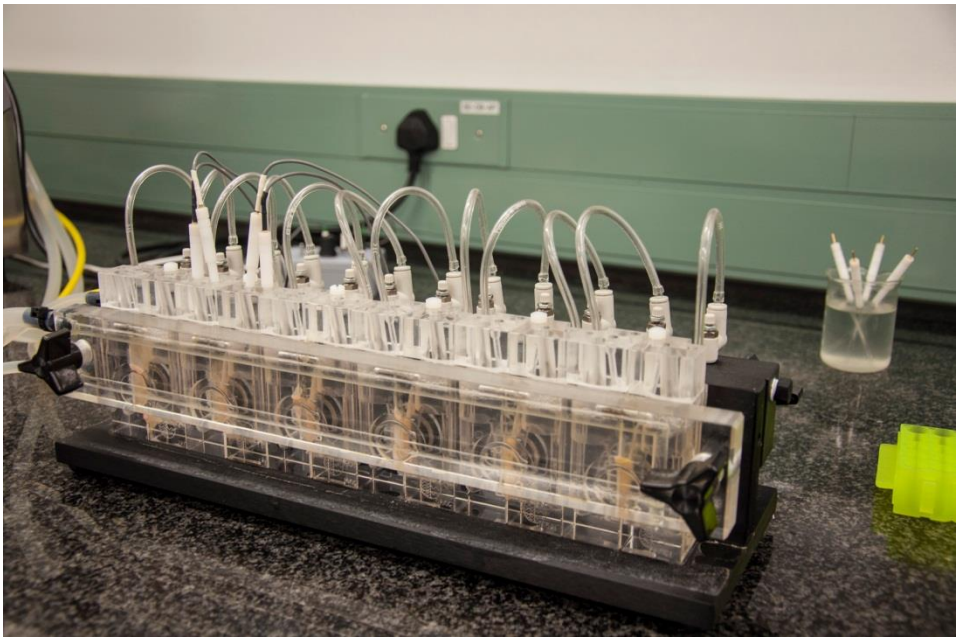


Figure 3.4: Photograph illustrating the assembled Sweetana-Grass diffusion apparatus with excised pig intestinal tissues mounted between half-cells, which are clamped in the heating block with gas lines attached

3.3.4 *In vitro* permeation studies

The KRB was removed from the donor chamber of each cell and replaced with pre-heated drug-containing medium. The TEER was measured at the start and end of the equilibration period and also at 20 min intervals during the transport study. Transport samples (200 µl) were withdrawn at 20 min intervals over a total period of 120 min. These samples were analysed with a validated high performance liquid chromatography (HPLC) analytical method.

3.4 Solubility studies

3.4.1 Sample preparation

A supersaturated solution of each drug was prepared in each of the selected media (i.e. KRB, Phos, FeSSIF and FaSSIF). This was accomplished by adding 2 g of each drug to 10 ml of each medium and mixing it in a 50 ml Corning Centristar® flask. These flasks were then sealed by using Parafilm® and placed into a pre-heated rotating dissolution bath for 24 hours. Samples (1 ml) were taken from the flasks by filtering through 0.45 µm membranes and diluted 1000x with purified water for the highly soluble drugs (abacavir and lamivudine); and 12.5x for furosemide and 5x for dapsone. Six samples of each solution were prepared and analysed with the same HPLC method used for analysing the transport study samples.

3.5 HPLC method validation

3.5.1 High performance liquid chromatography analysis method

The HPLC instrument and chromatographic details that were used for the sample analysis are summarised in Table 3.2.

Table 3.2: Analytical instrument and chromatographic conditions

Analytical Conditions	**Description**
Analytical instrument	Agilent® 1100 series HPLC equipped with a gradient pump, UV detector and Chemstation Rev. A.10.02 data acquisition and analysis software
Column	Venusil XBP C18, 150 x 4.6 mm, 5 µm
Mobile phase	Acetonitrile / 0.2% Tri-Ethylamine adjusted to pH 7, 10:90 initial ratio, gradient to 55/45 after 5 min, hold to 8 min, re-equilibrate
Flow rate	1 ml/min
Sample injection volume	20 µl
Detection wavelength	280 nm
Retention times	lamivudine: 4.47 min abacavir: 6.17 min furosemide: 6.45 min dapsone: 7.41 min
Stop time	12 min
Solvents	KRB Phos FeSSIF FaSSIF

3.5.2 Specificity

Specificity is the ability to identify and assess the compound of interest, or analyte, in the presence of other compounds that may be found in the sample (Shabir, 2004; UNODC, 2009). All possible components that could be present should form separate peaks when using the selected HPLC method. All of the transport media (KRB, Phos, FeSSIF and FaSSIF) were analysed by diluting 20 ml of transport medium to 100 ml by using a 70 %

purified water/ethanol mixture. A volume 1 ml of the diluted transport medium was transferred to a HPLC vial and injected into the HPLC column for analysis.

A mixture of the four drugs were also prepared (10 mM of each drug was used) in a 70 % water/ethanol solution and analysed with the same method. This tested the ability of the HPLC method to distinguish between the solutes and the analytes, as well as testing the method's retention time for the different drugs.

3.5.3 Linearity, limit of detection (LOD) and limit of quantification (LOQ)

The ability of an analytical method to determine the concentration of an analyte in a sample across a specific concentration range by means of a mathematical formula is known as the linearity (USP, 2014). The peak of known concentrations of the test drugs (abacavir, lamuvidune, dapsone, and furosemide) was plotted and linear regression was performed on the resulting curve. For a curve to be considered linear, a regression coefficient (R-squared) value of 0.99 or better is required (USP, 2014).

The LOD is defined as the lowest known concentration of analyte which is registered on the analytical instrument which is typically three times greater than the baseline noise, but not necessarily quantifiable in terms of the exact concentration. The LOQ is defined as the lowest concentration of analyte, which is registered on the analytical instrument that can be accurately quantified. As a general rule the LOQ is typically 10 times greater than the baseline noise (USP, 2014; Lavanya *et al.,* 2014; Shabir, 2004). The LOD and LOQ are determined by using the following equations:

$$LOD = \frac{3.3\sigma}{S} \qquad \textit{Eq. 3.1}$$

$$LOQ = \frac{10\sigma}{S} \qquad \textit{Eq. 3.2}$$

Where: σ is the standard deviation of the response and S is the slope of the calibration curve of the analyte.

For the determination of the linearity, LOD and LOQ a stock solution was prepared containing a concentration of 10 mg/100 ml of each of the selected drugs in each of the test media. The stock solution was then diluted to 1 mg/100 ml, 100 µg/100 ml and 10 µg/100 ml. Each of these diluted solutions was injected into the HPLC in different volumes, namely 5, 10, 20, 30, 40 and 50 µl respectively. The linear regression coefficient for each test compound was better than 0.99 and the LOQ value of each was lower than the lowest value obtained during the transport studies.

3.5.4 Precision

The precision of an analytical method can be described as the concurrence between a series of measurements following multiple sampling of the same sample solution (ICH, 1996). This can be divided into intra- and inter-day precision.

Intra-day precision describes the precision of an analytical method over a short time interval (less than 24 hours) (ICH, 1996). An analytical method is described as precise if the inter-day variation renders a percentage relative standard deviation (%RSD) value of less than 5%. This was achieved by analysing the same solution in six different injections on the same day (Shabir, 2004).

Intra-day precision measures the repeatability of the method by analysing the same samples on successive days. The %RSD for this should be less than 10% (Shabir, 2004).

3.5.5 Accuracy

The accuracy of an analytical method is determined by measuring how close the experimental and true values are to one another. Two stock solutions with concentrations of 1 µg/ml and of 10 µg/ml were prepared for each of the test compounds in a water/methanol solution and analysed by injecting in duplicate different volumes: 50 µl of the 1 µg/ml solution and 10, 20, 30, 40 and 50 µl of the 10 µg/ml solution onto the HPLC column. This mimics different stock solution concentrations. The theoretical concentration of each drug was mathematically determined and expressed as a percentage of the known concentration. A HPLC analytical method is considered to be accurate when the recovery is 100 ± 2% (ICH, 1996).

3.6 Data processing and statistical analysis

3.6.1 Percentage transport

The percentage transport of each model drug in each of the selected media was calculated at 20 min intervals (Equation 3.3). These percentage transport values were then plotted as a function of time.

$$\% \text{ Transport} = \frac{\text{Peak area of sample at specific time interval}}{\text{Peak area of initial solution}} \times 100 \qquad \textit{Eq. 3.3}$$

3.6.2 Apparent permeability coefficient (P_{app})

The P_{app} is defined as the initial flux of a compound through a membrane, normalised by membrane surface area and donor concentration (Equation 3.4).

$$P_{app} = \frac{dQ}{dt} x \frac{1}{A.C_0.60} \qquad \textit{Eq. 3.4}$$

With dQ/dt (g/s) the increase in the amount of the selected drug in the acceptor chamber over time, A (cm^2) the effective surface area of the excised tissue and C_0 the initial drug concentration in the donor chamber. It is necessary to convert the time in minutes to time in seconds, since P_{app} is expressed in $cm.s^{-1}$ (Yee, 1997; Grès *et al.,* 1998).

3.6.3 Efflux ratio (ER)

The ER is determined by comparing the P_{app} values in both directions to determine the extent of the efflux of the selected drug in the different media. The ER was calculated with the aid of equation 3.5.

$$ER = \frac{P_{app}\ (BL\text{-}AP)}{P_{app}\ (AP\text{-}BL)} \qquad \textit{Eq. 3.5}$$

The transport from the apical to basolateral direction is represented by P_{app} (AP-BL) and from the basolateral to apical direction by P_{app} (BL-AP) (Tarirai *et al.,* 2012).

3.6.4 Statistical analysis of data

Data obtained from the transport studies were statistically analysed by using analysis of variance techniques (ANOVA). Tukey's Honest Statistical Difference (HSD) post-hoc test was used to analyse non-parametric data. The analysis was performed by using Statistica (Statsoft®) software. Differences was deemed statistically significant if $p \leq 0.05$.

CHAPTER 4: Results and discussion

4.1 Introduction

Results obtained from the validation of the HPLC method are reported to serve as proof that the analytical method, which was used to analyse drug concentrations in the test samples generated from this study, produced reliable, repeatable and accurate results. Bi-directional transport of selected drugs was tested using a Sweetana-Grass diffusion apparatus. The transport data for each of the four selected drugs (i.e. abacavir, lamivudine, dapsone and furosemide) in each of the four selected media (i.e. KRB, Phos, FeSSIF and FaSSIF) are reported. P_{app} and ER values were calculated from the data, which were generated from the transport studies and statistical analysis was performed on the calculated values. Statistical analysis of the bi-directional transport data was performed to determine if statistically significant differences in the drug transport results were mediated by the different transport media. Differences in transport values were considered to be statistically significant if $p \leq 0.05$. Solubility testing was also performed on each of the selected drugs in the selected transport media.

4.2 Validation of HPLC method

The following results were obtained during the validation procedure of the HPLC analytical method, which was used to analyse the concentrations of the selected drugs in the test samples.

4.2.1 Specificity

An HPLC chromatogram of all four the selected drugs are given in Figure 4.1. From Figure 4.1 it is clear that separation of the selected drugs was obtained by the HPLC method uitlised in this study, which confirmed that no interference would occur during analysis of the mixed sample. The order of elution was as follows: lamivudine at 4.573 min, abacavir at 6.166 min, furosemide at 6.452 and dapsone at 7.408 min. Furthermore, relatively narrow and symmetrical peaks with minimal tailing were obtained for all four selected drugs.

Chromatograms of the selected transport media are given in Figure 4.2 to determine if any ingredients of the media have overlapping peaks with any of the selected drugs. The results clearly indicate that the different media used during the transport studies did not exhibit any peaks that could interfere with the peaks of the drugs (Figure 4.2).

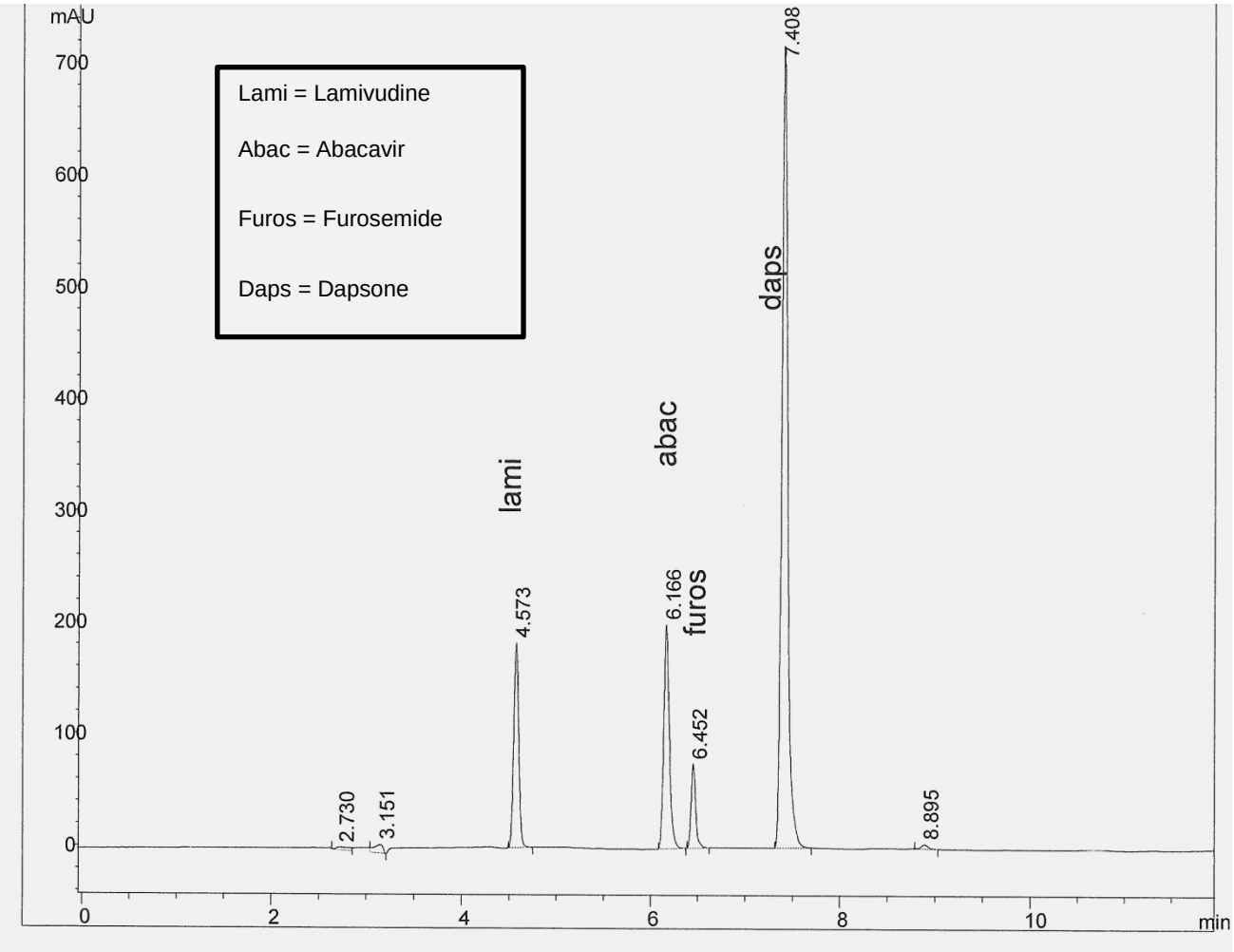
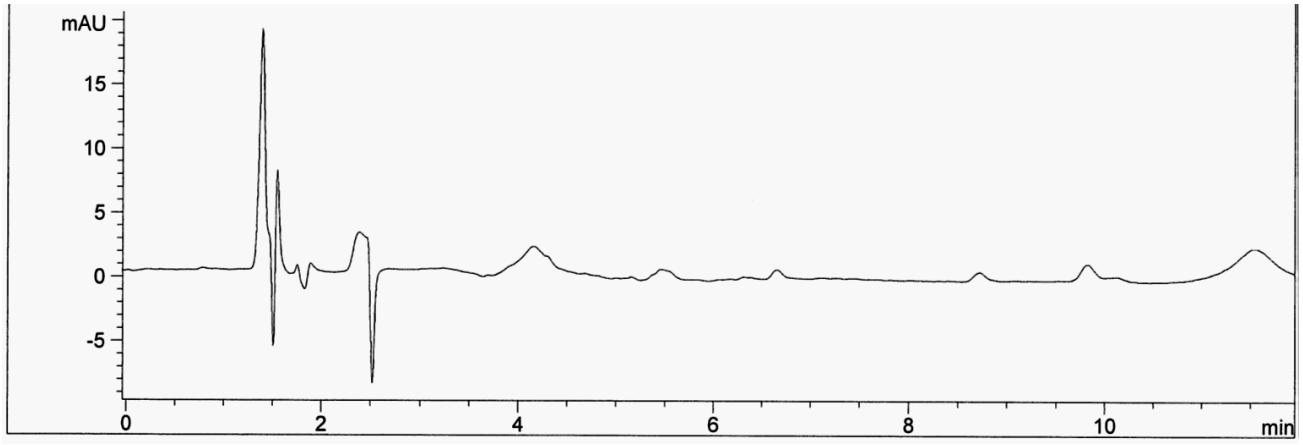
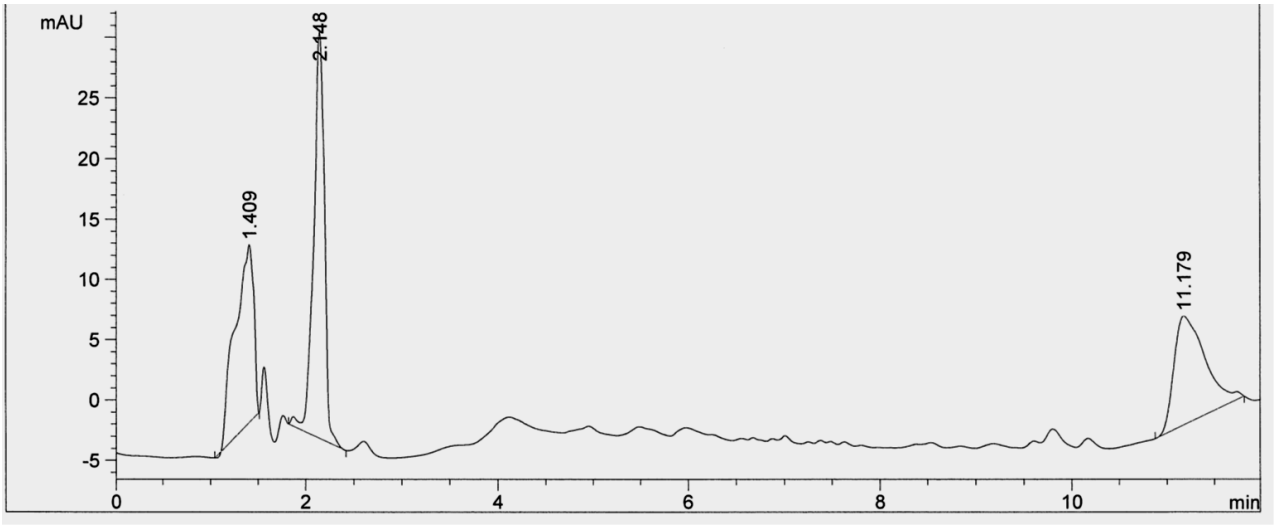


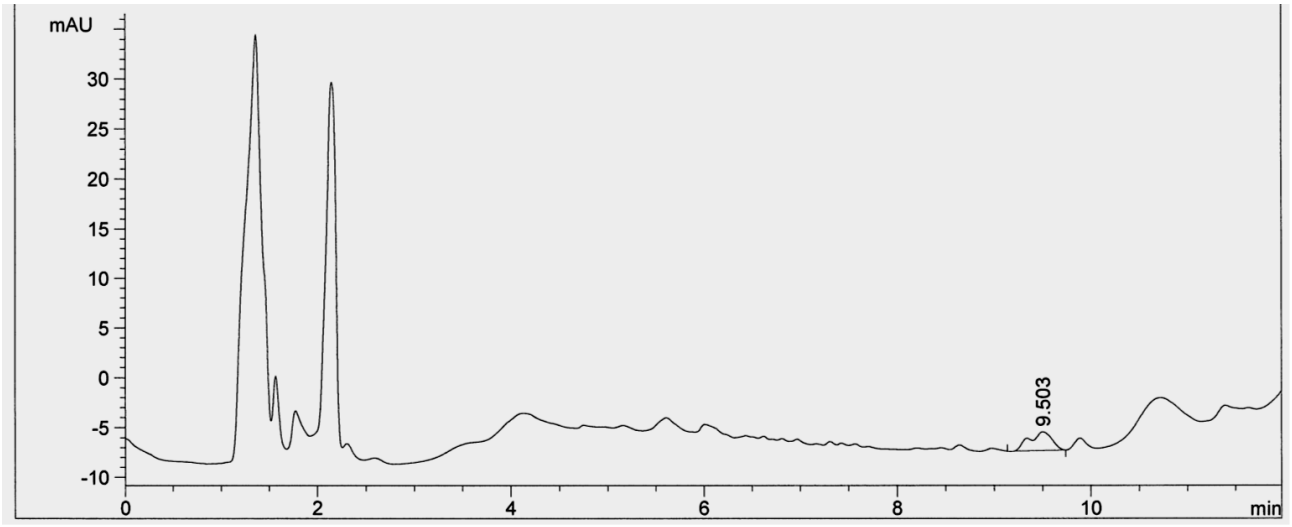
Figure 4.1: Chromatogram of selected drugs illustrating peak separation and retention times


mAU
15
10
5
0
-5
0
2
4
6
8
10
min

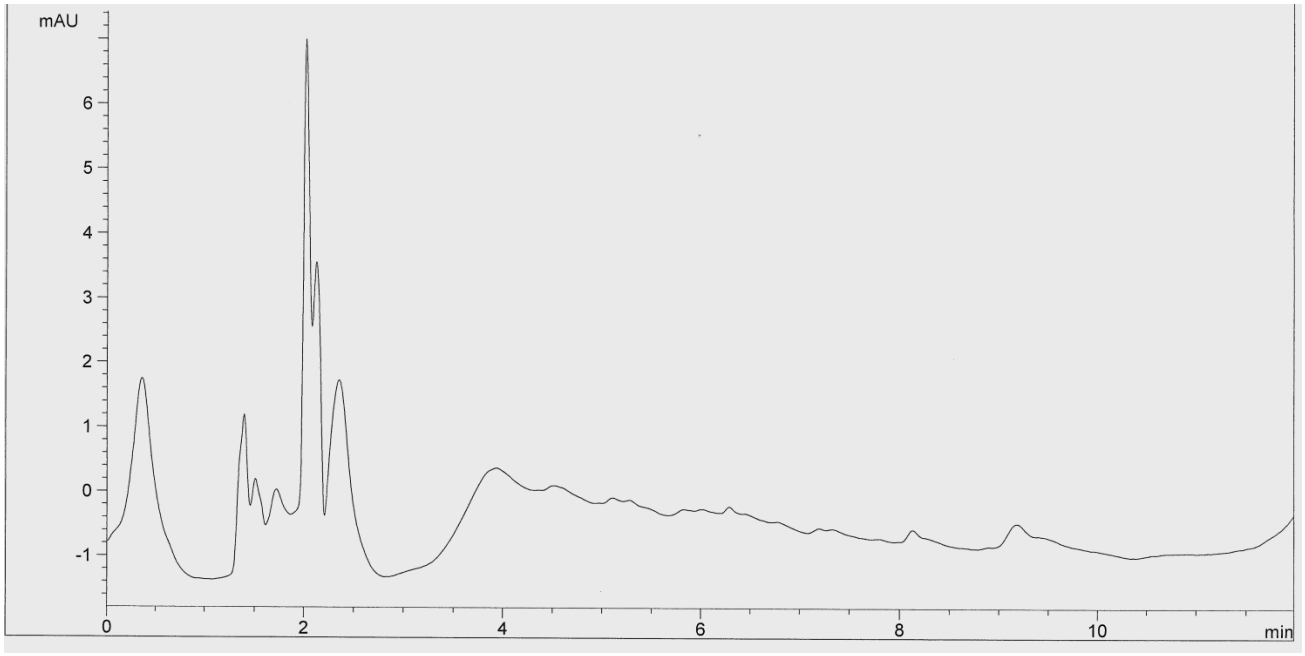
(A)


mAU
2.148
1.409
11.179
25
20
15
10
5
0
-5
0
2
4
6
8
10
min

(B)


mAU
9.503
30
25
20
15
10
5
0
-5
-10
0
2
4
6
8
10
min

(C)



(D)

Figure 4.2: HPLC chromatograms of A) Krebs Ringer bicarbonate buffer (KRB), B) Phosphate buffer (Phos), C) Fed state simulated intestinal fluid (FeSSIF) and D) Fasting state simulated intestinal fluid (FaSSIF)

4.2.2 Linearity

The peak areas obtained for a series of standard solutions of each of the selected drugs are shown in Tables 4.1 to 4.4. The raw data are presented as graphs and tables in **Addendum C**. The standard curves where peak area was plotted as a function of concentration for a series of standard solutions are shown in Figures 4.3 to 4.7. The linear regression values as well as mathematical equations describing these curves are included in the graphs.

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

Solution concentration µg/ml	Injection volume (µl)	Final concentration (µg/ml)	Peak area inj 1	Peak area inj 2	Average peak area
1.073	5	0.107	12.1	12.2	12.2
	10	0.215	24.0	23.9	24.0
	20	0.429	47.9	47.4	47.7
	30	0.644	71.4	71.7	71.5
	40	0.858	95.2	96.2	95.7
	50	1.073	119.9	119.7	119.8
10.73	10	2.146	242.0	241.7	241.8
	20	4.292	483.7	482.4	483.0
	30	6.438	723.1	723	723.3
	40	8.584	966	966	966.0
	50	10.730	1026	1207	1116.9

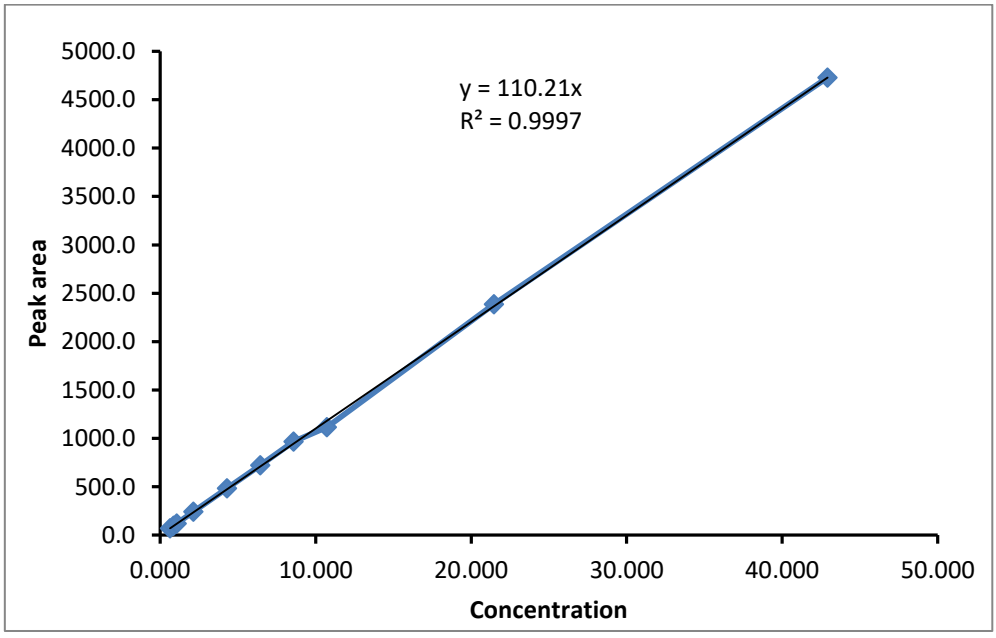


Figure 4.3: Standard curve (linear regression graph) where peak area was plotted as a function of concentration for a series of abacavir solutions

Table 4.2: Peak areas obtained from a series of lamivudine standard solutions

Solution concentration µg/ml	Injection volume (µl)	Final concentration (µg/ml)	Peak area inj 1	Peak area inj 2	Average peak area
1.122	5	0.112	10.8	10.7	10.7
	10	0.224	21.8	21.3	21.5
	20	0.449	43.4	45.7	44.5
	30	0.673	67.5	68.1	67.8
	40	0.898	90.3	89.9	90.1
	50	1.122	112.0	108.5	110.3
11.22	10	2.244	214.5	213.9	214.2
	20	4.488	429.0	431.6	430.3
	30	6.732	645.5	651	648.1
	40	8.976	875	868	871.5
	50	11.220	1082	1082	1082.3

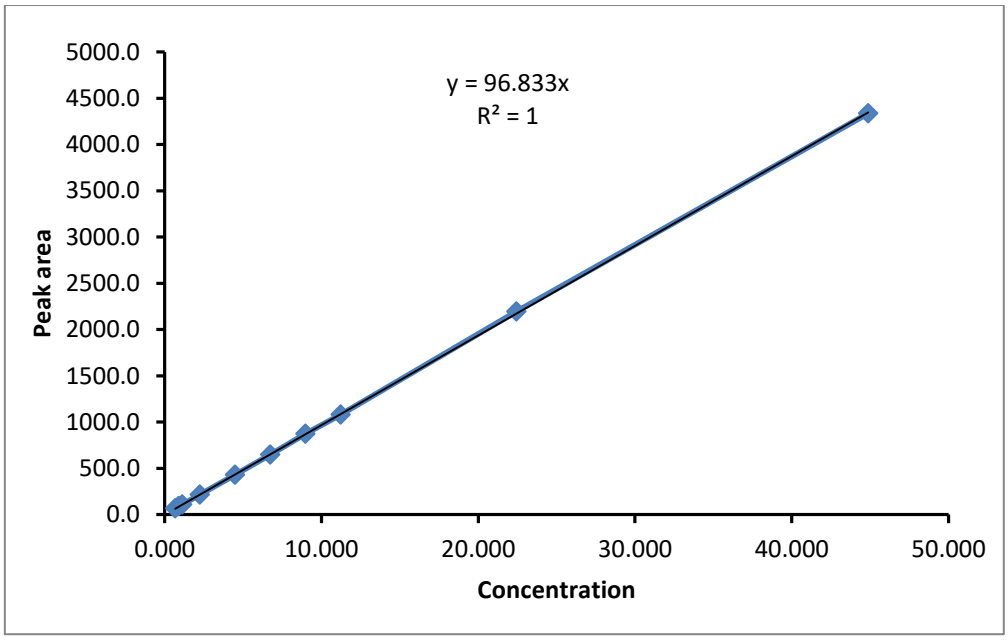


Figure 4.4: Standard curve (linear regression graph) where peak area was plotted as a function of concentration for a series of lamivudine solutions

Table 4.3: Peak areas obtained from a series of dapsone standard solutions

Solution concentration µg/ml	Injection volume (µl)	Final concentration (µg/ml)	Peak area inj 1	Peak area inj 2	Average peak area
0.994	5	0.099	38.4	22.7	30.5
	10	0.199	45.7	46.0	45.8
	20	0.398	91.3	91.1	91.2
	30	0.596	137.8	138.0	137.9
	40	0.795	184.6	185.2	184.9
	50	0.994	231.9	230.8	231.4
9.94	10	1.988	455.1	455.3	455.2
	20	3.976	914.3	915.4	914.8
	30	5.964	1374.4	1374	1374.0
	40	7.952	1833	1839	1835.7
	50	9.940	2292	2293	2292.6

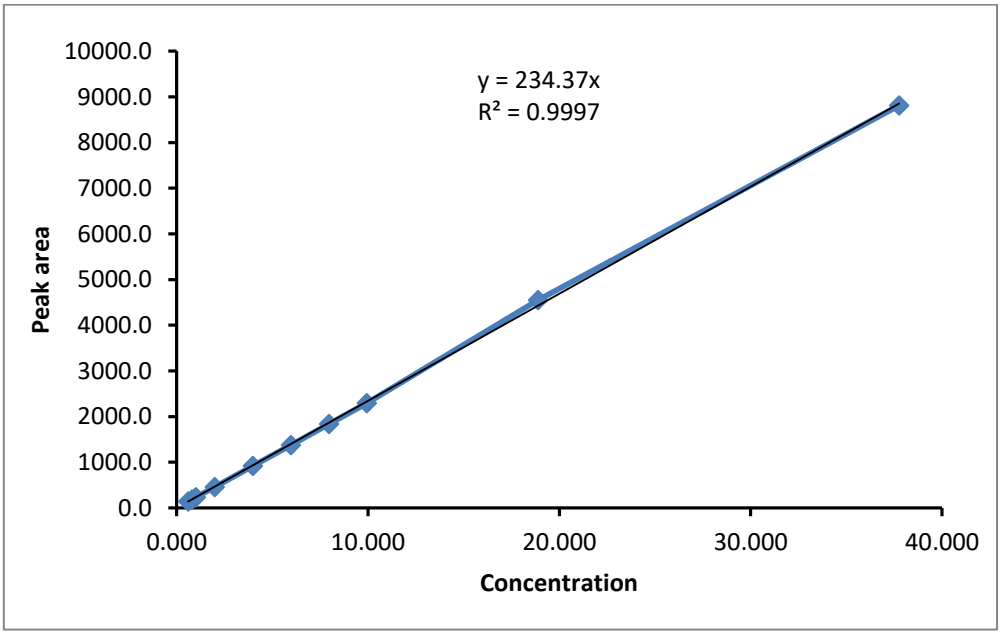


Figure 4.5: Standard curve (linear regression graph) where peak area was plotted as a function of concentration for a series of dapsone solutions

Table 4.4: Peak areas obtained from a series of furosemide standard solutions

Solution concentration µg/ml	Injection volume (µl)	Final concentration (µg/ml)	Peak area inj 1	Peak area inj 2	Average peak area
0.993	5	0.099	16.4	18.1	17.2
	10	0.199	34.5	36.1	35.3
	20	0.397	67.4	67.1	67.2
	30	0.596	100.0	100.4	100.2
	40	0.794	133.0	132.4	132.7
	50	0.993	165.3	160.2	162.8
9.93	10	1.986	313.1	313.7	313.4
	20	3.972	627.2	626.6	626.9
	30	5.958	939.3	941	940.2
	40	7.944	1253	1257	1254.9
	50	9.930	1567	1570	1568.8

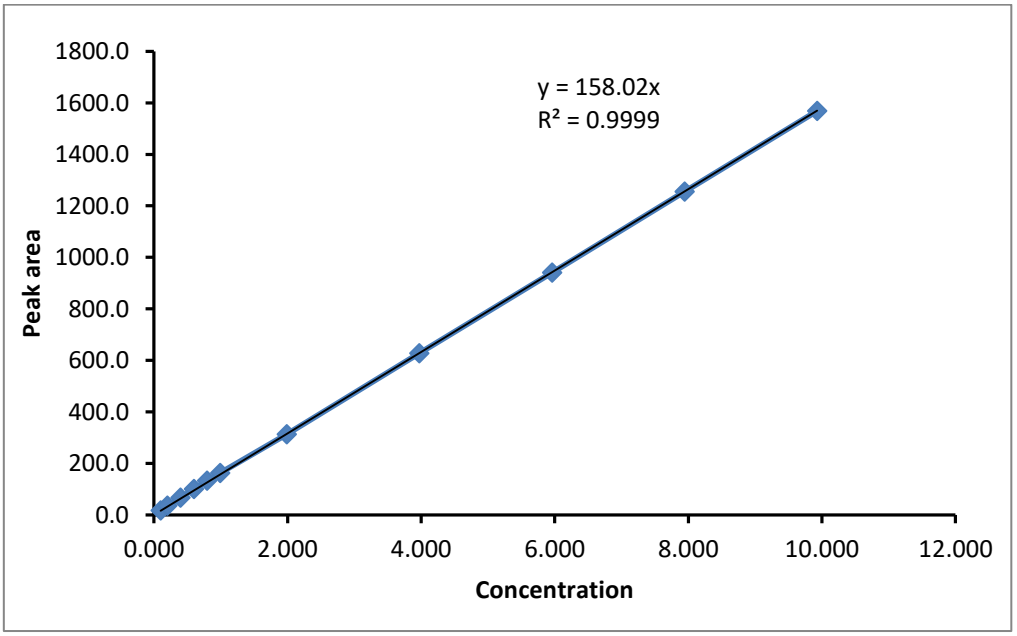


Figure 4.6: Standard curve (linear regression graph) where peak area was plotted as a function of concentration for a series of furosemide solutions

The linear regression coefficient (R^2) values obtained for all four selected drugs comply with the requirement for linearity of an R^2 value higher than 0.999 (Singh, 2013.

4.2.3 Accuracy

For an analytical method to be considered accurate, the mean percentage recovery is required to be between 98% and 102% (ICH, 2005). The percentage recovery values obtained by the HPLC analytical method for the selected drugs are shown in Table 4.5.

Table 4.5: Percentage recovery of the selected model drugs by HPLC analysis

Spiked Values (µg/ml)	Abacavir (% recovery)	Lamivudine (% recovery)	Dapsone (% recovery)	Furosemide (% recovery)
2	97.91	101.43	100.71	100.14
4	98.05	100.99	100.23	100.12
6	98.22	100.58	100.10	100.13
Average	98.06	101.00	100.35	100.13
SD	0.16	0.43	0.32	0.01
% RSD	0.16	0.42	0.32	0.01

All the average percentage recovery values were within the recommended limits of recovery and the method could therefore be considered accurate.

4.2.4 Limit of detection and limit of quantification

Equations 3.1 and 3.2 were used respectively for the calculation of the limit of detection

Table 4.6: Limit of detection and limit of quantification of the selected model drugs

Model drug	LOD (µg/ml)	LOQ (µg/ml)
Abacavir	0.002	0.007
Lamivudine	0.003	0.009
Dapsone	0.002	0.007
Furosemide	0.004	0.013

The initial drug concentration was 800 µg/ml, significantly higher than the LOQ for each drug.

4.2.5 Repeatability

According to the USP, a percentage relative standard deviation (%RSD) of <2% is required for a method to be considered repeatable.

From the data presented in Tables 4.6-4.10, it is clear that the HPLC analysis method delivered repeatable results in all media tested during a 24 h test period, which confirmed that the analytical method delivered acceptable intra-day repeatability. When comparing the recovery values obtained for each of the selected drugs in different media, the following %RSD values were obtained: 0.082% for abacavir, 0.12% for lamivudine, 0.63% for dapsone and 0.42% for furosemide. The HPLC analytical method delivered repeatable results in all media when six samples at a concentration of 32 µg/ml were injected into the HPLC column. The fact that each model drug delivered comparable results in the different media and on different days proves that the analytical method was capable of delivering inter-day repeatable results. Complete raw data are available as graphs and tables in **Addendum C**.

Table 4.7: Percentage relative standard deviation (%RSD) for repeated (n = 6) analysis of abacavir in the four selected media

	Peak area KRB	**Peak area Phos**	**Peak area FeSSIF**	**Peak area FaSSIF**
Average	3724.57	3084.00	3669.76	3676.76
SD	33.88	28.46	30.49	25.94
% RSD	0.91	0.92	0.83	0.71

Table 4.8: Percentage relative standard deviation (%RSD) for repeated (n = 6) analysis of lamivudine in the four selected media

	Peak area KRB	Peak area Phos	Peak area FeSSIF	Peak area FaSSIF
Average	3061.73	3030.97	3170.09	3166.88
SD	4.67	8.50	14.55	12.36
% RSD	0.15	0.28	0.46	0.39

Table 4.9: Percentage relative standard deviation (%RSD) for repeated (n = 6) analysis of dapsone in the four selected media

	Peak area KRB	Peak area Phos	Peak area FeSSIF	Peak area FaSSIF
Average	7589.03	7304.04	7153.93	7261.86
SD	134.46	12.47	46.81	33.19
% RSD	1.77	0.17	0.65	0.46

Table 4.10: Percentage relative standard deviation (%RSD) for repeated (n = 6) analysis of furosemide in the four selected media

	Peak area KRB	Peak area Phos	Peak area FeSSIF	Peak area FaSSIF
Average	5881.62	5393.09	5319.03	5415.85
SD	62.43	59.31	30.56	5.32
% RSD	1.06	1.10	0.57	0.10

4.2.6 Conclusion

The HPLC analytical method employed was sufficiently sensitive, repeatable and selective to ensure the accurate measurement of *in vitro* transport samples. The validation requirements were met, as described in the sections above.

4.3 Transport studies

The data generated from the *ex vivo* transport studies were used to calculate the P_{app} and ER values for each of the selected drugs in each of the transport media. The data discussion and comparison were done in terms of solubility, rather than the chronological order in the BCS. The data used to calculate the values are available as graphical representations in **Addendum C**.

4.3.1 Abacavir (BCS Class 1 drug)

The P_{app} values as well as the ER values of abacavir in the selected transport media are shown in Figure 4.7.

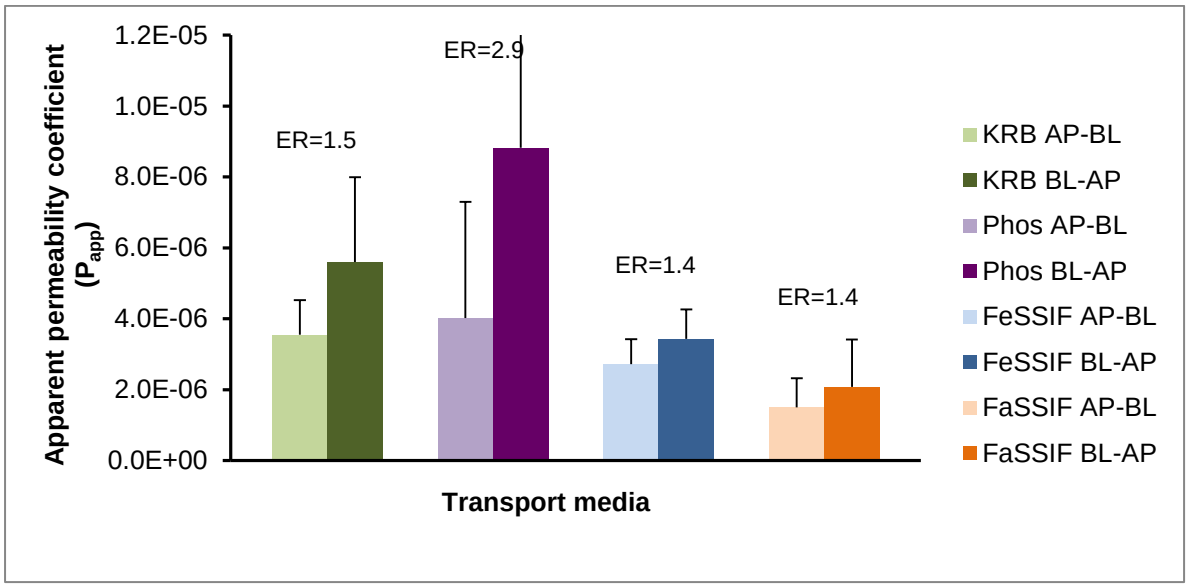


Figure 4.7: The P_{app} and ER values of abacavir in the respective transport media. AP-BL = apical to basolateral direction, BL-AP = basolateral to apical direction

Comparison of the P_{app} values of abacavir in the respective media, it is apparent that transport in the AP-BL direction in the KRB and Phos is similar, with greater transport in the BL-AP direction in Phos than obtained in KRB. This resulted in a relatively high ER for abacavir in Phos when compared to all the other media, although only statistically significantly higher than FaSSIF (Table 4.11). ER values of > 1 were obtained in all the media confirming active efflux of abacavir occurred, which was expected because it is a known substrate for P-glycoprotein. It is also noticeable that the overall transport is lower in the SIF's than in the buffers, but only BL-AP transport in FaSSIF was statistically significantly lower to that in Phos (Table 4.11). This may be due to the presence of bile components in

the SIF's that reduced free drug concentration by the formation of micelles and thereby reduced the amount of drug available for transport (Bergnic, Trontelj & Kristl, 2012).

From the transport results obtained for abacavir it seems that the transport medium composition had a relatively low impact because very few statistically significant changes were obtained in the bi-directional transport between the different media. This indicates that the permeation of BCS class 1 drugs (high solubility and high permeability) may be affected to a relatively low extent by transport medium composition during *ex vivo* transport studies.

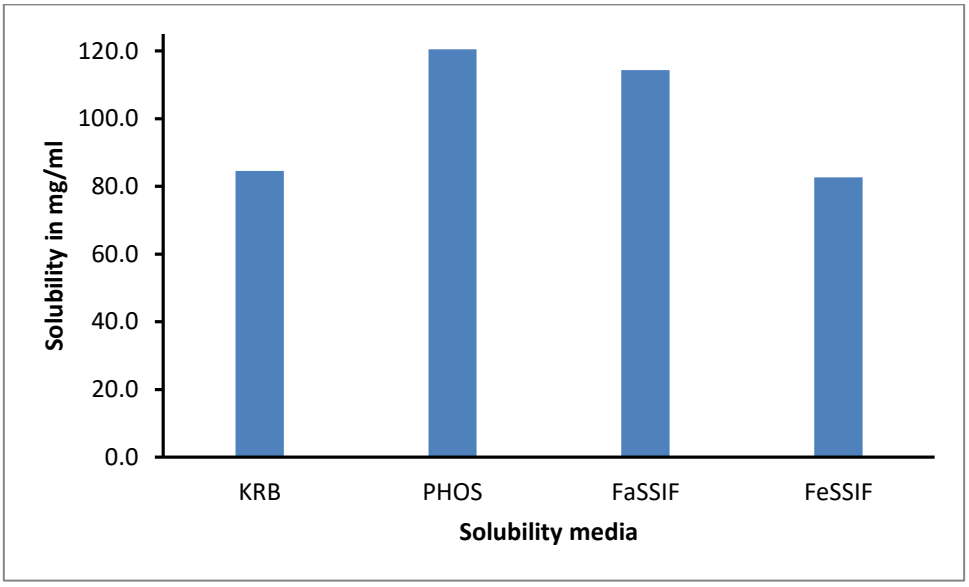


Figure 4.8: The solubility values of abacavir in the selected media

Solubility is an important consideration in the prediction of drug pharmacokinetics. This is especially true when using poorly soluble drugs, where solubility is the rate limiting step in the absorption process of the drug. The influence of transport medium composition on the solubility of drugs is an extremely important factor to consider. This is often overlooked in *in vitro* transport studies as the traditional buffers are optimised to ensure membrane integrity, not biorelevancy (Kleberg, Jacobsen & Müllertz, 2010). It is therefore important to consider the possible altering effects that transport medium composition may have on drug solubility during the preparation of experimental solutions intended for use in transport studies.

Abacavir exhibited better solubility in Phos than in any other of the selected media (figure 4.8). Due to the presence of bile components in the SIF's, a lower solubility was expected in these media than in the buffers. FaSSIF exhibited the second highest solubility, with a possible explanation being the relatively low concentration of bile components in this medium. As expected, abacavir exhibited the lowest solubility in FeSSIF, probably due to the high concentration of bile components in this medium. Abacavir exhibited a relatively

low solubility in KRB, which should be further investigated in order to understand the mechanism behind this phenomenon.

No apparent correlation was therefore found between the permeability and solubility of this compound in the respective media. This may be due to the fact that maximum solubility of abacavir at 37ºC was much larger than the concentration used to conduct the permeation studies (i.e. 800 µg/ml).

Table 4.11: The p-values generated by statistical analysis of abacavir bi-directional transport and efflux ratio values in the respective media

Transport direction	Medium	KRB	Phos	FeSSIF	FaSSIF
AP-BL	**KRB**		1.00	1.00	0.61
AP-BL	**Phos**	1.00		0.98	0.29
AP-BL	**FeSSIF**	1.00	0.98		0.99
AP-BL	**FaSSIF**	0.61	0.29	0.99	
BL-AP	**KRB**		0.72	0.98	0.60
BL-AP	**Phos**	0.72		0.07	0.01
BL-AP	**FeSSIF**	0.98	0.07		1.00
BL-AP	**FaSSIF**	0.60	0.01	1.00	
ER	**KRB**		1.00	1.00	1.00
ER	**Phos**	1.00		1.00	1.00
ER	**FeSSIF**	1.00	1.00		1.00
ER	**FaSSIF**	1.00	1.00	1.00	

The only statistically significant difference noted with abacavir as model drug was between Phos and FaSSIF in the BL-AP direction (Table 4.11). This may be explained by the very high concentration of bile components in the FaSSIF. FaSSIF also exhibited a pronounced difference in AP-BL transport when compared to the buffers. Micelle formation due to the presence of bile components may reduce the amount of free drug available for transport.

This reduction in the free drug fraction may account for the statistically significant difference (Müller *et al.,* 2013; Ingels *et al.,* 2004).

Statistically, the transport of abacavir in the selected buffers (i.e. Phos and KRB) differed very little from one another (p = 1.00). The same was true when comparing transport of abacavir in the selected SIFs (i.e. FeSSIF and FaSSIF). It is important to keep in mind that micelle formation may negatively influence the permeation of hydrophilic drugs such as abacavir, but the opposite effect is usually obtained for poorly soluble, lipophilic drugs.

The ER values for abacavir were similar in three of the selected media and the higher value in Phos may be explained by potential modulation of the active efflux transporter, P-gp, by a component in the Phos buffer. However, this needs to be investigated further to be conclusive with respect to what causes a higher efflux transport of abacavir in this transport medium.

4.3.2 Lamivudine (BCS Class 3 drug)

The P_{app} values as well as the ER values of lamivudine in the selected transport media are shown in Figure 4.9.

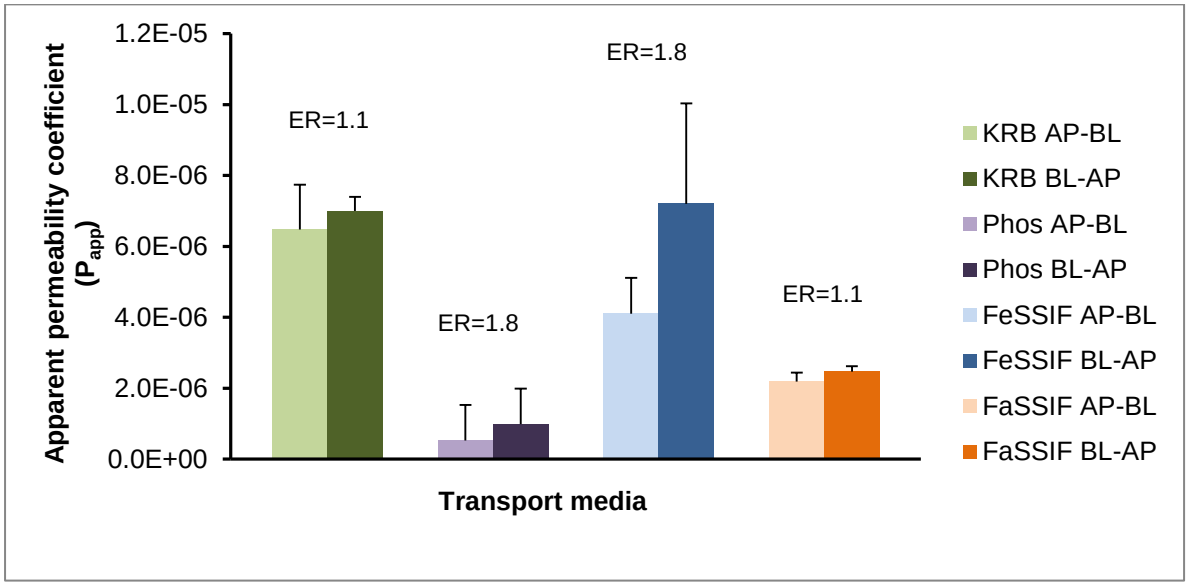


Figure 4.9: The P_{app} and ER values of abacavir in the respective transport media. AP-BL = apical to basolateral direction, BL-AP = basolateral to apical direction

Lamivudine displayed pronounced differences in terms of bi-directional transport in the various media, which were statistically significantly different in many instances (Table 4.12). When comparing the AP-BL transport in the different media, it is clear that lamivudine

exhibited the highest permeation in KRB and the lowest in Phos (figure 4.6). The ER value for lamivudine was similar in Phos and FeSSIF, while it was lower in KRB and FaSSIF.

According to the BDDCS, class 3 drugs are susceptible to absorptive transporter effects, but can also be modulated by efflux transporters. Any differences in the extent or rate of transport of lamivudine between the selected media can therefore most probably be ascribed to the effect of the medium on drug transporters (Benet, 2009), but the difference may also be explained to some extent in terms of paracellular transport that changed due to changes in the tight junctions.

Lamivudine is absorbed via one or more absorptive transporter pathways as well as partly by means of paracellular uptake via the tight junctions. It was confirmed by research done by Reis *et al.,* (2013) that lamivudine is partly transported by the paracellular pathway. Phos increased the TEER of the tissue over the duration of the study. Since high TEER values may be indicative of "tighter" intercellular junctions, lower paracellular transport was expected. TEER values over 120 minutes are given as graphical representations in **addendum C.**

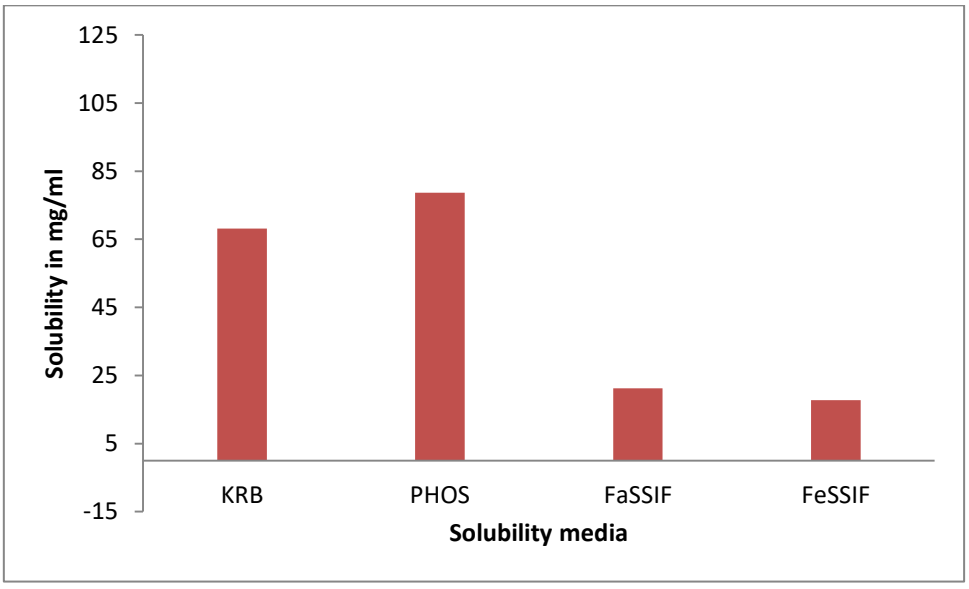


Figure 4.10: The solubility values of lamivudine in the selected media

Lamivudine showed greater solubility in the buffers (Phos and KRB) than in the SIF's with the highest solubility in Phos. When comparing the solubility of lamivudine in the SIF's, the highest solubility was obtained in FaSSIF. This is expected due to the hydrophilic nature of the drug and FeSSIF has a considerably higher concentration of bile components in its composition than FaSSIF.

Although the solubility was considerably lower in the SIF media, a direct correlation was not observed with the transport. This can possibly be explained by the fact that lamivudine is a highly soluble drug and the concentrations used in the transport study was far below the saturation solubility as well as the fact that it is mainly absorbed by means of active transporters. The medium composition therefore had an effect on lamivudine's transport possibly as a result of transporter modulation rather than the effect on its solubility.

Table 4.12: The p-values generated by statistical analysis lamivudine bi-directional transport and efflux in the respecive media

Transport medium	Medium	KRB	Phos	FeSSIF	FaSSIF
AP-BL	**KRB**		0.00	0.38	0.00
AP-BL	**Phos**	0.00		0.02	0.86
AP-BL	**FeSSIF**	0.38	0.02		0.71
AP-BL	**FaSSIF**	0.00	0.86	0.71	
BL-AP	**KRB**		0.02	1.00	0.21
BL-AP	**Phos**	0.02		0.02	1.00
BL-AP	**FeSSIF**	1.00	0.02		0.17
BL-AP	**FaSSIF**	0.21	1.00	0.17	
ER	**KRB**		1.00	1.00	1.00
ER	**Phos**	1.00		1.00	1.00
ER	**FeSSIF**	1.00	1.00		1.00
ER	**FaSSIF**	1.00	1.00	1.00	

Due to the statistically significant difference in the P_{app} values of lamivudine between Phos, KRB and FeSSIF, it may be concluded that one or more of the constituents of Phos had interfered with the transporter uptake of lamivudine (table 4.12). The transporter which may have influenced lamivudine uptake includes OAT1, OCT, BCRP and P-gp (Reis *et al.,* 2013). The OAT transporter functions by exchanging an intracellular dicarboxylate with an organic ion. *In vivo,* the dicarboxylate is returned to the intracellular space by a Na/dicarboxylate co-transporter (NaDC3). This transporter is dependent on a sodium ion concentration gradient

across the membrane and since no sodium ions were present in Phos, this transporter could not function properly (Sekine, Miyazaki & Endou, 2006).

The statistically significant differences which were observed in the P_{app} values, both AP-BL and BL-AP, between the aqueous buffers and SIF's may be explained further by the formation of micelles in the presence of bile salts and lecithin. Since only the free fraction of the drug is available for transport any reduction in the free drug concentration will have a negative influence on drug permeation. Micelle formation could possibly have mediated a reduction in the free fraction of the drug, which in turn caused a reduction in drug transport in the SIF's (Ingels *et al.,* 2004; Müller *et al.,* 2013).

4.3.3 Dapsone (BCS Class 2 drug)

The P_{app} values as well as the ER values of dapsone in the selected transport media are shown in Figure 4.11.

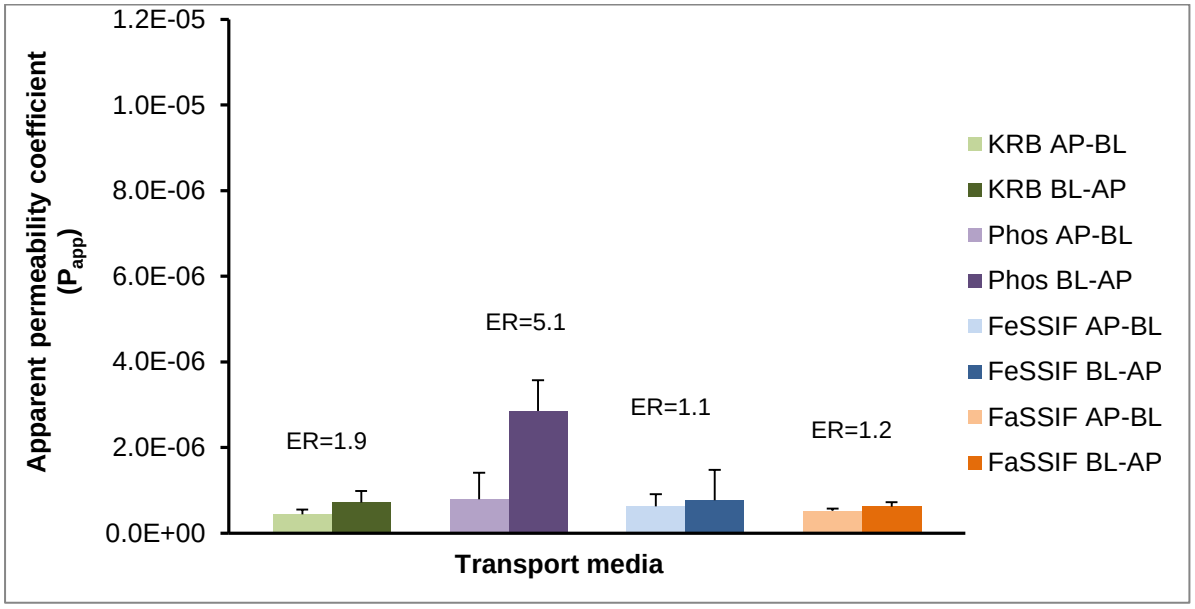


Figure 4.11: The P_{app} and ER values of dapsone in the respective transport media. AP-BL = apical to basolateral direction, BL-AP = basolateral to apical direction

Dapsone exhibited considerably lower overall transport than the BCS class 1 and 3 compounds (i.e. abacavir and lamivudine), even though it is a drug with good permeability. This relatively low transport can most probably be attributed to the low solubility of this drug. It remained in suspension in all the selected media at the concentration used in this study. It is important to keep in mind that transport of dapsone in the AP-BL direction occurred by means of passive diffusion. Only molecules in solution can get transported across the

intestinal tissue, which allows molecules to dissolve from the suspended particles. Dissolution is thus the rate limiting step in the absorption of dapsone (Amidon *et al.,* 1995).

It is apparent that similar P_{app} values were obtained in all the selected media in the AP-BL direction for dapsone, but a pronouncedly higher P_{app} value was obtained in the BL-AP direction in Phos. This may be due to an unknown stimulatory effect of the buffer on the efflux transporters. A similar effect on BL-AP transport was noted in the BCS class 1 drug in Phos as well. No satisfactory explanation could be found from the literature, thus further study on this phenomenon is warranted in order to reach a conclusive answer.

Dapsone, a BCS class 2 drug, was expected to be extremely sensitive to the effect of efflux transporters (Wu & Benet, 2005) and that is probably why a relatively high ER value was obtained in Phos (ER = 5.1). This is due to the extremely low solubility but good permeability of the model drug as discussed above. Any molecules in solution available for transport will move through the intestinal tissue from the donor to the acceptor side. If the molecule is a substrate for efflux transporters, any molecule that diffuses into the intestinal membrane will be promptly pumped back into the intestinal lumen. Due to the low solubility of the drug, the efflux transporters will most likely not get easily saturated (Custodio, Wu & Benet, 2007). The lower ER values in the SIF's when compared to the buffers may be explained by the inhibitory effect of sodium taurocholate on P-gp. This inhibitory effect was shown to be concentration dependent, therefore it can be concluded that competitive transporter inhibition occur (Deferme *et al.,* 2003, Ingels *et al.,* 2001).

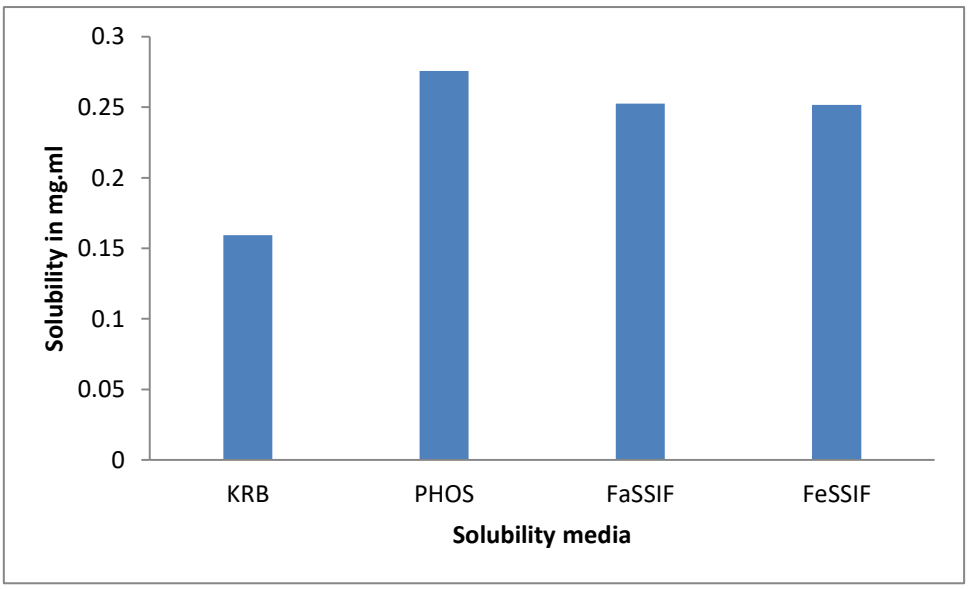


Figure 4.12: The solubility values of dapsone in the selected media

Dapsone exhibited relatively low solubility in all the selected media, which was far below the concentration used in the permeation study (figure 4.12). This is also the only selected drug where a correlation between solubility and transport could be observed. Dapsone showed the highest solubility in Phos, and also exhibited the highest transport in both the AP-BL and BL-AP direction in this medium. Dapsone also exhibited higher solubility in the SIF's than in the KRB, which can be explained by the lipophilicity of this drug and the fact that the SIFs contained lipid components.

Table 4.13: The p-values generated by statistical analysis dapsone bi-directional transport and efflux in the respecive media

Dapsone	Medium	KRB	PB	FeSSIF	FaSSIF
AP-BL	**KRB**		1.00	1.00	1.00
AP-BL	**Phos**	1.00		1.00	1.00
AP-BL	**FeSSIF**	1.00	1.00		1.00
AP-BL	**FaSSIF**	1.00	1.00	1.00	
BL-AP	**KRB**		1.00	1.00	1.00
BL-AP	**Phos**	1.00		1.00	1.00
BL-AP	**FeSSIF**	1.00	1.00		1.00
BL-AP	**FaSSIF**	1.00	1.00	1.00	
ER	**KRB**		0.01	0.00	0.00
ER	**Phos**	0.01		0.81	0.83
ER	**FeSSIF**	0.00	0.81		1.00
ER	**FaSSIF**	0.00	0.83	1.00	

The low ER and statistical conformance of the SIF's underlines the inhibitory effect taurocholate has on P-gp. The efflux in the Phos buffer differs markedly from the efflux in all other media, exhibiting the highest ER. This may be due to an unknown factor influencing the efflux transporters. Efflux in the KRB are markedly lower than in the Phos, and markedly

higher than in the SIF's, providing a middle ground that underlines the extremely low efflux of the SIF's and the extremely high efflux exhibited by the Phos

4.3.4 Furosemide (BCS Class 4 drug)

The P_{app} values as well as the ER values of furosemide in the selected transport media are shown in Figure 4.16.

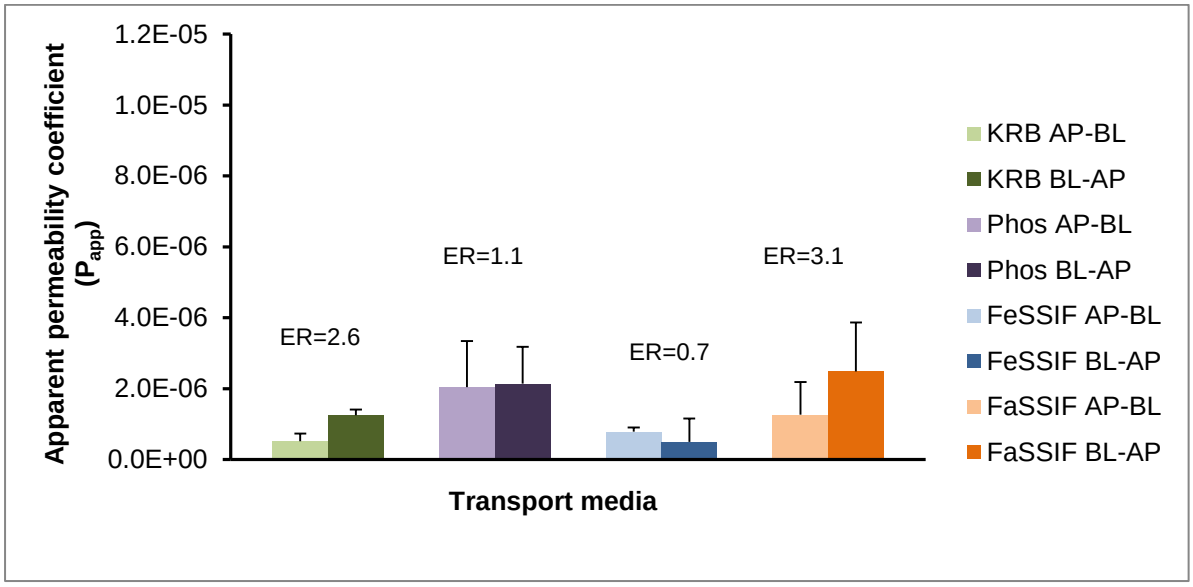


Figure 4.13: The P_{app} and ER values of furosemide in the respective transport media. AP-BL = apical to basolateral direction, BL-AP = basolateral to apical direction

Furosemide exhibited the highest permeation when applied in Phos and exhibited the lowest transport in the FeSSIF when comparing the transport in the selected media. The largest ER value for furosemide was observed in FaSSIF. Furosemide showed almost no efflux in Phos (ER close to 1), while an ER value below 1 was obtained in the FeSSIF medium. A study conducted by Ingels *et al,* (2004) reported a taurocholate concentration dependent reduction in efflux. Since FeSSIF has the highest concentration of taurocholate, inhibition of P-gp and other efflux carriers was expected, however, furosemide showed the highest efflux in this medium. This can possibly be explained by the fact that the FaSSIF was applied to the basolateral side in the BL-AP transport study, while efflux transporters are allocated on the apical side of the intestinal epithelium.

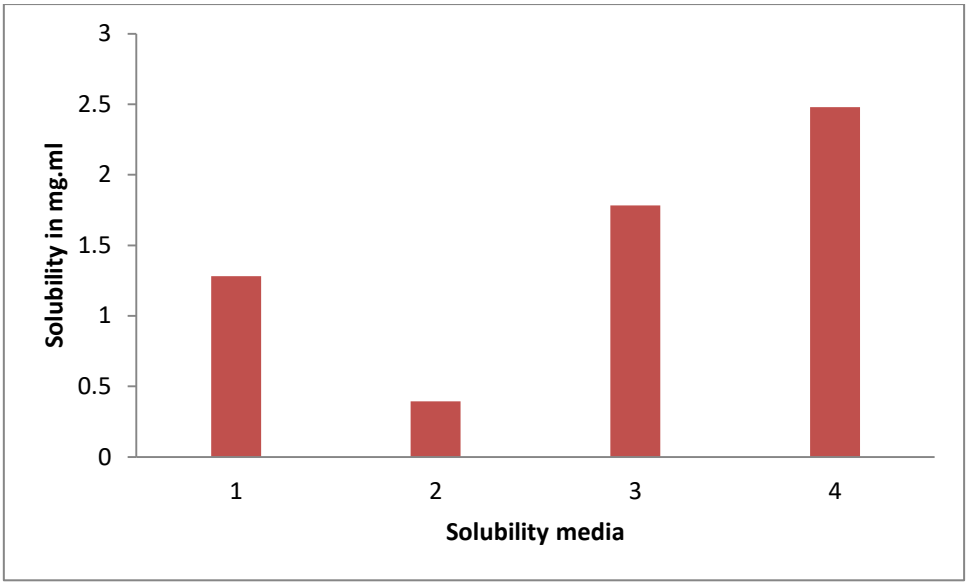


Figure 4.14: The solubility values of furosemide in the selected media

Furosemide exhibited lower solubility in the buffers than in the SIF's. This underlines the lipophilicity of this drug. An interesting observation is that the buffer in which the lowest solubility of furosemide was obtained, exhibited the highest overall transport (i.e. in the AP-BL and BL-AP directions) across the excised intestinal tissue. Class 4 compounds such as furosemide are very susceptible to changes in the medium composition on the solubility of the drug as well as the permeability of the membrane.

Table 4.14: Statistical analysis of furosemide bi-directional transport and efflux in the respective media

Furosemide	Medium	KRB	PB	FeSSIF	FaSSIF
AP-BL	**KRB**		0.92	1.00	1.00
AP-BL	**Phos**	0.92		0.98	1.00
AP-BL	**FeSSIF**	1.00	0.98		1.00
AP-BL	**FaSSIF**	1.00	1.00	1.00	
BL-AP	**KRB**		1.00	1.00	1.00
BL-AP	**Phos**	1.00		1.00	1.00
BL-AP	**FeSSIF**	1.00	1.00		0.99
BL-AP	**FaSSIF**	1.00	1.00	0.99	
ER	**KRB**		1.00	1.00	1.00
ER	**Phos**	1.00		1.00	1.00
ER	**FeSSIF**	1.00	1.00		1.00
ER	**FaSSIF**	1.00	1.00	1.00	

The transport of furosemide (BCS Class 4 drug) showed no statistically significant difference between the selected media.

4.4 TEER measurements

The measured TEER values obtained during the transport studies were used as an indication of tissue viability and integrity. Differences in the composition of the transport medium employed or the addition of a transport enhancing compound in the formulation may also have an influence on the measured TEER values by means of modulating the tight junctions (Lindhart & Bechgaard, 2002). The final TEER values (after 120 min) for the selected drugs and media are shown in Table 4.15. All the TEER data are available as a graphical representations in **Addendum C**.

Table 4.15: Average TEER values after 120 min for the selected drugs and transport media

TEER values in Ω.cm^{-2} after 120 min.		
Drug/medium	**TEER AP-BL**	**TEER BL-AP**
ABA/KRB	86.03	56.96
ABA/Phos	58.74	56.37
ABA/FeSSIF	90.78	91.85
ABA/FaSSIF	58.74	56.43
LAM/KRB	73.51	56.96
LAM/Phos	48.06	59.99
LAM/FeSSIF	51.62	48.06
LAM/FaSSIF	49.31	52.15
DAPS/KRB	50.37	45.75
DAPS/Phos	98.43	85.44
DAPS/FeSSIF	23.14	23.14
DAPS/FaSSIF	55.18	61.05
FUROS/KRB	55.18	62.83
FUROS/Phos	75.29	76.01
FUROS/FeSSIF	56.96	44.50
FUROS/FaSSIF	56.96	60.52

From the literature, the minimum TEER value for intact excised rat intestinal tissue after 120 min was found to be 20 Ω.cm^2 (Da Silva *et al.,* 2015). All the TEER values exceeded 20 Ω.cm^2 at 120 min, and the intestinal segments were therefore considered to be viable. The average final TEER value in the AP-BL direction was 61.77 ± 17.68 Ω.cm^2 and 58.63 ± 15.24 Ω.cm^2 in the BL-AP direction. Although a greater reduction in the TEER values was evident in the SIF, the pig model was robust enough to ensure repeatability of the test results (Westerhout *et al.,* 2014). In some of the experiments where Phos was used as the transport medium, the TEER value has increased which indicated modulation of tight

junctions in such a way that the openings closed when compared to the initial state. A table with the percentage reduction as well as graphical depictions of the fluctuations in the TEER values for each drug/medium combination is shown in **addendum C.**

4.5 Summary of results

The bi-directional transport studies of three of the selected drugs showed that active drug efflux had occurred in all test media. The only exception was furosemide where no efflux occurred when using FeSSIF and negligible efflux when using KRB media. The high-solubility drugs (abacavir & lamivudine) showed markedly higher P_{app} values than the low solubility drugs (dapsone & furosemide). Lamivudine showed markedly smaller P_{app} values in the Phos than in any of the other selected transport media.

Abacavir exhibited lower P_{app} values in the SIF than in the buffer solutions. Lamivudine exhibited markedly lower P_{app} values in Phos than in any of the other media. Dapsone showed efflux in all the media, which has not been shown experimentally before (not published in the literature), to the knowledge of the author. The BDDCS predicted that efflux transporters may have a pronounced effect on the transport of Class 2 drugs (Wu & Benet, 2005), which was observed for dapsone when it was applied in Phos. Furosemide (a BCS class 4 drug) exhibited efflux to variable extents in the different buffer solutions, underlining the possibility that variations in transport media composition may alter drug uptake (Custodio, Wu & Benet, 2008).

The transport of the model drugs was lower in the SIF, with the exception of lamivudine. This trend was also reported in a previous study which investigated the effect of medium composition on drug transport (Ingels *et al.,* 2004).

4.6 Conclusion

It can be concluded that transport medium composition has a large effect on drug permeability, solubility and transporter effects (efflux and uptake). It is imperative to take this into consideration when designing a permeation study in order to reduce the variables that may influence drug permeation. KRB exhibited repeatable permeation data, while Phos exhibited the least predictable results as indicated by standard deviations between replicates. In general, the results indicated that when buffers are used as transport media, intestinal membrane permeability may be overestimated. It is clear, however, that future mechanistic studies are required in order to fully explain all the effects that transport media composition has on the permeation of drug molecules across a membrane.

Chapter 5: Final conclusions and future recommendations

5.1 Introduction

Improved *in vitro* models for intestinal drug permeation studies are necessary to simulate *in vivo* intestinal conditions more accurately. In addition to accurate representation of the luminal conditions and *in vivo* transport processes, these improved models need to be less time consuming and more cost effective.

Shortcomings of the current models in use include low throughput, high cost (if purpose-bred laboratory animals are used) and the employment of non-biorelevant transport media such as Phosphate or saline buffers. Various studies have been done to test the effect of media composition alterations on cell monolayers such as Caco-2, MDCK and HT-29, but *ex vivo* studies across excised intestinal tissues are lacking certain elements to adequately simulate normal *in vivo* conditions. These include pH of the medium, medium composition and stirring to simulate bowel peristalsis (Annaert *et al.,* 2009, Bergnic, Trontelj & Kristl, 2012; Da Silva *et al.,* 2015).

In vitro models are required to comply with the 3R ethics concept in research, but if the data generated from these models do not correlate with *in vivo* data, it will not be useful. Slaughter of animals for meat production at an abattoir provide opportunities to source tissue from waste products (e.g. intestinal tissue), which complies with the 3R concept, making it an ideal candidate for use in permeation studies. Shortcomings of this model include limited data to compare it with other *in vitro* models in terms of drug permeation, lack of confirmation of Paneth cell presence and differences in Peyer's patch expression and distribution. The latter has led to the exclusion of Peyer's patches during permeation studies (Gonzalez, Moeser & Blikslager, 2015). The viability of the excised intestinal tissue must also be ensured during the permeability study as degradation may influence results (Pietzonka *et al.,* 2001).

5.2 Final conclusions

Drug permeation data obtained from this study were repeatable as indicated by the relatively low standard deviations within experimental groups. The HPLC analytical method complied with validation specifications of linearity, selectivity, precision and accuracy.

For highly soluble compounds (BCS class 1 and 3, which were represented by abacavir and dapsone, respectively), the most repeatable permeability results were obtained in KRB and for poorly soluble compounds (BCS class 2 and 4, which were represented by lamivudine

and furosemide, respectively) the most repeatable permeability results were obtained in FeSSIF. The Phosphate buffer is not recommended for any drug permeability tests, as possible interaction with intestinal transporters was evident with drugs used in this study. This includes the inhibition of uptake transporters as well as the "activation" of efflux transporters. This effect may merely be an extension in the uptake transporter inhibition by limiting the amount of drug absorbed from the apical side of the membrane, thereby decreasing the amount of drug absorbed. When testing the permeation from the basolateral side the efflux transporter will still function unimpeded, thereby enhancing the efflux effect in the Phosphate buffer. The simulated intestinal fluids, on the other hand, may inhibit p-gp due to the presence of taurocholate in these media, but as taurocholate is present in *in vivo* conditions, this may represent the living organism better.

The model compounds showed differences in solubility in the different media, with class 3 and 4 compounds exhibiting pronounced differences. The highly soluble. but poorly permeable class 3 compound (i.e. dapsone) showed a markedly higher solubility in the aqueous buffers than in the SIFs, with the opposite occurring in the case of the class 4 compound (i.e. furosemide). This may cause class 4 compounds to act as class 3 compounds in the SIF transport media (Custodio, Wu & Benet, 2008). The class 1 compound (i.e. abacavir) was influenced by the lipid components in the SIF's underlining the importance of media selection and the suitability of the medium to the study. The solubility of the class 3 compound (i.e. lamivudine) differed markedly between the aqueous salt buffers and the SIF's. Since both these drugs are highly soluble, it is doubtful that any clinical significant difference will be noted *in vivo.*

From the data obtained in this study, it is clear that transport medium composition influenced not only drug solubility, but also drug transport across excised pig intestinal tissues. Although some of the transport changes could be related to changes in efflux, further studies need to be done in order to fully explain the physico-chemical interactions, which may be responsible for the solubility changes in each of the transport media.

5.3 Future recommendations

These studies are recommended for follow-up research, in order to better characterise the effect of transport media composition on drug permeation:

- Studies which include biorelevant media and known transport protein substrates are required to pinpoint the specific transporters which are influenced/modulated by certain components in the transport media.

- pH dependant studies in each transport medium to integrate the ionisation of different compounds on drug permeation.

- Pig's intestinal transport data must be compared to other published data using other *in vitro* techniques (Caco-2, rat's intestine, PAMPA).

- The design of a medium throughput system for screening the effect of each component present in biorelevant media (Westerhout *et al.,* 2014; Da Silva *et al.,* 2015).

- Studies to determine the toxicity profile of the SIF as bile components are known to be toxic on excised tissue (Berginc, Trontelj & Kristl, 2012).

- More studies are required to elucidate the specific interactions between the various transport media and the different uptake and efflux mechanisms found in intestinal tissue. Integration of these results will aid in the design of an improved *in vitro* transport model. Effort also has to be made in order to develop a system that allow for a large number of intestinal segments to be tested at once. This will allow for larger studies in order to generate larger data sets.

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Addendum A: Conference proceedings

ABSTRACT

Effect of transport medium composition on *in vitro* drug permeation across excised pig intestinal tissue

HJ Heystek, JH Hamman and JD Steyn

Centre of Excellence for Pharmaceutical Sciences, North-West University, Potchefstroom, 2531, South Africa

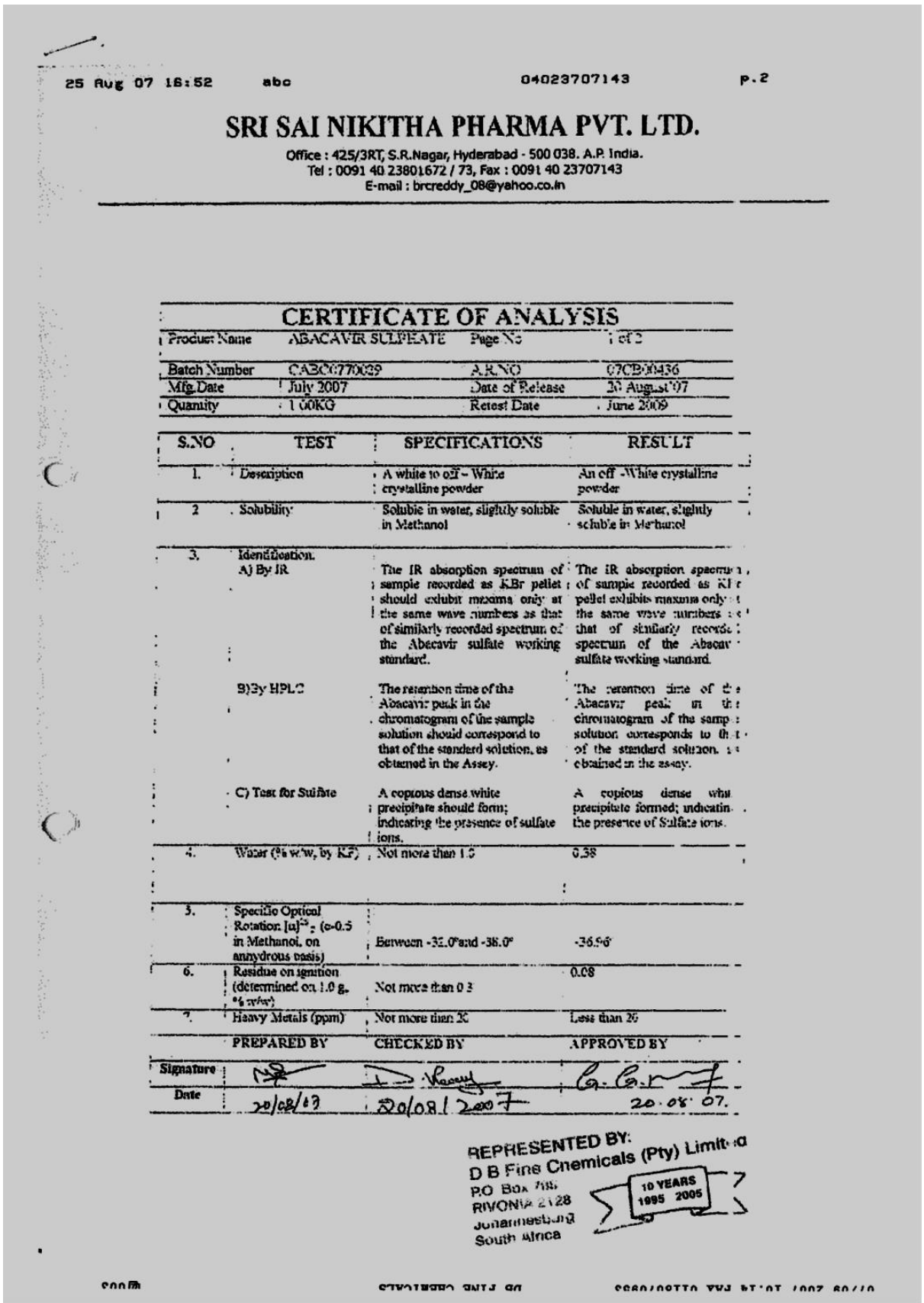
Purpose: To determine the effect of the type of transport medium on the bi-directional transport of four selected drugs across excised animal tissues in a Sweetana-Grass diffusion apparatus.

Methods: Jejunum tissue was excised from slaughtered pigs at Potchefstroom abattoir and transported in ice-cold Krebs-Ringer Bicarbonate (KRB) buffer to the laboratory. The serosa was removed by blunt dissection, and pieces of the jejunum were mounted on half cells of the diffusion chambers. Bi-directional transport experiments were conducted with different transport media namely Krebs-Ringer bicarbonate buffer, Phosphate buffer, fasting state simulated intestinal fluid (FaSSIF) and fed state simulated intestinal fluid (FeSSIF) for each of the selected drugs namely abacavir, dapsone, lamivudine and furosemide. Samples (200 µl) were withdrawn from the acceptor chambers at 20 min intervals and analysed by means of a validated high-performance liquid chromatography method. The apparent permeability coefficient (P_{app}) values as well as efflux ratio values were calculated from the transport data.

Results: The transport results (i.e. the apparent permeability coefficient or P_{app} values) showed that the transport medium composition had a pronounced effect on the extent and rate of transport of the selected drugs across both directions of the excised pig intestinal tissues and therefore also on the degree of drug efflux. The results indicated that KRB, FeSSIF and FaSSIF rendered more predictable and repeatable transport of drugs across the intestinal epithelium than the commonly used Phosphate buffer.

Conclusion: The choice of transport medium for *in vitro* drug permeability studies is an important factor that should be considered to render more realistic and predictable transport data.

Addendum B: Certificates of Analysis



25 Aug 07 16:52 abc 04023707143 p.2

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 : brcreddy_08@yahoo.co.in

CERTIFICATE OF ANALYSIS

Product Name	ABACAVIR SULPHATE	Page No	1 of 2
Batch Number	CABC0770039	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 recorded 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 Assay.	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 1.0	0.38
5.	Specific Optical Rotation $[\alpha]_D^{25}$ (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 0.3	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
P.O Box 788
RIVONIA 2128
Johannesburg
South Africa

10 YEARS
1995 2005

XIAMEN MCHEM LABORATORIES LTD.
厦门迈克制药有限公司
TEL: +86-592-2218988 FAX: 2218616 2218615
Email: Mchem@mchem.net Http://www.mchem.net

CERTIFICATE OF ANALYSIS

PRODUCT NAME	**LAMIVUDINE**
BATCH NO.	**040803**
QUANTITY	**100g**
MANUFACTURER	**XIAMEN MCHEM LABORATORIES LTD.**
DATE OF MANUFACTURE	**AUG. 2004**
DATE OF EXPIRY	**AUG. 2007**

TESTS	SPECIFICATIONS	RESULTS
Description	A white or off-white crystalline powder.	Complies
Solubility	70mg/ml, in water at 20℃ Sparingly soluble in methanol and ethanol	Complies
Identification	IR should comply with the reference standard spectrum. UV should comply with the reference standard spectrum	Complies Complies
Specific Optical Rotation (C=1, MeOH)	136°~-145°	142.4°
Heavy Metals	≤20ppm	Complies
Melting Range	172~178℃	175~177.5℃
Residual on ignition	≤0.25%	0.07%
Water by K.F.	≤0.5%	0.06%
Loss on Drying	≤1.0%	0.03%
Related Substance by HPLC	Single Impurity: ≤0.1% Total Impurities: ≤0.5%	Complies Complies
Residual solvents: **Methanol** **Methylene dichloride** **Hexane** **Toluene**	 ≤3000ppm ≤600ppm ≤290ppm ≤890ppm	 ND ND ND ND
Assay by titration	98.0%~102.0% ($C_8H_{11}N_3O_3S$)	99.90%
Conclusion	The material complies with the In-House standard.	

Quality Manager: 陈至波 **Rechecker:** 洪亭钢 **Analyst:** 林亚芬

Markings: Kirsch Pharma South Africa (PTY) Ltd
Gewel Street, Isando
KPSA 2436/11/2004

厦门迈克制药有限公司
Xiamen Mchem Laboratories Ltd.

Director

RIA INTERNATIONAL LLC

11 Melanie Lane # 17, East Hanover, NJ 07936
Ph: (973) 581-1282 Fax: (973) 581-1283
E-mail: ria@riausa.net web: www: riausa.net

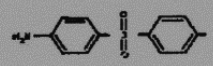
DB2954

CERTIFICATE OF ANALYSIS

REPRESENTED BY:
DB Fine Chemicals (Pty) Ltd
P.O. Box 786 Rivonia 2128
Johannesburg South Africa
Tel: +27 (0)11 807 2991 Fax: +27 (0)11 807 0953
Established Since 1995

DAPSONE BP

CAS # 80-08-0
MF # $(H_2NC_6H_4)_2SO_2$
MW # 248.30
Batch # 20100128
Code # 92307



Mfg Date: 01/2010
Exp Date: 01/2012
Country of Origin: China

TEST	SPECIFICATION	RESULT
Appearance	White crystalline powder	Complies
Melting point	175-181°C	178.5°C
pH	6.5 – 7.5	6.8
Loss on Drying	≤ 0.2%	0.05%
Color of solution	<30	Complies
Iron (Fe)	<0.5ppm	0.2ppm
Iron and other substance		
Above 0.5mm	0	0
0.5 – 0.2mm	<5	2
Below 0.2mm	<30	20
Fe	0 – 1	1
Assay (HPLC)	>99.5%	99.8%

Conclusion: This product complies with BP

Addendum C: *In vitro* Transport of model compounds in the selected medium

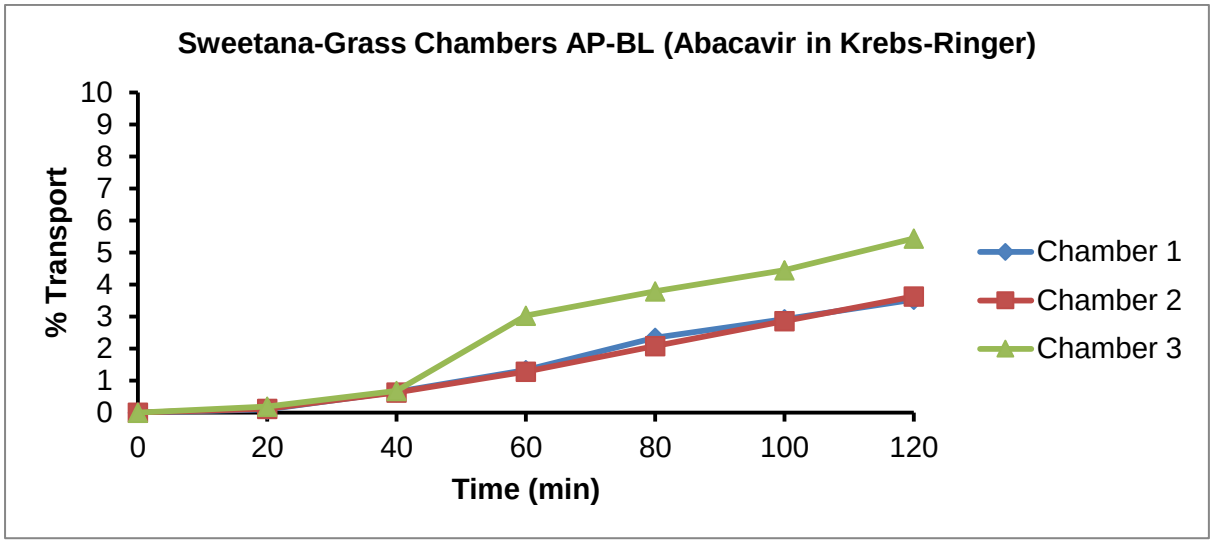


Figure C1: Percentage transport versus time of Abacavir in KRB (AP-BL)

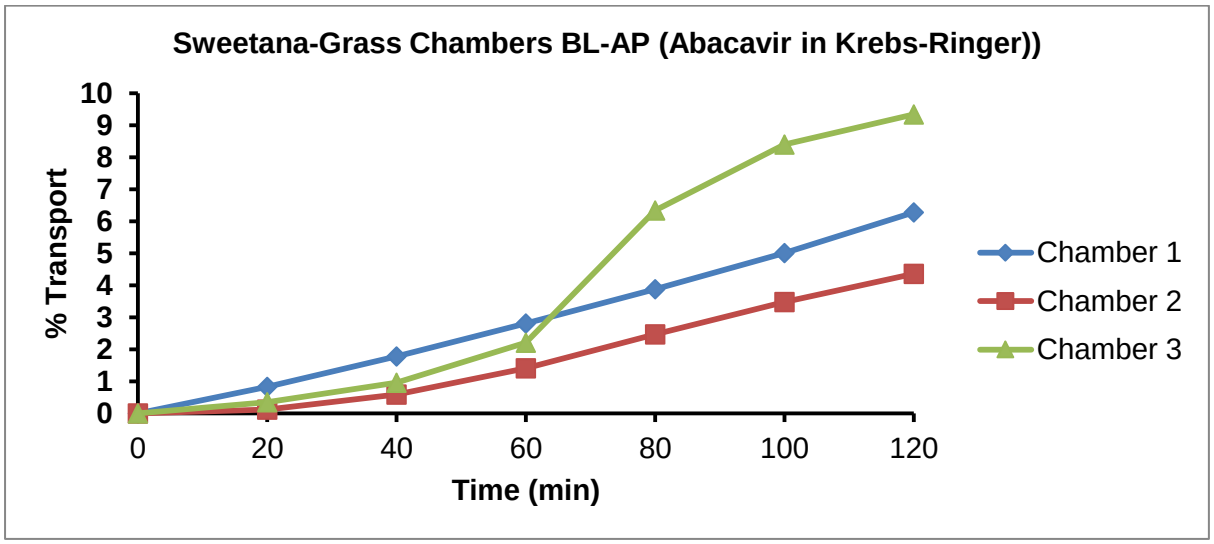


Figure C2: Percentage transport versus time of Abacavir in KRB (BL-AP)

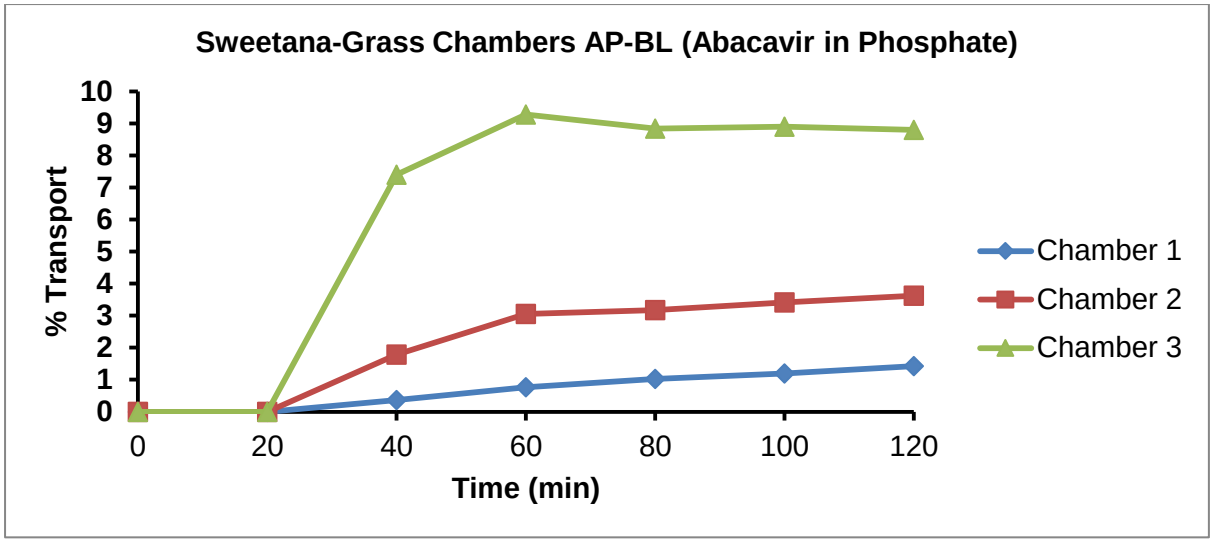


Figure C3: Percentage transport versus time of Abacavir in Phos (AP-BL)

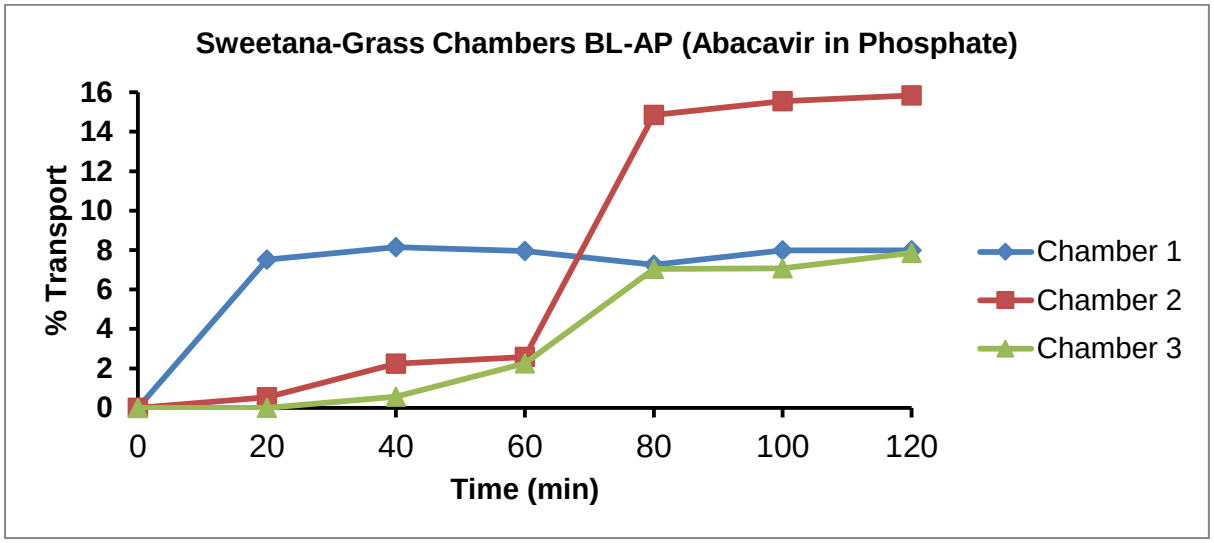


Figure C4: Percentage transport versus time of Abacavir in Phos (BL-AP)

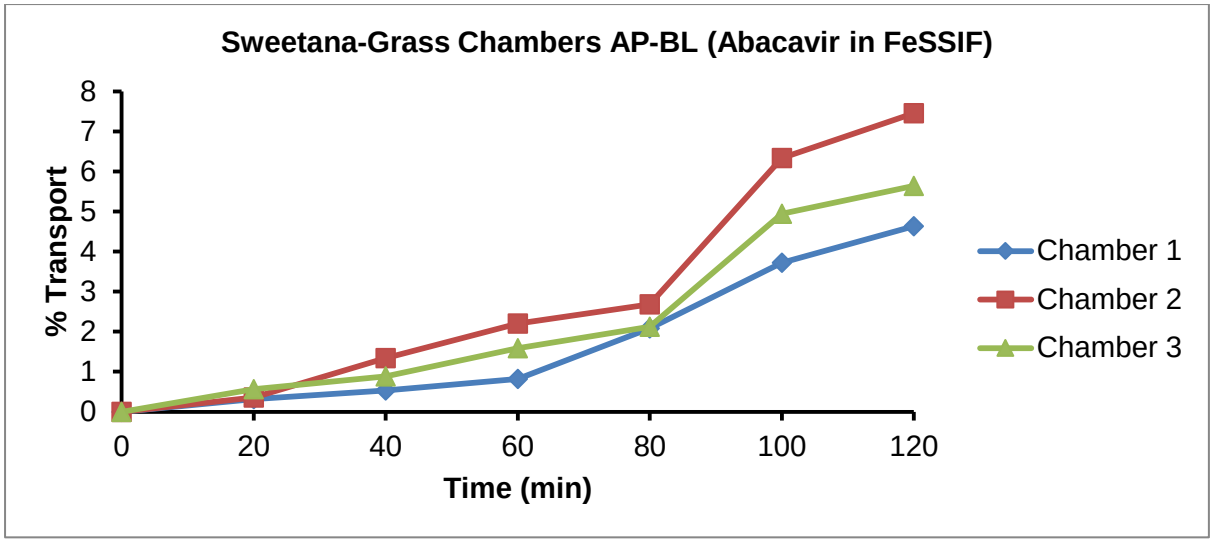


Figure C5: Percentage transport versus time of Abacavir in FeSSIF (AP-BL)

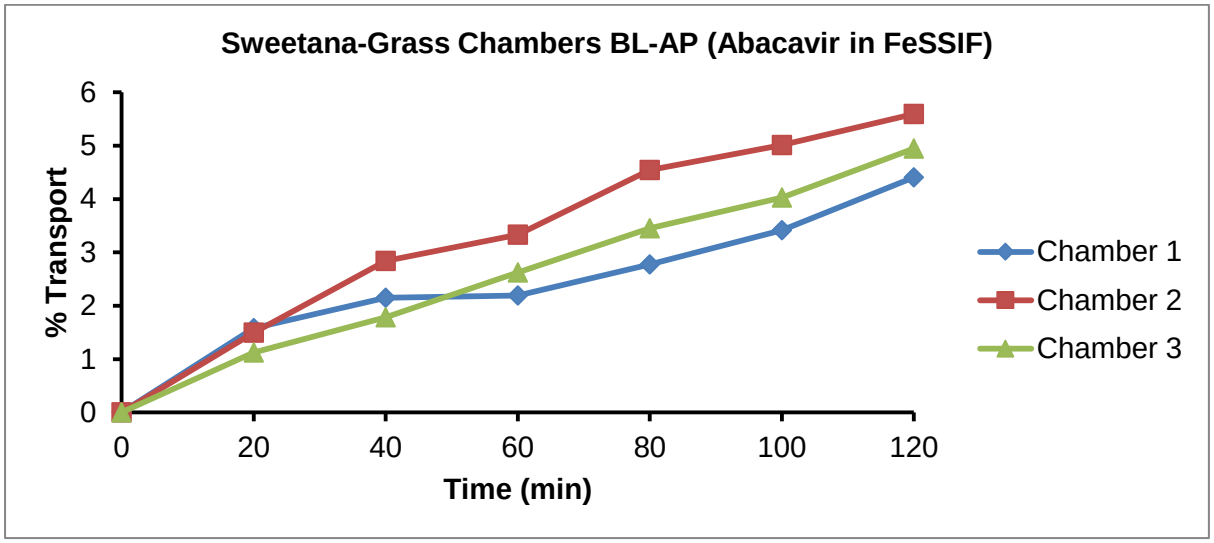


Figure C6: Percentage transport versus time of Abacavir in FeSSIF (BL-AP)

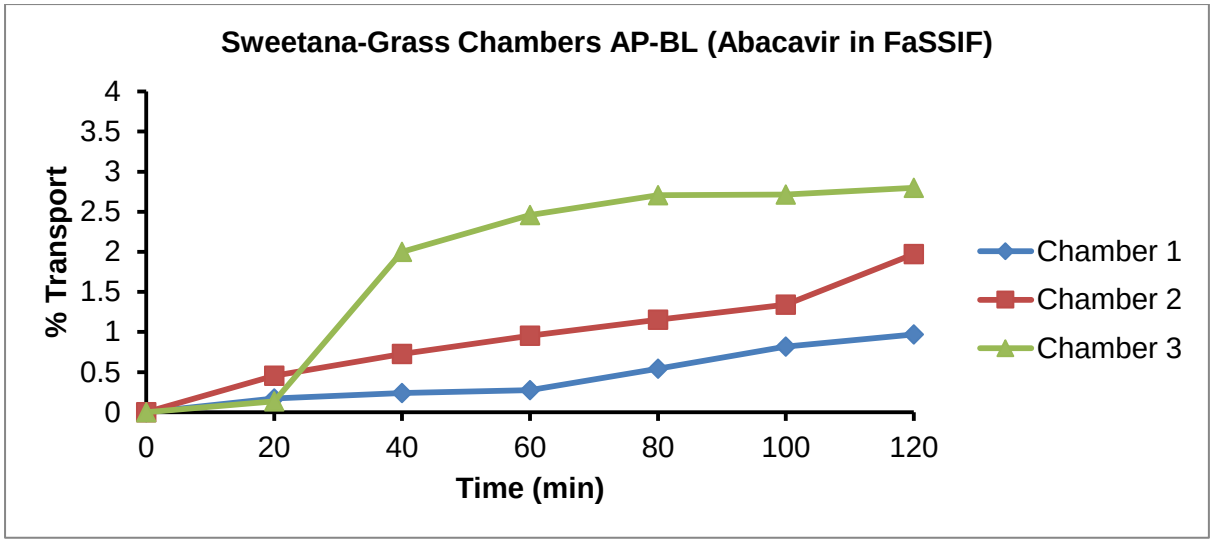


Figure C7: Percentage transport versus time of Abacavir in FaSSIF (AP-BL)

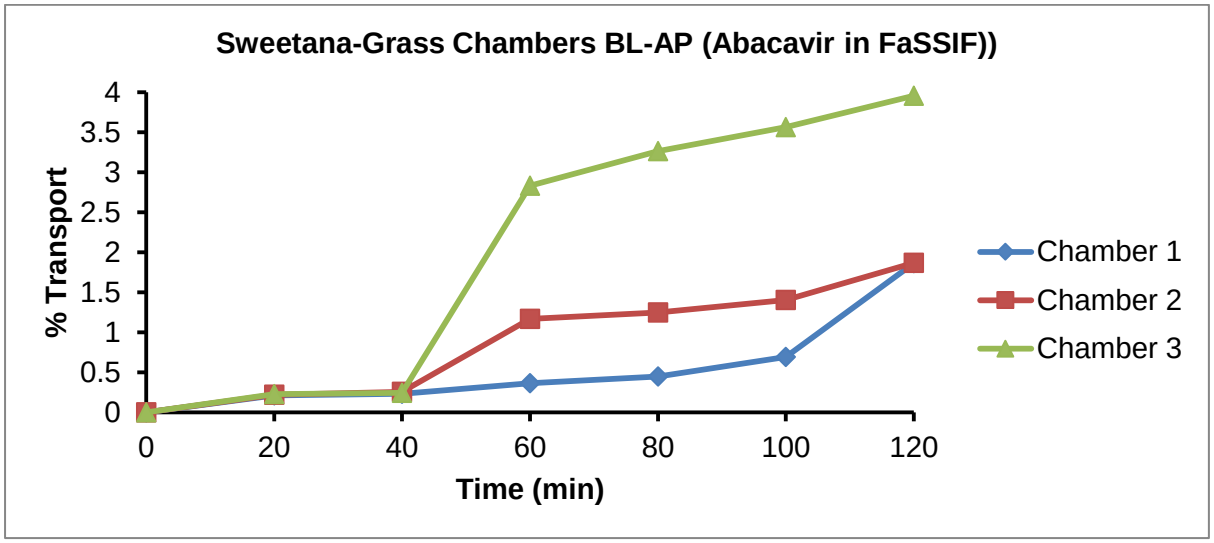


Figure C8: Percentage transport versus time of Abacavir in FaSSIF (BL-AP)

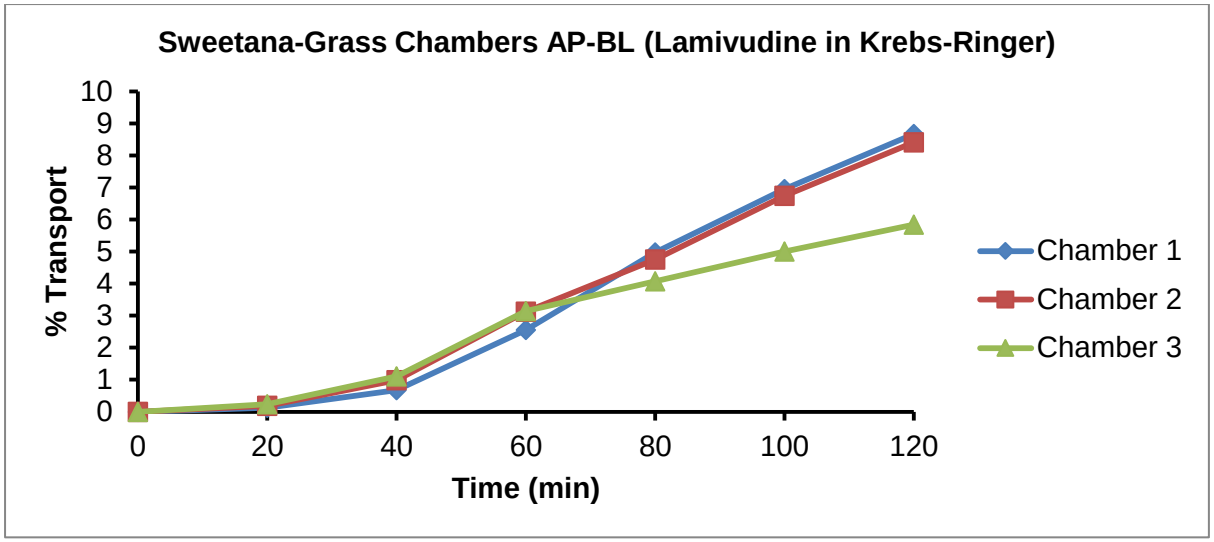


Figure C9: Percentage transport versus time of Lamivudine in KRB (AP-BL)

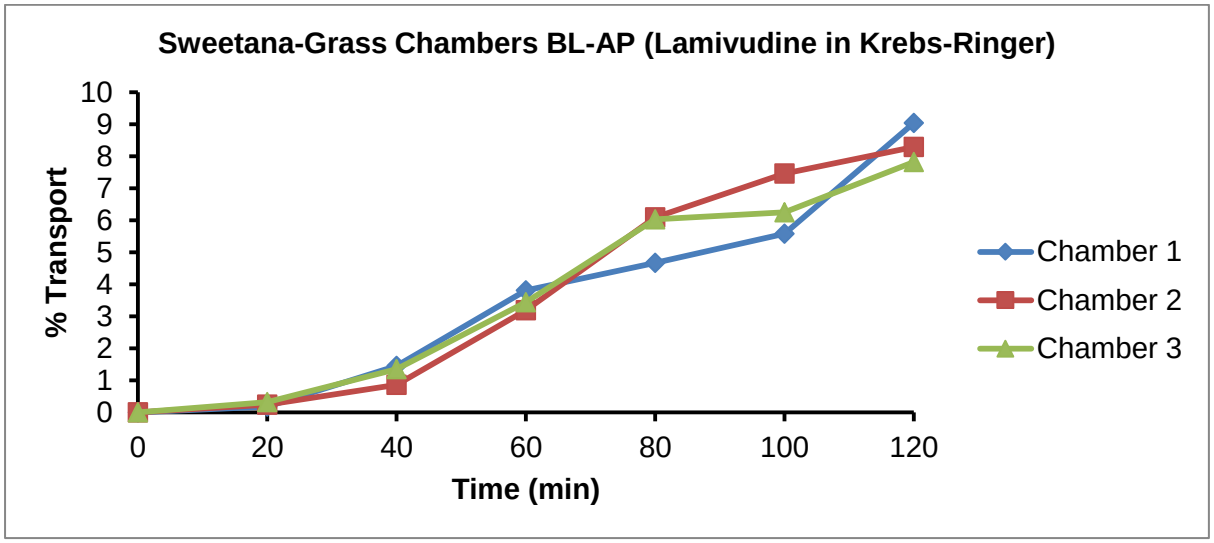


Figure C10: Percentage transport versus time of Lamivudine in KRB (BL-AP)

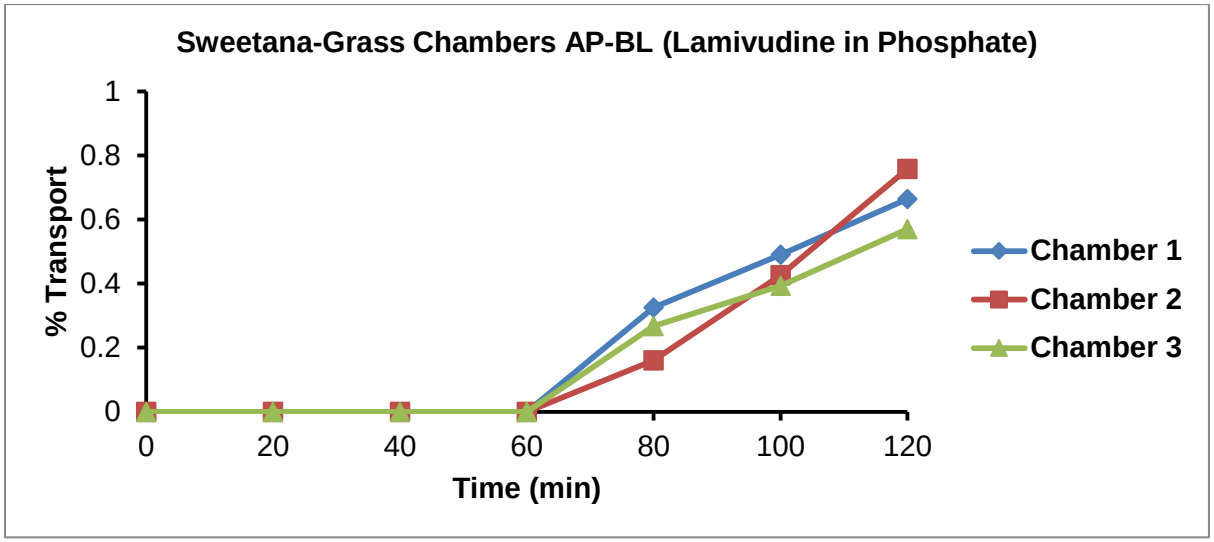


Figure C11: Percentage transport versus time of Lamivudine in Phos (AP-BL)

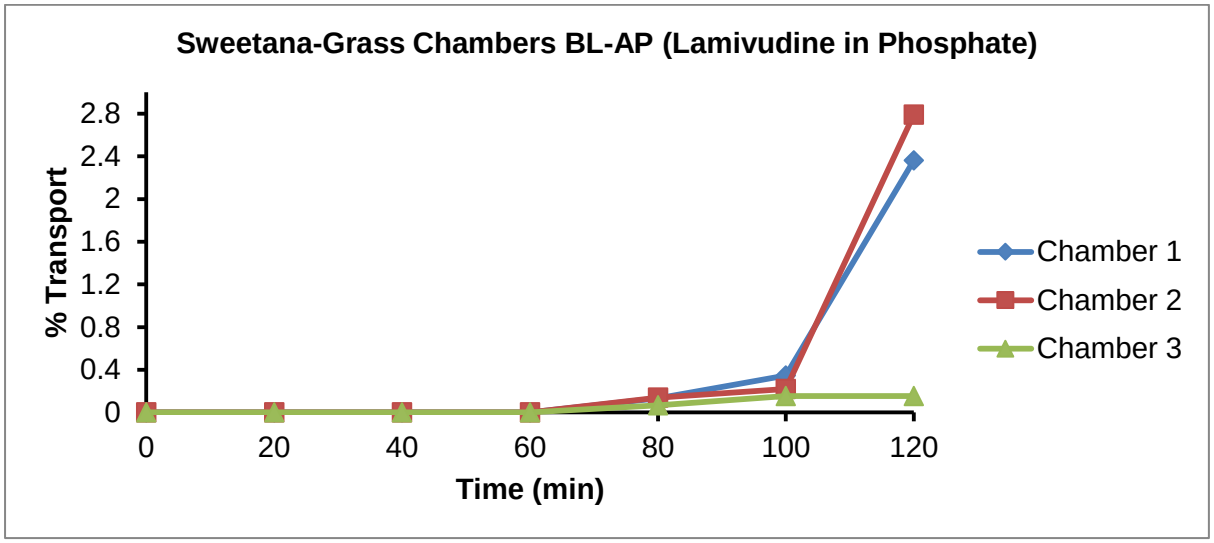


Figure C12: Percentage transport versus time of Lamivudine in Phos (BL-AP)

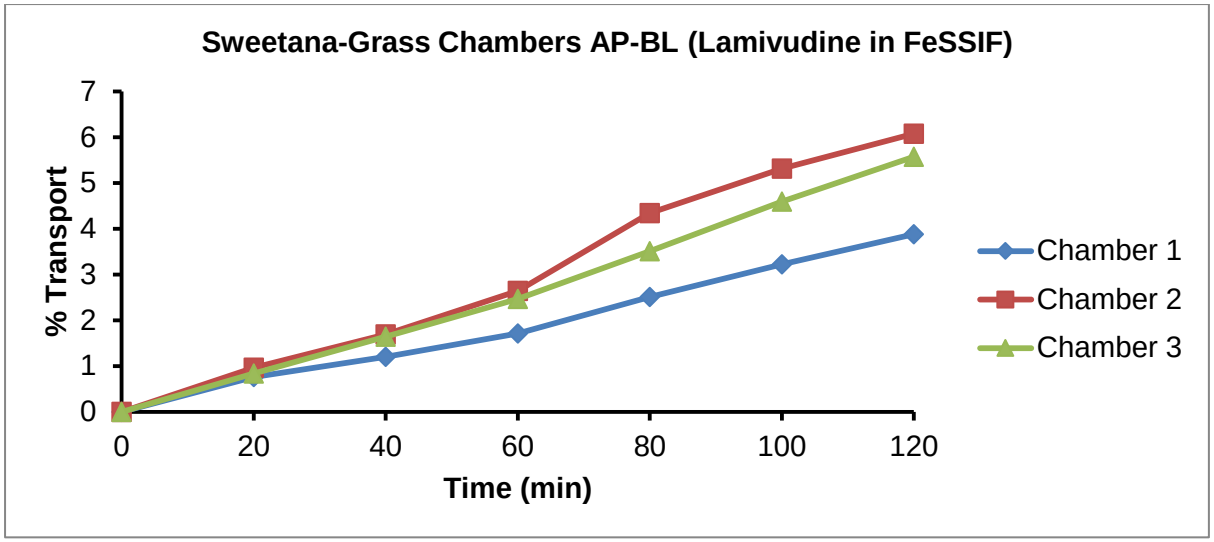


Figure C13: Percentage transport versus time of Lamivudine in FeSSIF (AP-BL)

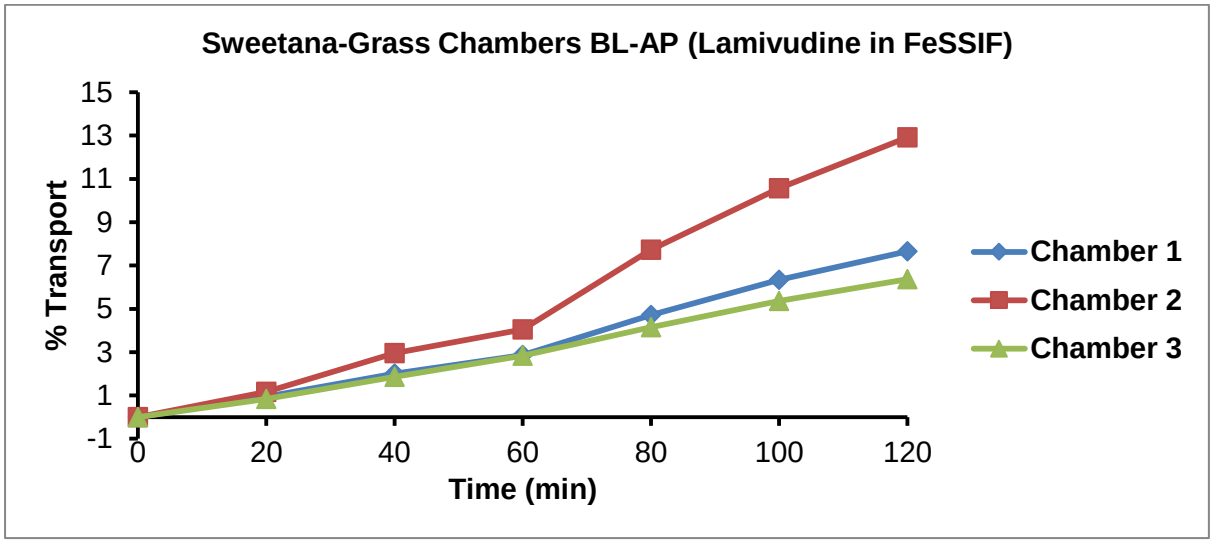


Figure C14: Percentage transport versus time of Lamivudine in FeSSIF (BL-AP)

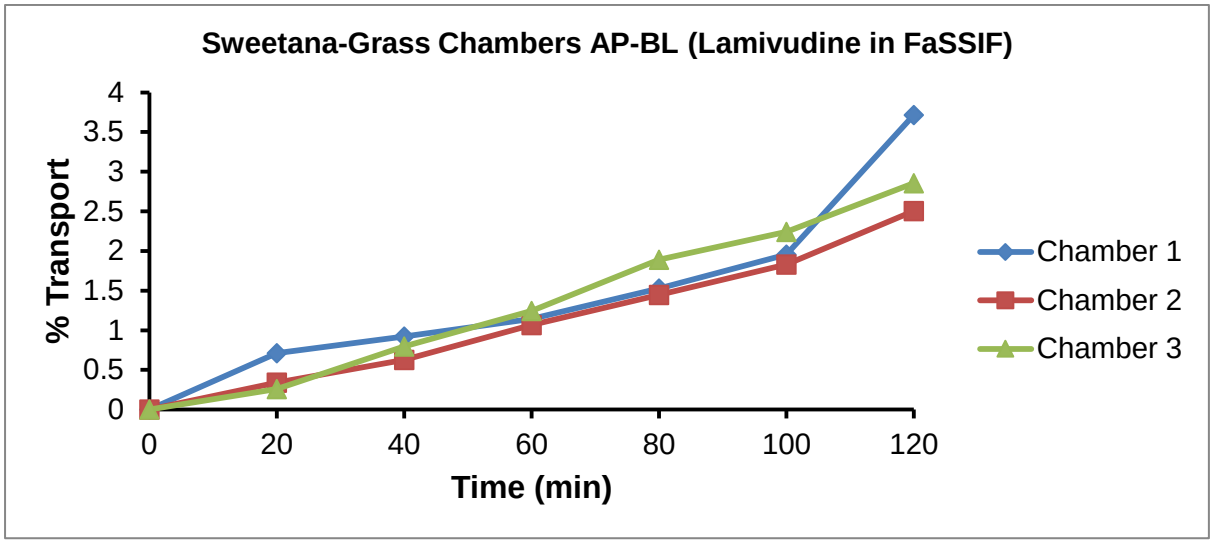


Figure C15: Percentage transport versus time of Lamivudine in FaSSIF (AP-BL)

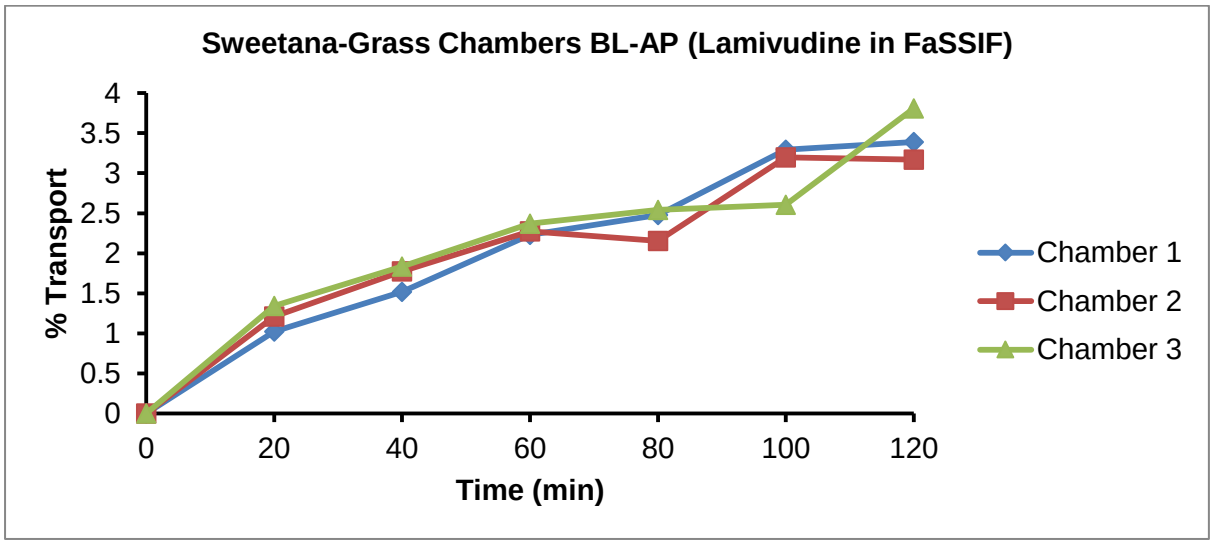


Figure C16: Percentage transport versus time of Lamivudine in FaSSIF(BL-AP)

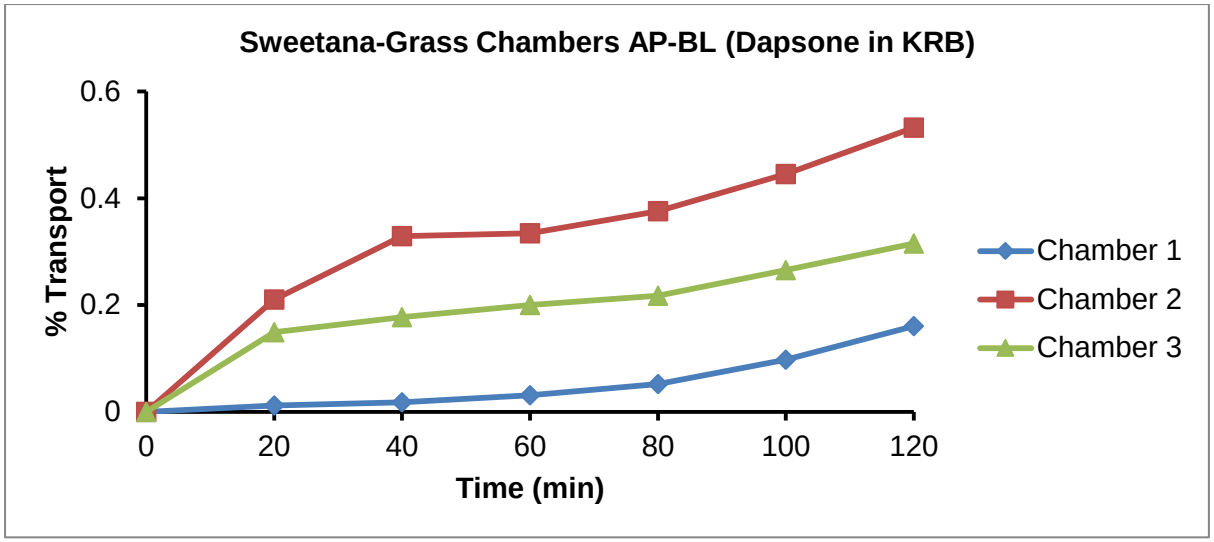


Figure C17: Percentage transport versus time of Dapsone in KRB (AP-BL)

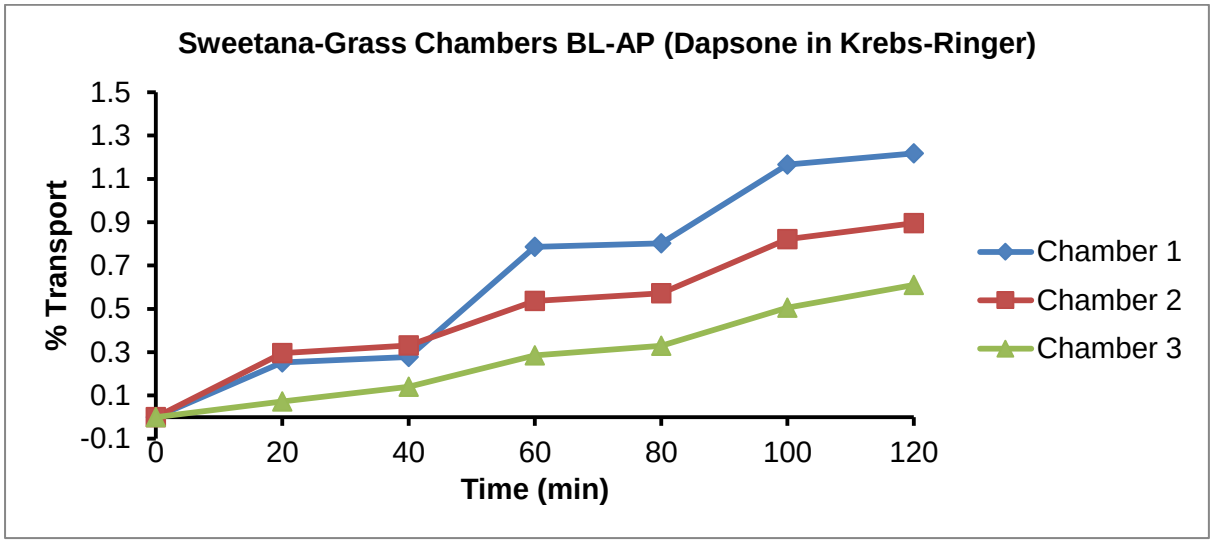


Figure C18: Percentage transport versus time of Dapsone in KRB (BL-AP)

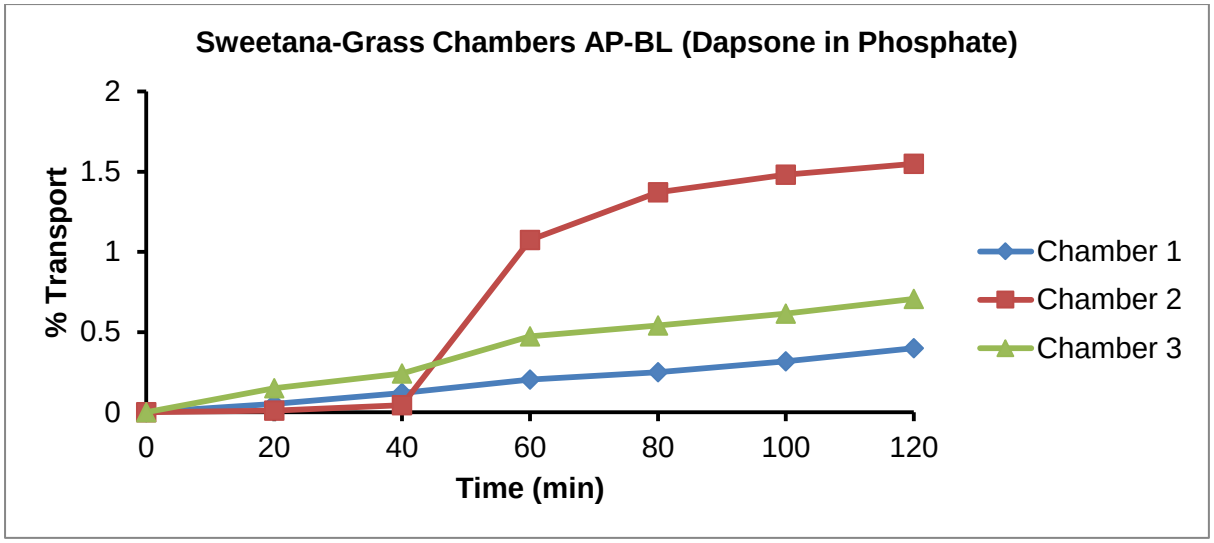


Figure C19: Percentage transport versus time of Dapsone in Phos (AP-BL)

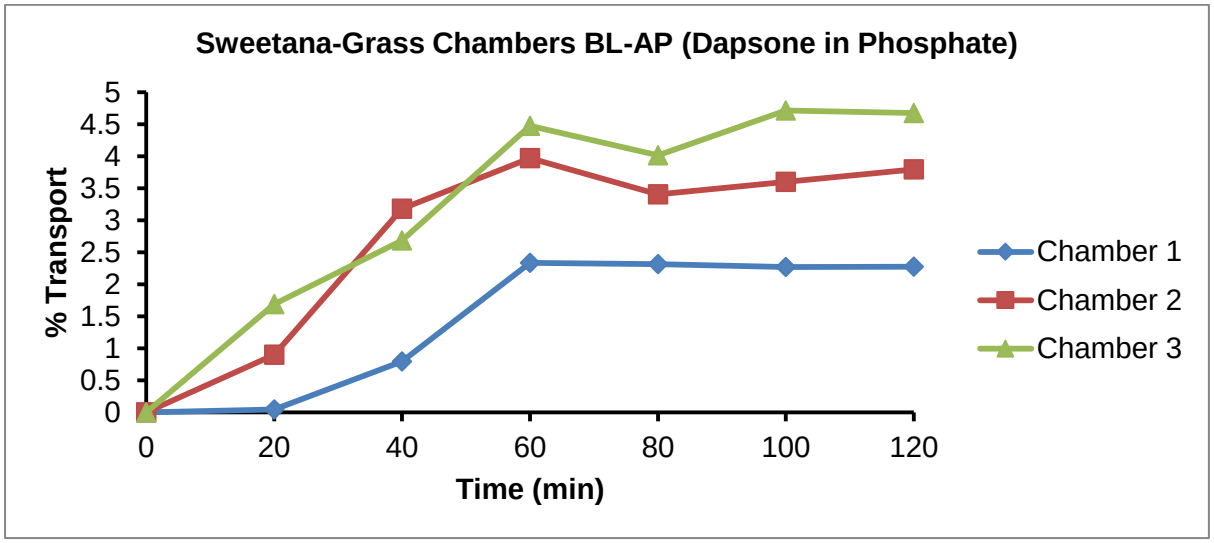


Figure C20: Percentage transport versus time of Dapsone in Phos (BL-AP)

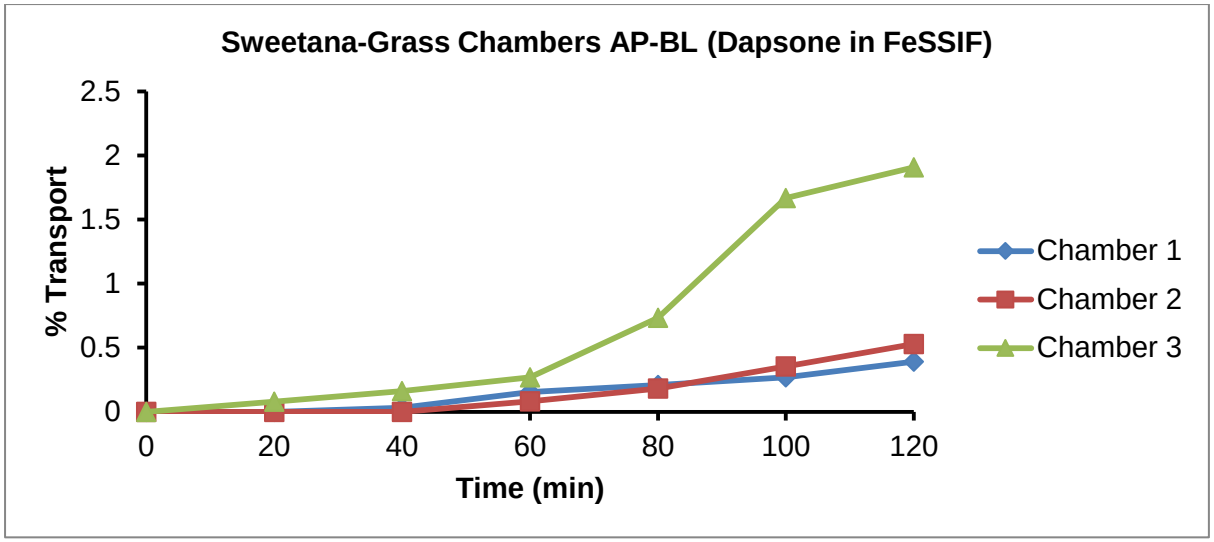


Figure C21: Percentage transport versus time of Dapsone in FeSSIF (AP-BL)

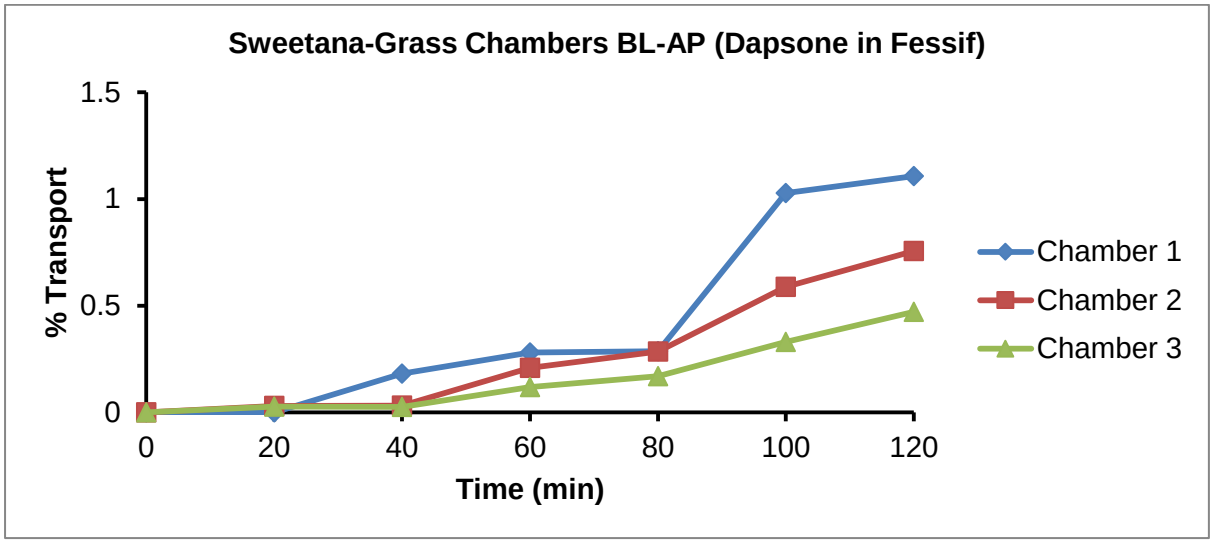


Figure C22: Percentage transport versus time of Dapsone in FeSSIF (BL-AP)

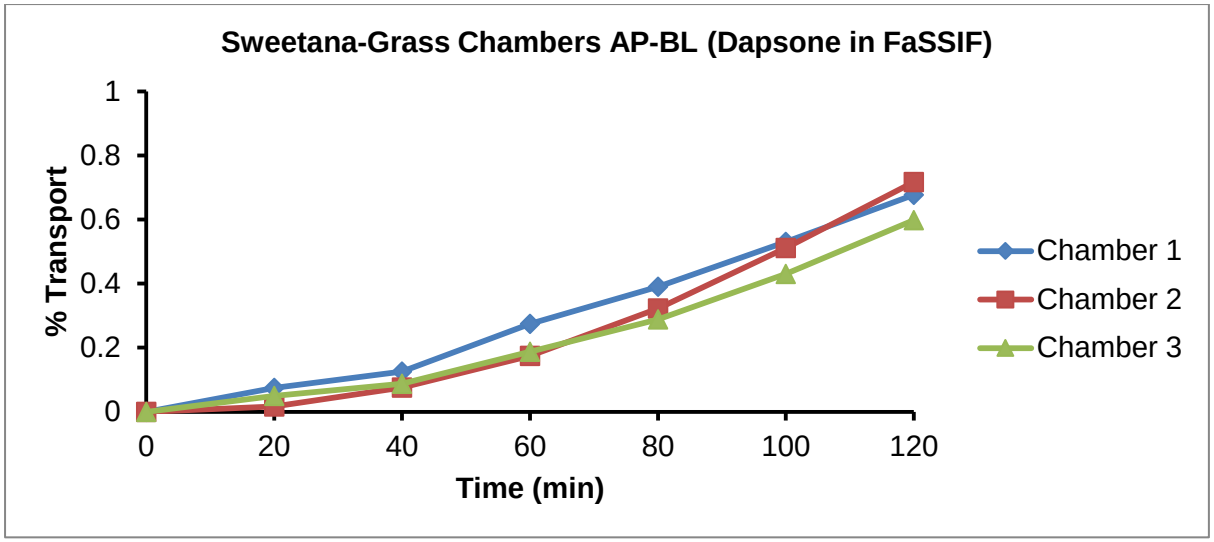


Figure C23: Percentage transport versus time of Dapsone in FaSSIF (AP-BL)

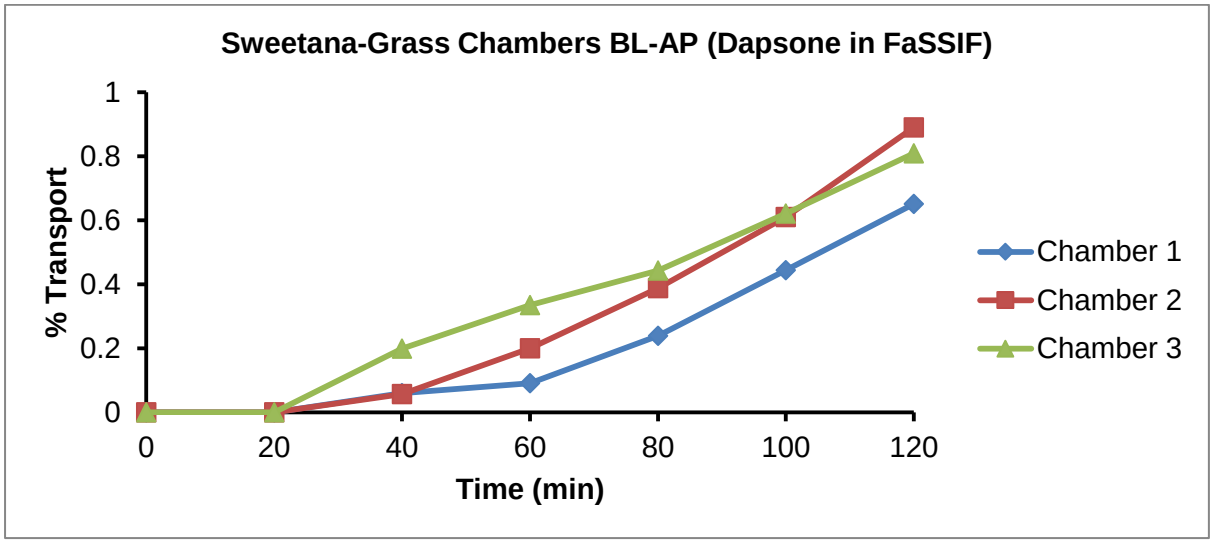


Figure C24: Percentage transport versus time of Dapsone in FaSSIF (BL-AP)

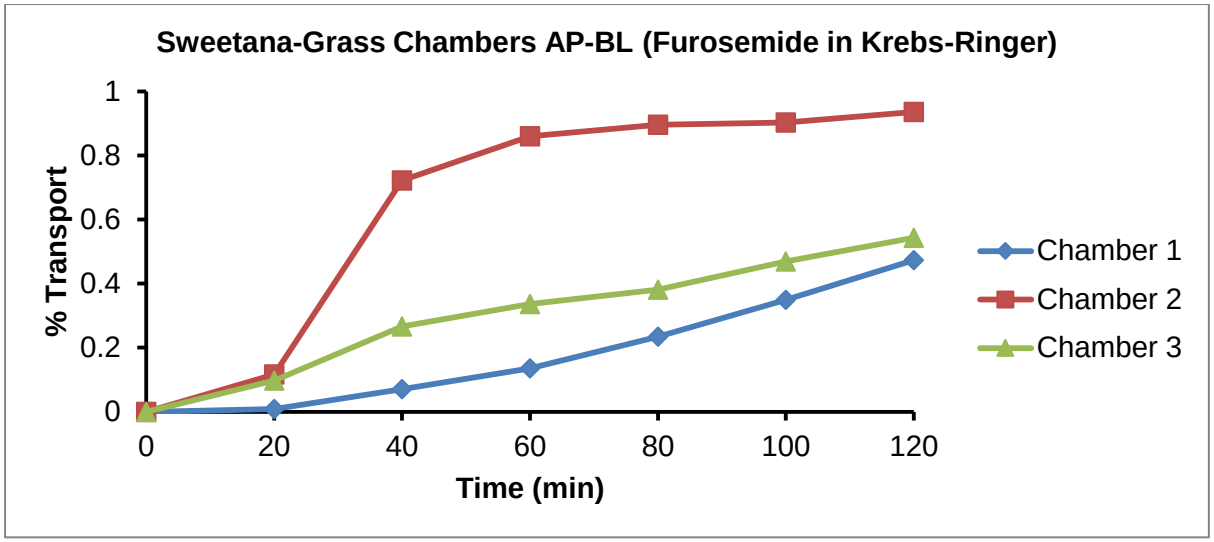


Figure C25: Percentage transport versus time of Furosemide in KRB (AP-BL)

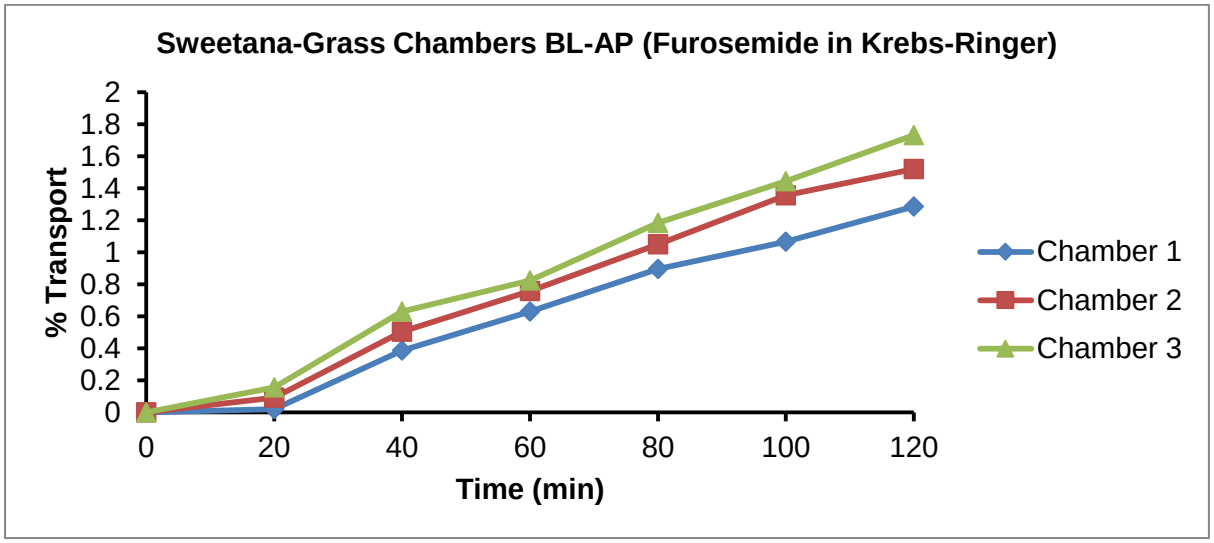


Figure C26: Percentage transport versus time of Furosemide in KRB (BL-AP)

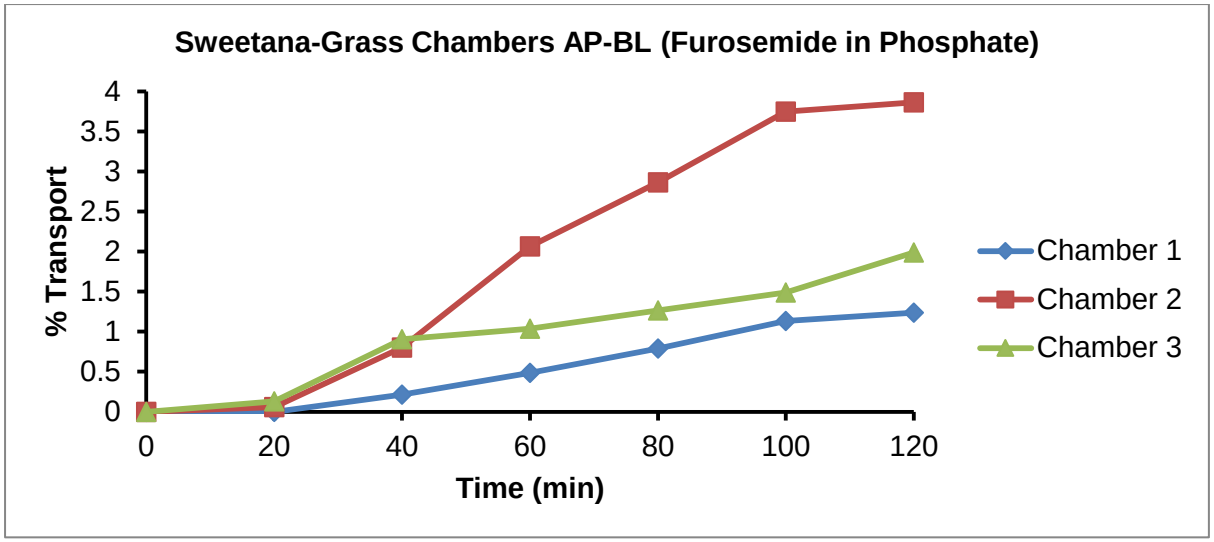


Figure C27: Percentage transport versus time of Furosemide in Phos (AP-BL)

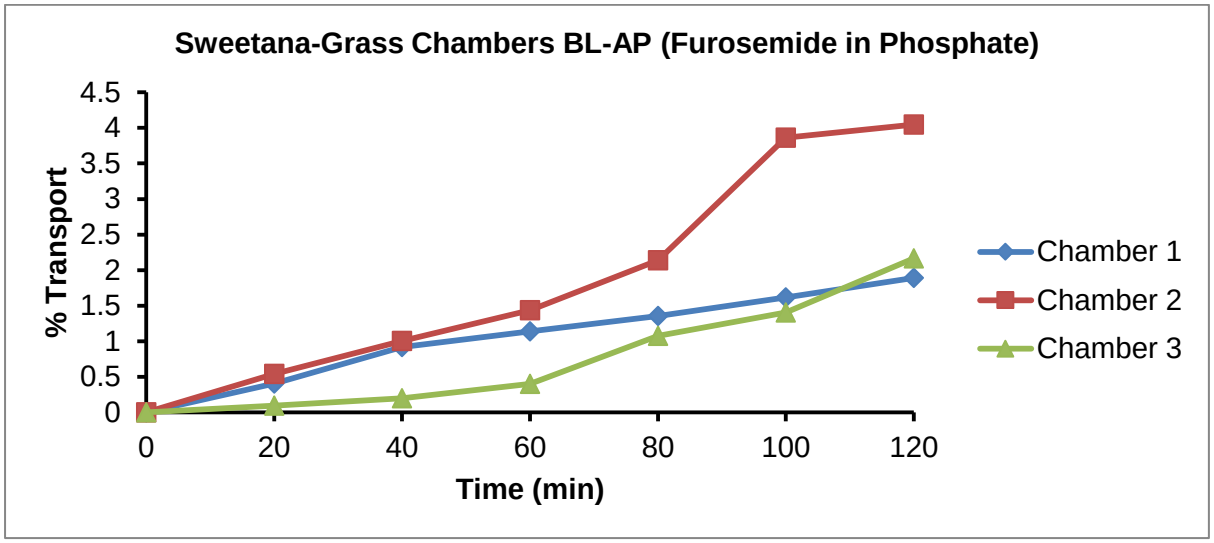


Figure C28: Percentage transport versus time of Furosemide in Phos (BL-AP)

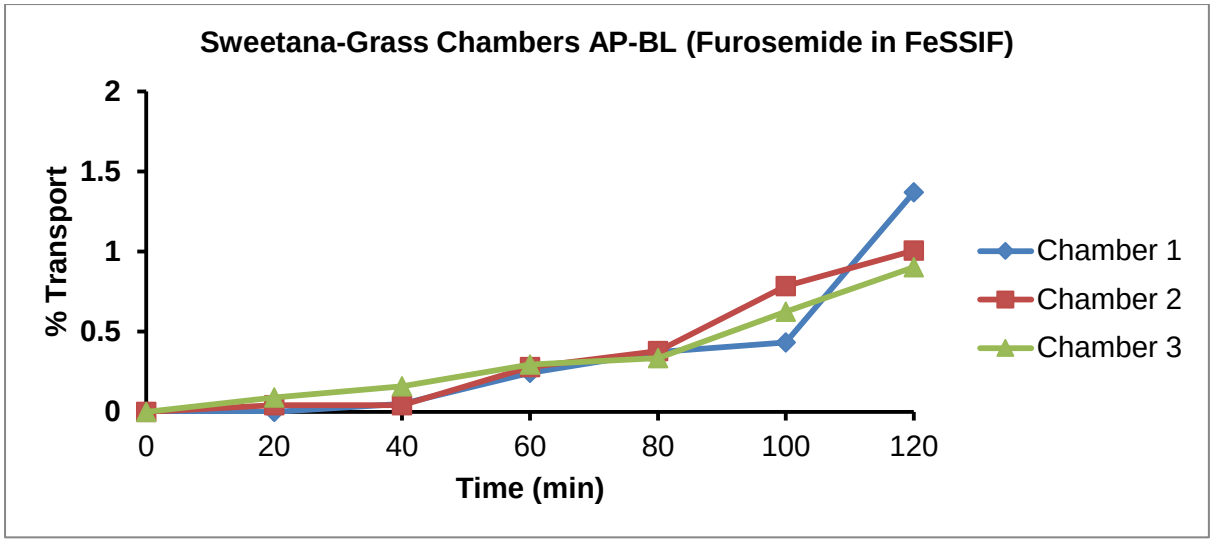


Figure C29: Percentage transport versus time of Furosemide in FeSSIF (AP-BL)

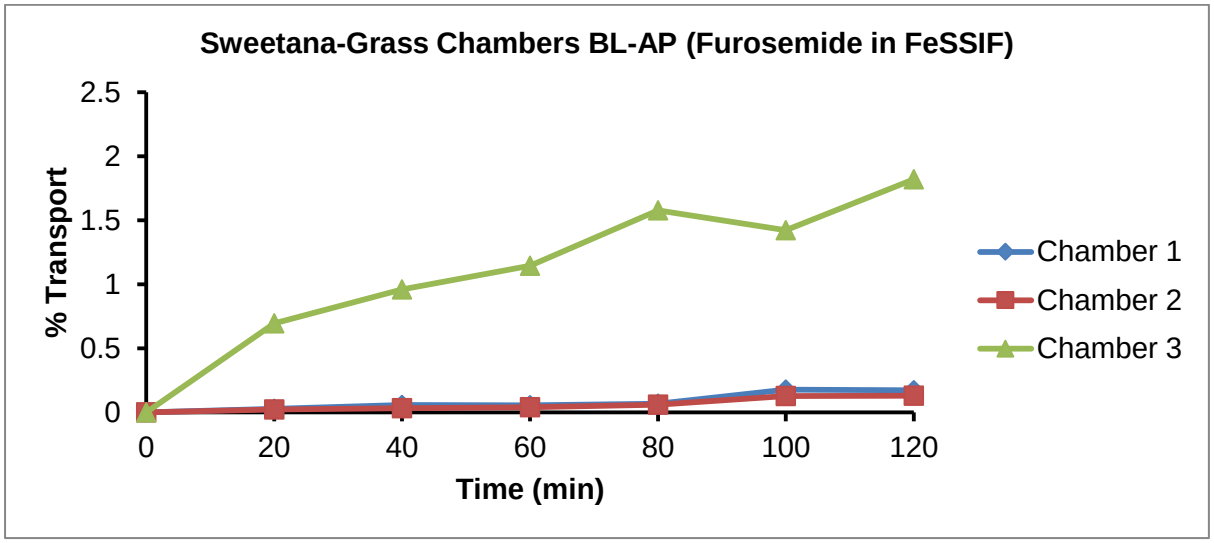


Figure C30: Percentage transport versus time of Furosemide in FeSSIF (BL-AP)

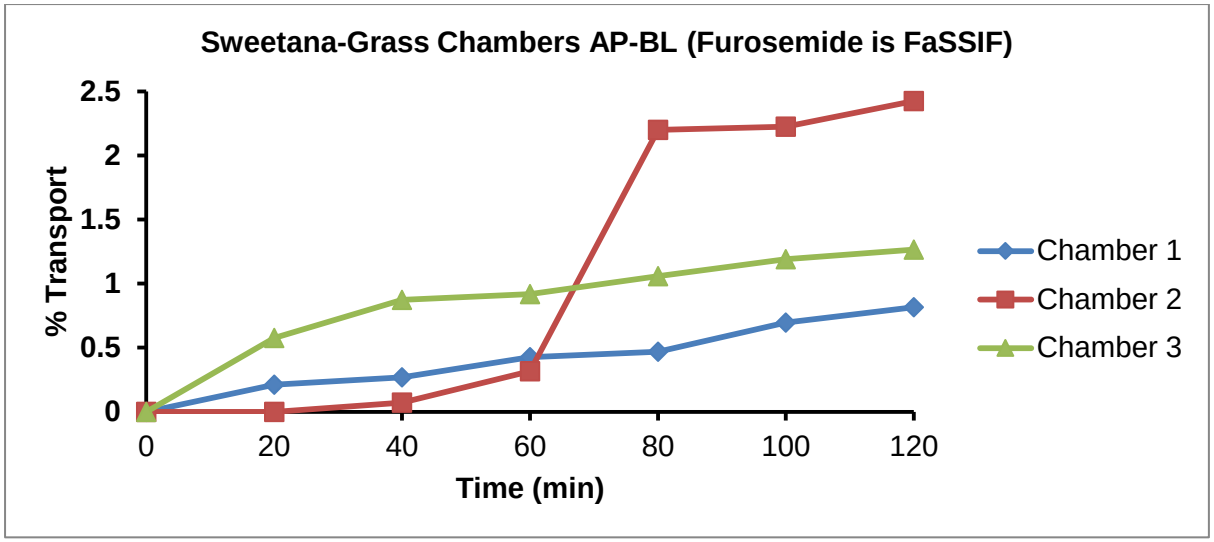


Figure C31: Percentage transport versus time of Furosemide in FaSSIF (AP-BL)

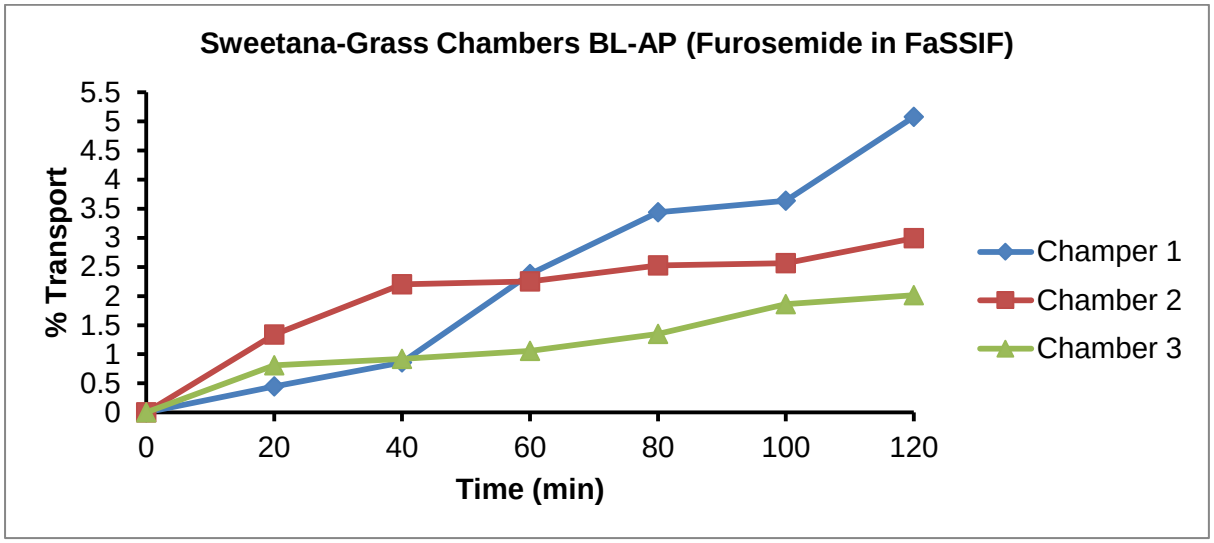


Figure C32: Percentage transport versus time of Furosemide in FaSSIF (BL-AP)

Table C1: Average P_{app} and ER of the test compounds in different media

Drug	Medium	Avg ER	Avg P_{app} a-b	Avg P_{app} b-a
Abacavir	**Krebs-Ringer Bicarbonate**	1.5	3.55E-06	5.60E-06
Abacavir	**Phosphate**	3.0	4.02E-06	8.83E-06
Abacavir	**FeSSIF**	1.4	2.72E-06	3.43E-06
Abacavir	**FaSSIF**	1.4	1.50E-06	2.08E-06
Lamivudine	**Krebs-Ringer Bicarbonate**	1.1	6.47E-06	7.00E-06
Lamivudine	**Phosphate**	1.8	5.21E-07	9.87E-07
Lamivudine	**FeSSIF**	1.8	4.10E-06	7.20E-06
Lamivudine	**FaSSIF**	1.1	2.18E-06	2.46E-06
Dapsone	**Krebs-Ringer Bicarbonate**	1.9	4.37E-07	7.17E-07
Dapsone	**Phosphate**	5.1	7.87E-07	2.85E-06
Dapsone	**FeSSIF**	1.1	6.29E-07	7.71E-07
Dapsone	**FaSSIF**	1.2	5.21E-07	6.22E-07
Furosemide	**Krebs-Ringer Bicarbonate**	2.6	5.19E-07	1.25E-06
Furosemide	**Phosphate**	1.1	2.04E-06	2.14E-06
Furosemide	**FeSSIF**	0.7	7.86E-07	5.01E-07
Furosemide	**FaSSIF**	3.1	1.26E-06	2.48E-06

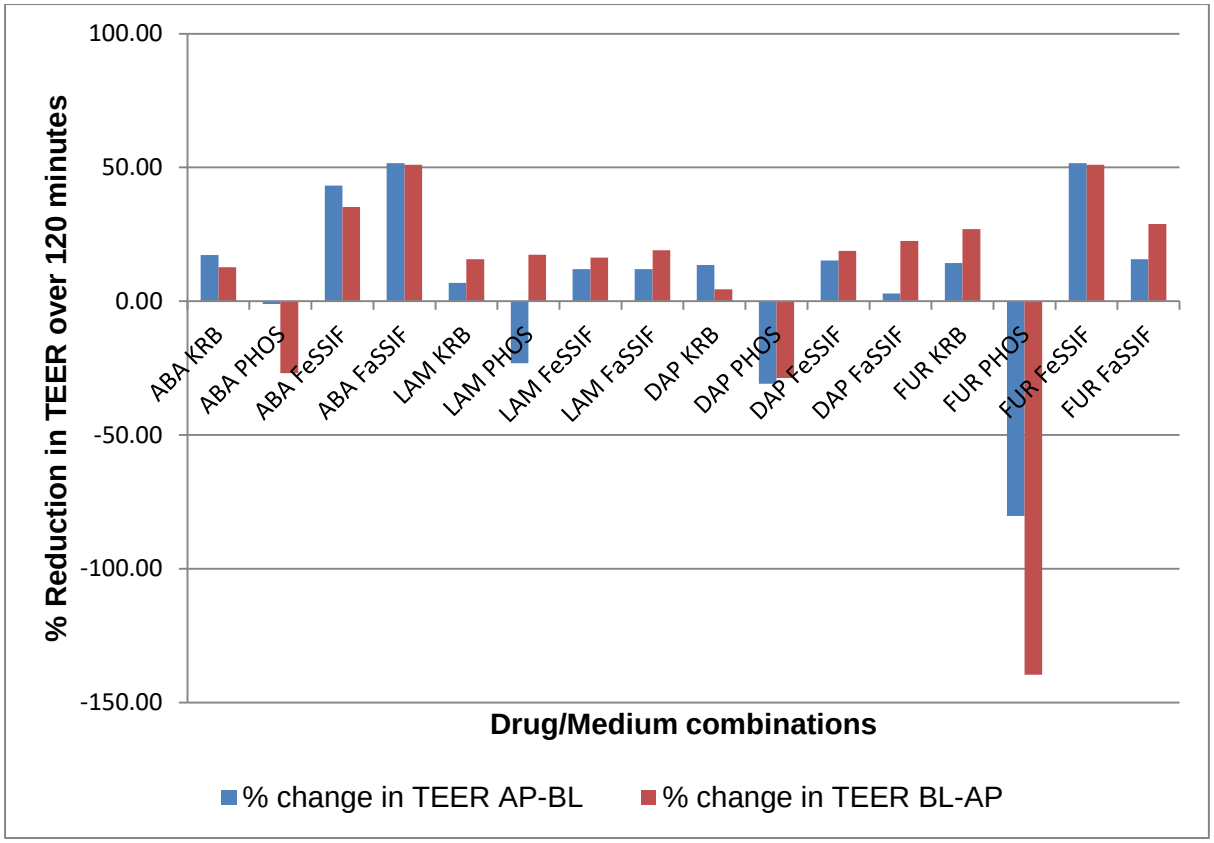


Figure C33: Average reduction in TEER values over 120 min. Negative values signify an increase in the TEER value over time.

Table C2: Average TEER reduction over 120 min.

Drug /Medium	% Reduction in TEER AP-BL	% Reduction in TEER BL-AP
ABA/KRB	17.24	12.65
ABA/PHOS	-1.06	-26.92
ABA/FeSSIF	43.23	35.16
ABA/FaSSIF	51.61	51.02
LAM/KRB	6.80	15.65
LAM/PHOS	-23.17	17.35
LAM/FeSSIF	11.96	16.35
LAM/FaSSIF	12.00	19.09
DAP/KRB	13.48	4.49
DAP/PHOS	-30.91	-28.68
DAP/FeSSIF	15.22	18.75
DAP/FaSSIF	2.83	22.50
FUR/KRB	14.29	26.90
FUR/PHOS	-80.28	-139.62
FUR/FeSSIF	51.61	51.02
FU/ FaSSIF	15.70	28.89

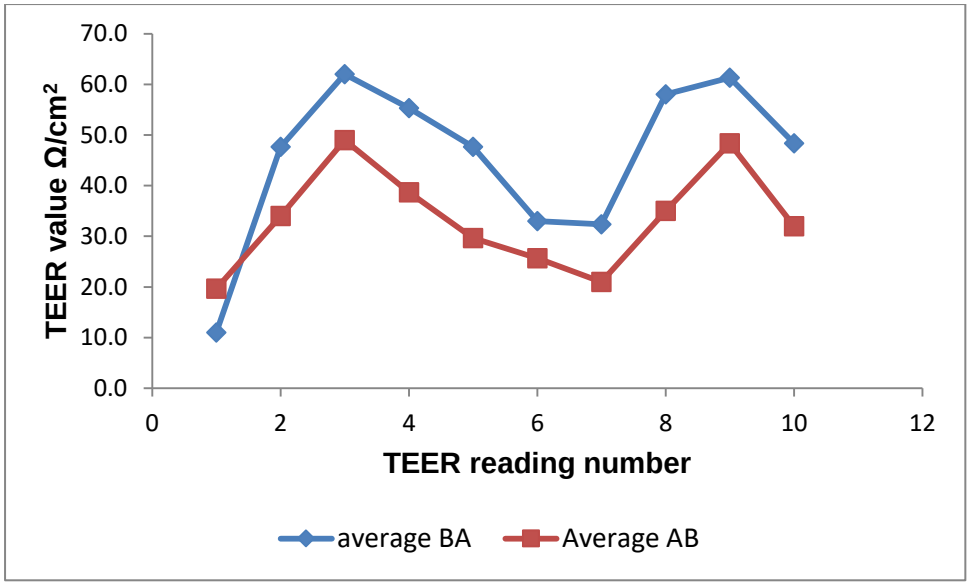


Figure C34: TEER values of ABA in KRB from initial buffer submersion. Drug containing buffer was added at reading 4.

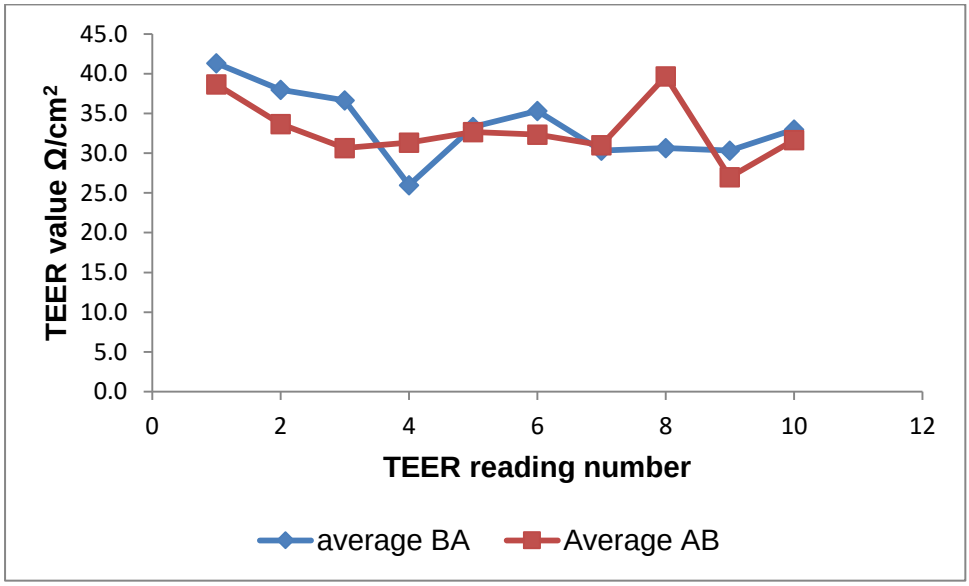


Figure C35: TEER values of ABA in Phos from initial buffer submersion. Drug containing buffer was added at reading 4.

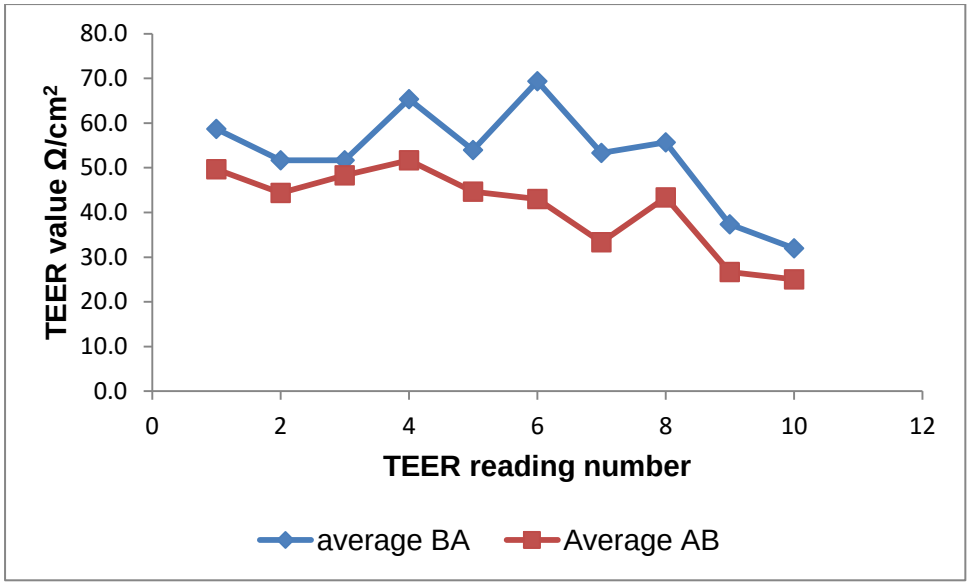


Figure C36: TEER values of ABA in FeSSIF from initial buffer submersion. Drug containing buffer was added at reading 4.

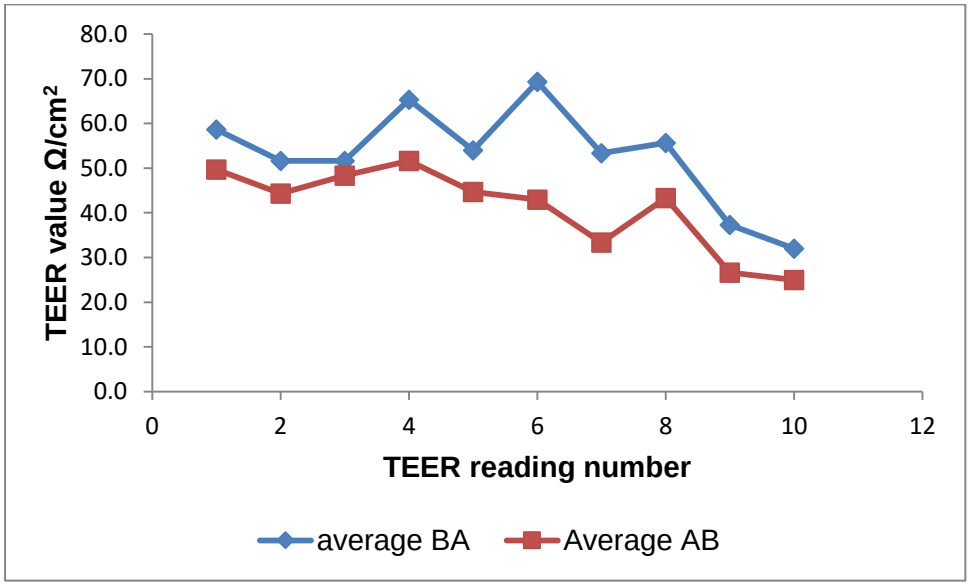


Figure C37: TEER values of ABA in FaSSIF from initial buffer submersion. Drug containing buffer was added at reading 4.

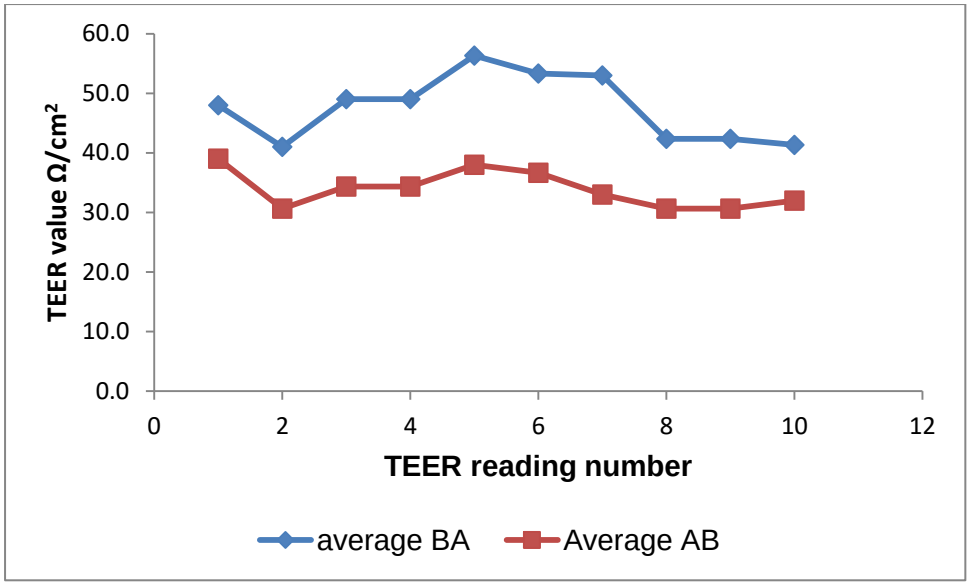


Figure C38: TEER values of LAM in KRB from initial buffer submersion. Drug containing buffer was added at reading 4.

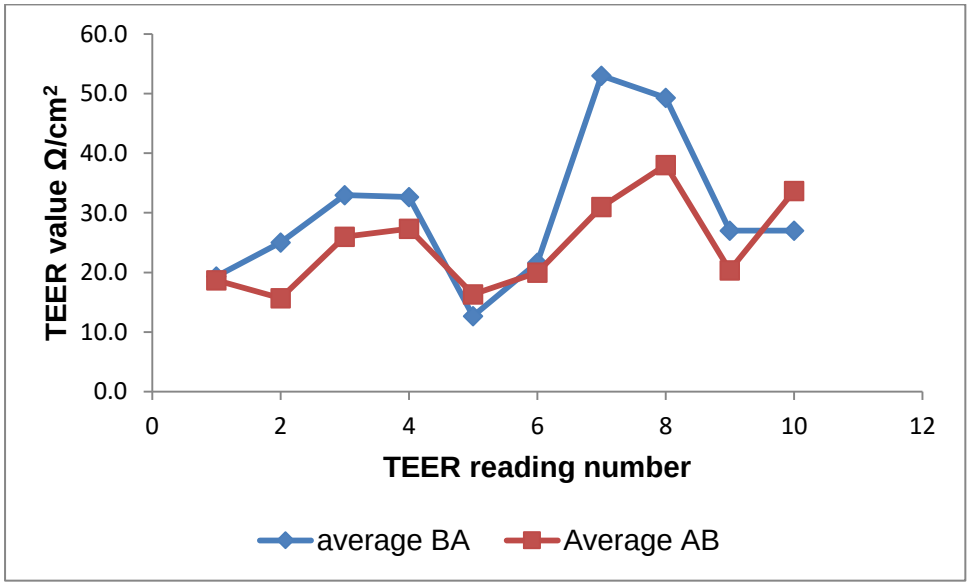


Figure C39: TEER values of LAM in Phos from initial buffer submersion. Drug containing buffer was added at reading 4.

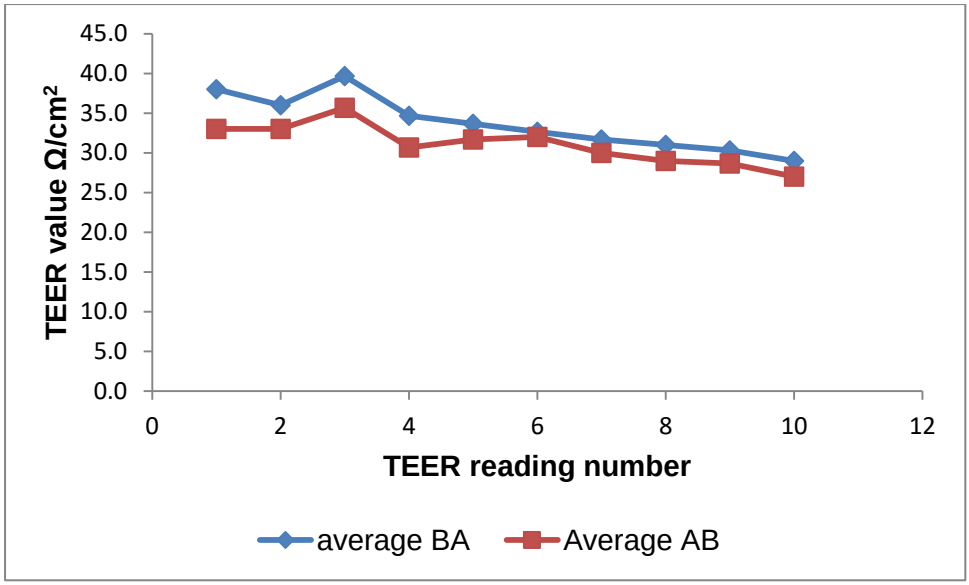


Figure C40: TEER values of LAM in FeSSIF from initial buffer submersion. Drug containing buffer was added at reading 4.

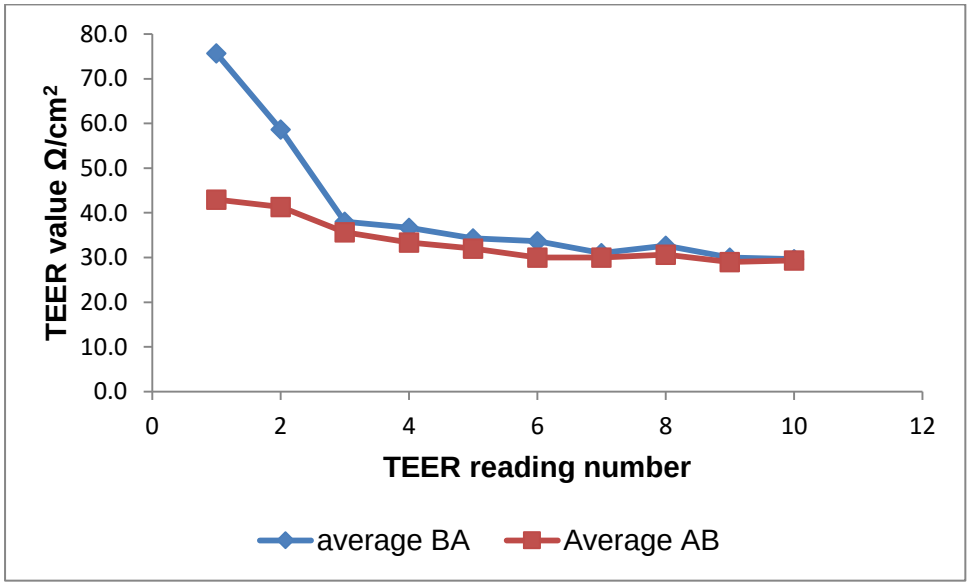


Figure C41: TEER values of LAM in FaSSIF from initial buffer submersion. Drug containing buffer was added at reading 4.

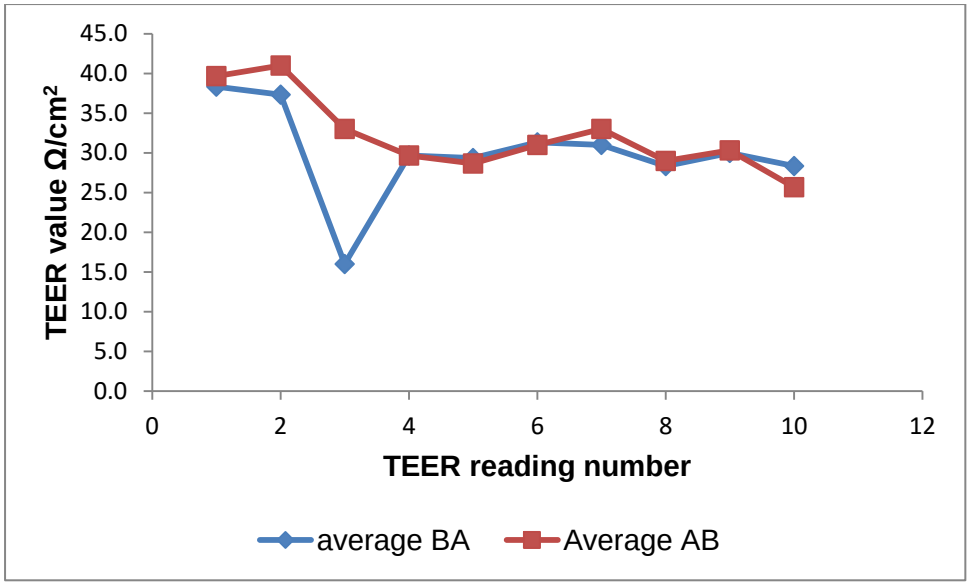


Figure C42: TEER values of DAPS in KRB from initial buffer submersion. Drug containing buffer was added at reading 4.

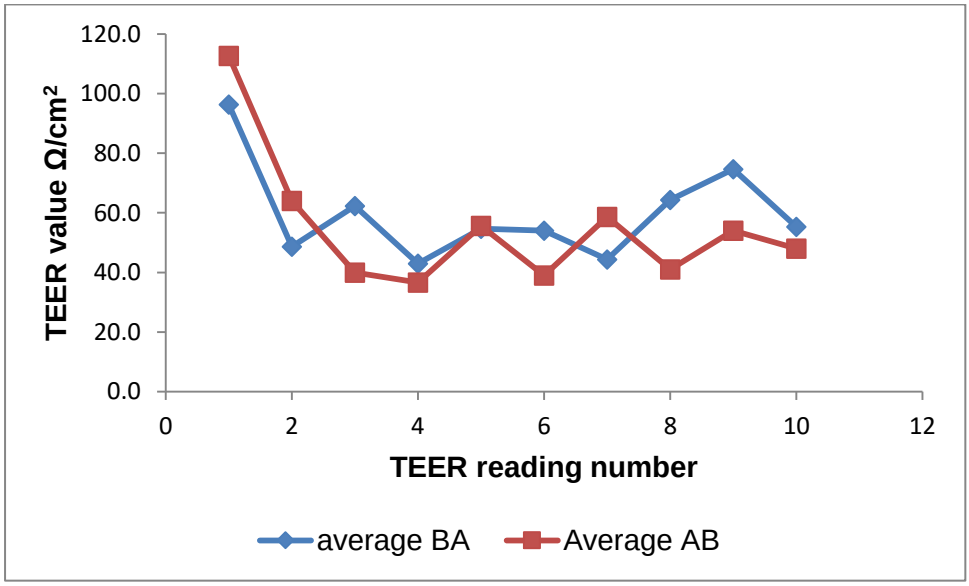


Figure C43: TEER values of DAPS in Phos from initial buffer submersion. Drug containing buffer was added at reading 4.

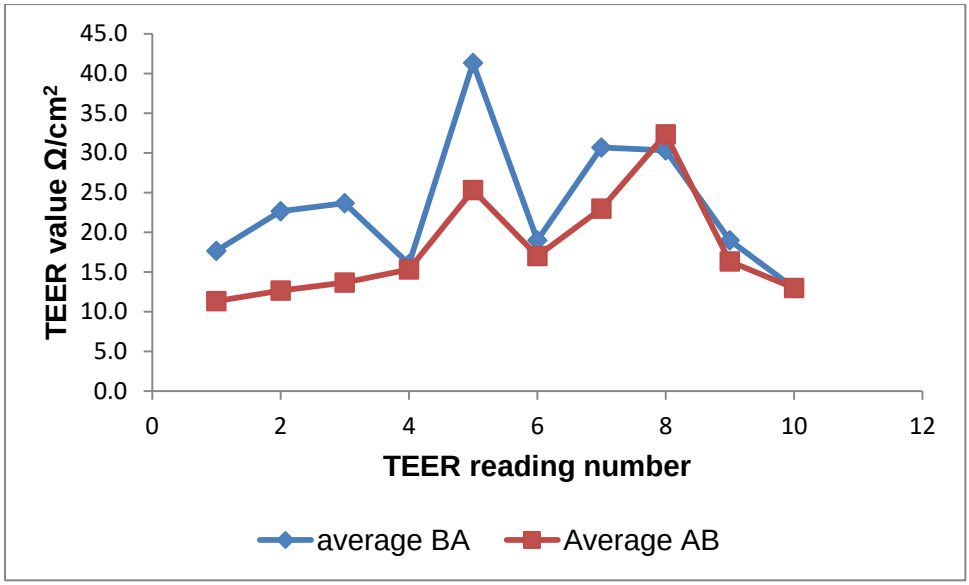


Figure C44: TEER values of DAPS in FeSSIF from initial buffer submersion. Drug containing buffer was added at reading 4.

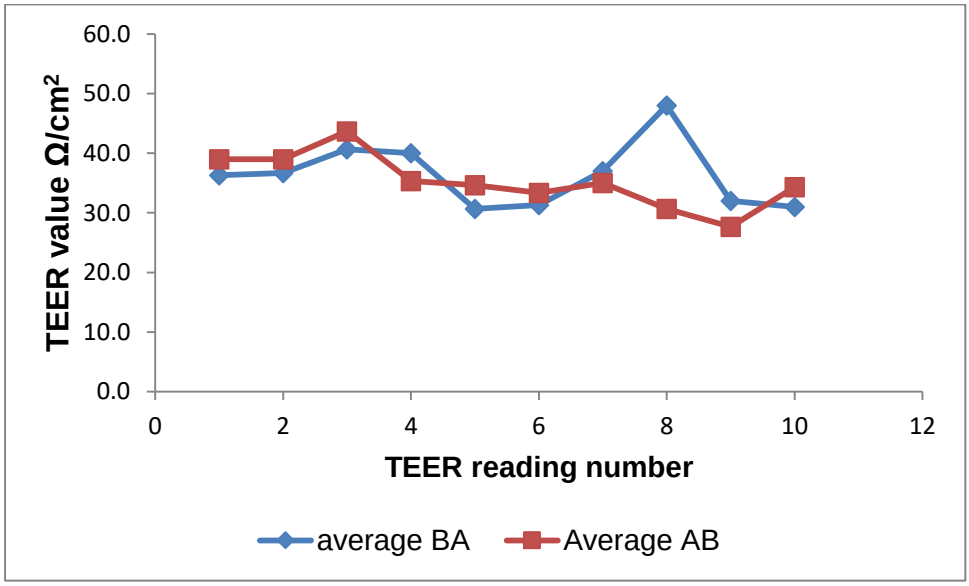


Figure C45: TEER values of DAPS in FaSSIF from initial buffer submersion. Drug containing buffer was added at reading 4.

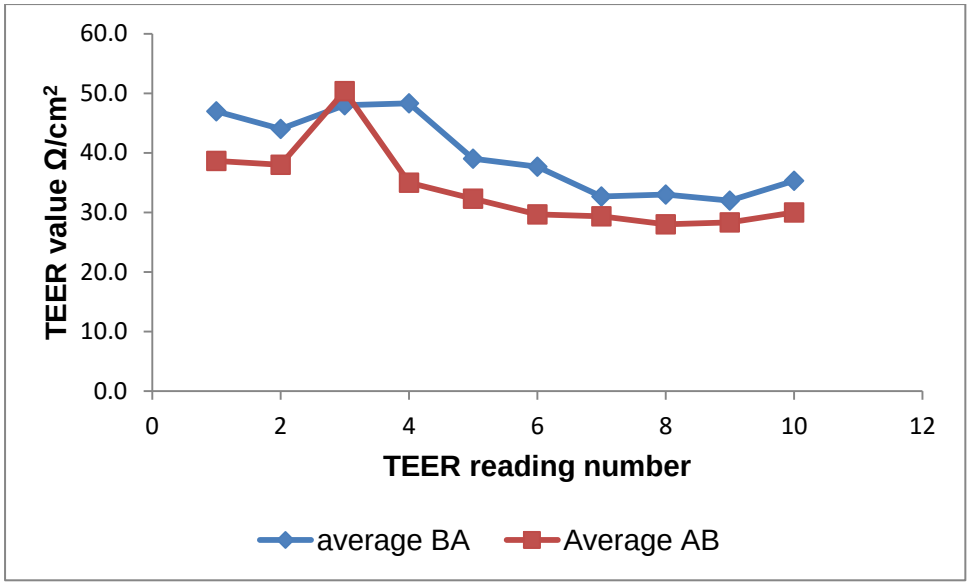


Figure C46: TEER values of Furos in KRB from initial buffer submersion. Drug containing buffer was added at reading 4.

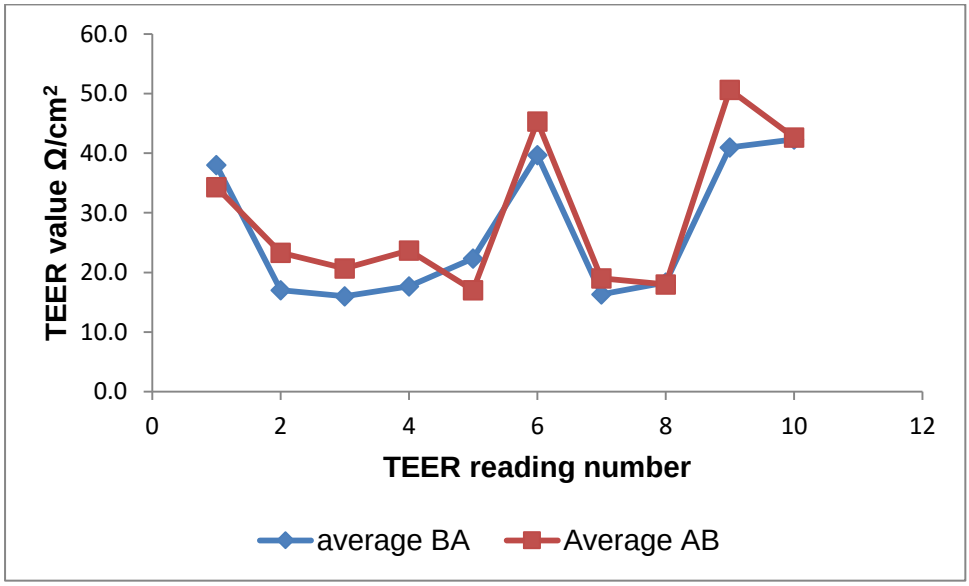


Figure C47: TEER values of Furos in Phos from initial buffer submersion. Drug containing buffer was added at reading 4.

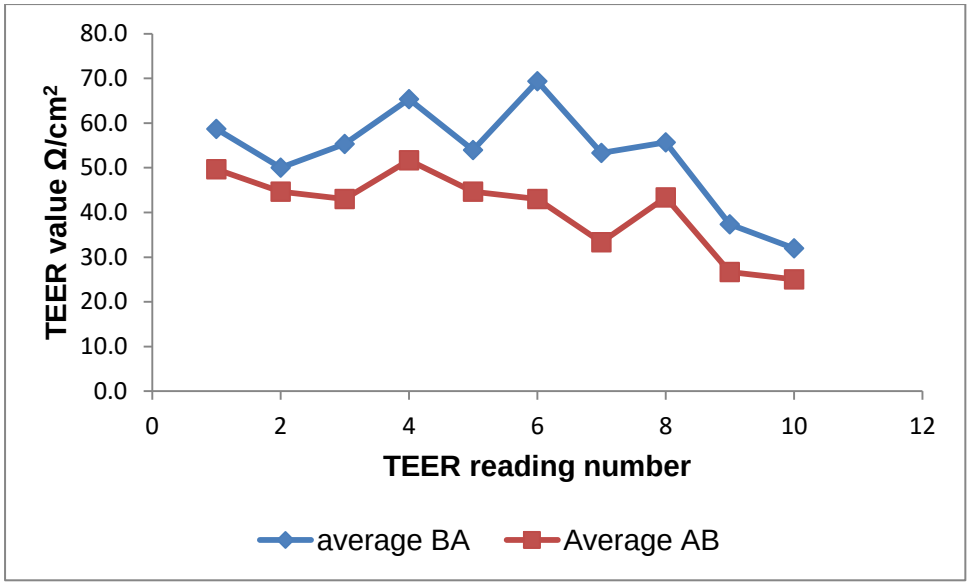


Figure C48: TEER values of Furos in FeSSIF from initial buffer submersion. Drug containing buffer was added at reading 4.

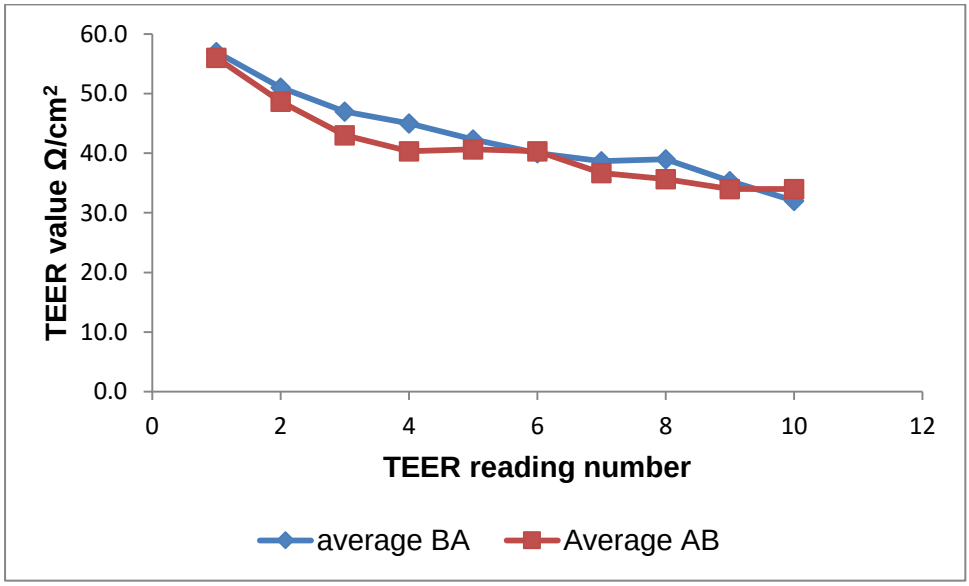


Figure C49: TEER values of Furos in FaSSIF from initial buffer submersion. Drug containing buffer was added at reading 4.

Table C3: Average solubility of test compounds in different media

	Test Compound solubility (mg/ml)			
Medium	**Abacavir**	**Lamivudine**	**Dapsone**	**Furosemide**
KRB	84.58	68.18	0.16	1.28
PHOS	120.47	78.69	0.28	0.39
FaSSIF	114.31	21.25	0.25	1.78
FeSSIF	82.64	17.78	0.25	2.48

Table C4: Statistical data: AP-BL transport

Papp AP-BL					
Drug	Medium	KRB	PB	FeSSIF	FaSSIF
Abacavir	Krebs-Ringer Bicarbonate		1.000000	0.999796	0.610274
Abacavir	Phosphate	1.000000		0.978262	0.289563
Abacavir	FeSSIF	0.999796	0.978262		0.987390
Abacavir	FaSSIF	0.610274	0.289563	0.987390	
Lamivudine	Krebs-Ringer Bicarbonate		0.000158	0.378599	0.002607
Lamivudine	Phosphate	0.000158041		0.021218	0.864344
Lamivudine	FeSSIF	0.378599049	0.021218		0.705822
Lamivudine	FaSSIF	0.002606913	0.864344	0.705822	
Dapsone	Krebs-Ringer Bicarbonate		0.999999	1.000000	1.000000
Dapsone	Phosphate	0.999999		1.000000	1.000000
Dapsone	FeSSIF	1.000000	1.000000		1.000000
Dapsone	FaSSIF	1.000000	1.000000	1.000000	
Furosemide	Krebs-Ringer Bicarbonate		0.923274	1.000000	0.999944
Furosemide	Phosphate	0.923274		0.983522	0.999904
Furosemide	FeSSIF	1.000000	0.983522		1.000000
Furosemide	FaSSIF	0.999944	0.999904	1.000000	

Table C5: Statistical data: BL-AP transport

Papp BL-AP					
Drug	Medium	KRB	PB	FeSSIF	FaSSIF
Abacavir	Krebs-Ringer Bicarbonate		0.719992	0.981831	0.596145
Abacavir	Phosphate	0.719992		0.065034	0.006867
Abacavir	FeSSIF	0.981831	0.065034		0.999872
Abacavir	FaSSIF	0.596145	0.006867	0.999872	
Lamivudine	Krebs-Ringer Bicarbonate		0.024011	1.000000	0.213442
Lamivudine	Phosphate	0.024011		0.017294	0.999649
Lamivudine	FeSSIF	1.000000	0.017294		0.166078
Lamivudine	FaSSIF	0.213442	0.999649	0.166078	
Dapsone	Krebs-Ringer Bicarbonate		1.000000	0.997566	0.994388
Dapsone	Phosphate	1.000000		0.987007	0.976031
Dapsone	FeSSIF	0.997566	0.987007		1.000000
Dapsone	FaSSIF	0.994388	0.976031	1.000000	
Furosemide	Krebs-Ringer Bicarbonate		0.999999	1.000000	0.999961
Furosemide	Phosphate	0.999999		0.998852	1.000000
Furosemide	FeSSIF	1.000000	0.998852		0.991877
Furosemide	FaSSIF	0.999961	1.000000	0.991877	

Table C6: Statistical data: ER

ER					
Drug	Medium	KRB	PB	FeSSIF	FaSSIF
Abacavir	Krebs-Ringer Bicarbonate		0.999991	1.000000	1.000000
Abacavir	Phosphate	0.999991		0.999957	0.999963
Abacavir	FeSSIF	1.000000	0.999957		1.000000
Abacavir	FaSSIF	1.000000	0.999963	1.000000	
Lamivudine	Krebs-Ringer Bicarbonate		1.000000	1.000000	1.000000
Lamivudine	Phosphate	1.000000		1.000000	1.000000
Lamivudine	FeSSIF	1.000000	1.000000		1.000000
Lamivudine	FaSSIF	1.000000	1.000000	1.000000	
Dapsone	Krebs-Ringer Bicarbonate		0.010411	0.000178	0.000184
Dapsone	Phosphate	0.010411		0.804755	0.832977
Dapsone	FeSSIF	0.000178	0.804755		1.000000
Dapsone	FaSSIF	0.000184	0.832977	1.000000	
Furosemide	Krebs-Ringer Bicarbonate		0.999985	0.999747	1.000000
Furosemide	Phosphate	0.999985		1.000000	0.999656
Furosemide	FeSSIF	0.999747	1.000000		0.997428
Furosemide	FaSSIF	1.000000	0.999656	0.997428	

Addendum D: Ethical approval

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Ethics Committee
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ETHICS APPROVAL OF PROJECT

The North-West University Research Ethics Regulatory Committee (NWU-RERC) hereby approves your project as indicated below. This implies that the NWU-RERC 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: Excised pig buccal and intestinal tissues as in vitro models for pharmacokinetic studies

Project Leader: Prof Sias Hamman

Ethics number:

N	W	U	-	0	0	0	2	5	-	1	5	-	A	5
Institution				Project Number						Year			Status	

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

Approval date: 2015-04-16 **Expiry date**: 2020-04-15

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-RERC:*
  - *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-RERC. 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-RERC and new approval received before or on the expiry date.*
- *In the interest of ethical responsibility the NWU-RERC 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-RERC 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

Linda du Plessis

Digitally signed by Linda du Plessis
DN: cn=Linda du Plessis, o=NWU, Vaal Triangle Campus, ou=Vice-Rector: Academic, email=linda.duplessis@nwu.ac.za, c=US
Date: 2015.04.20 20:35:13 +02'00'

Prof Linda du Plessis
Chair NWU Research Ethics Regulatory Committee (RERC)

Addendum E: Other works co-authored

- Joubert, R., Steyn, J.D., Heystek, H.J., Steenekamp, J.H., Du Preez, J.L. & Hamman, J.H. 2016. *In vitro* oral drug permeation models: the importance of taking physiological and physico-chemical factors into consideration. *Expert Opinion on Drug Delivery*: 1-9.