A STUDY TO CORRELATE DRUG ABSORPTION IN HUMANS (F_a) TO *IN VITRO* INTESTINAL PERMEATION USING THE SWEETANA-GRASS DIFFUSION MODEL

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ABSTRACT

Background: The oral route still remains the most popular method for drug delivery as it is convenient, cost efficient and has high patient compliance. However, for a drug to be successfully administered *via* this route it must be able to cross the intestinal membranes with a rate high enough (permeability) in order to enter the blood stream to produce the desired reaction. So, although a new chemical entity may possess pharmacological activity, it may not have the necessary physicochemical properties to be absorbed to such an extent that it will be of clinical value. For this reason it is necessary to have the means to accurately predict the *in vivo* permeability of drugs *in vitro* in a cost efficient manner. Permeability can be predicted by: (i) physicochemical characterisation, (ii) *in silico*, (iii) *in vitro*, (iv) *in situ* or (v) *in vivo* methods. Of these methods, *in vivo* studies are the most accurate, however, they have several drawbacks such as being time consuming, have high costs and ethical approval has to be obtained. Physicochemical characterisation and *in silico* methods are the easiest and cheapest to perform, but lack the physical interactions that occur at the membrane interface, thus lacking in reliability and close correlation with the *in vivo* situation. *In vitro* methods using intestinal tissue provides a compromise between the physicochemical characterisation and *in vivo* testing, as it is easy to perform and mimics the membrane interface found *in vivo*.

Aim: The aim of this study was to determine the permeability of selected drugs using rat intestine mounted in Sweetana-Grass diffusion chambers and compare the permeability observed with the absorption of the same drugs in humans using the F_a parameters in order to determine the feasibility of using this *in vitro* method to predict *in vivo* permeability. Two different methods of intestinal tissue preparation were also investigated to optimise the method further.

Methods: Validations of the various analytical methods used to determine the selected drugs quantitatively were performed. Jejunal tissue was prepared in one of two ways. It was either used as is (unstripped) or the serosal muscle layer was removed (stripped). The transport of caffeine, furosemide, verapamil, ketoprofen, propranolol, carbamazepine, promethazine, paracetamol, acyclovir and ranitidine was determined using a vertical diffusion chamber system, mounted with either stripped or unstripped tissue. All the studies were done in the

apical to basolateral direction. Permeability was expressed by the calculation of the apparent permeability co-efficient (P_{app}).

Results: The permeability of all drugs studied was lower than found in previous studies with Caco-2 cells. This can be expected because of the difference in the composition of the rat intestine and the Caco-2 cell monolayer as well as the longer path length the drugs have to travel during the absorption process through the jejunal tissue. The data show a non-linear relationship between F_a and P_{app}. For the stripped method of tissue preparation the r^2 value obtained was 0.0579 indicating poor correlation. For the unstripped method of tissue preparation the r^2 value was 0.2877, indicating poor but improved correlation compared to the stripped method of tissue preparation. Graphs used to indicate a correlation between P_{app} and F_a showed aciclovir to be an outlier. Removal of this drug from the equation gave an r^2 of 0.4013 for the stripped tissue preparation and 0.4623 for unstripped tissue preparation indicating a much better correlation between transport across rat intestine and fraction absorbed in humans.

Conclusion: It was possible to determine the P_{app} values for ten selected drugs in this study by using the *in vitro* Sweetana-Grass diffusion method. These P_{app} values showed a non-linear relationship between F_a and P_{app} in rat jejunum, as has been observed in other studies. When comparing the methods of tissue preparation, the unstripped method of tissue preparation gave a better correlation between P_{app} and F_a (r^2 = 0.0579 compared to r^2 = 0.2877). If the outlier aciclovir is excluded, the correlation improves significantly (stripped r^2 = 0.4013 unstripped r^2 = 0.4623). A possible reason for the high permeability of aciclovir observed in this study may be the fact that its transport is concentration dependant. Also, the absorption of aciclovir varies across species, thus not making it ideal for a study comparing transport across rat intestine to absorption in humans. This method of intestinal transport prediction does show a correlation with fraction absorbed in humans, however a larger number of drugs, with a wider spread of F_a values should be evaluated before it can be used to predict *in vivo* absorption with confidence.

OPSOMMING

Agtergrond: Die orale roete is steeds die mees gewilde metode vir geneesmiddelaflewering omdat dit gerieflik en koste-effektief is en pasiënte goed daarmee saamwerk. Vir 'n geneesmiddel om suksesvol deur hierdie roete afgelewer te kan word, moet dit egter die intestinale membrane kan oorsteek teen 'n tempo hoog genoeg (permeabiliteit) sodat dit die bloedstroom kan bereik om die gewenste effek uit te oefen. Dit kan dus wees dat hoewel 'n nuwe chemiese entiteit farmakologiese aktiwiteit besit, dit nie die nodige fisies-chemiese eienskappe het om tot so 'n mate geabsorbeer te word om van kliniese waarde te wees nie. Om hierdie rede is dit nodig om 'n manier te hê waarmee die *in vivo*-permeabiliteit op 'n koste-effektiewe wyse *in vitro* bepaal kan word. Permeabiliteit kan deur (i) fisies-chemiese karakterisering, of met (ii) *in silico-*, (iii) *in vitro-*, (iv) *in situ-* of (v) *in vivo*-metodes voorspel word. Van hierdie metodes is *in vivo*-studies die mees akkurate, hoewel dit verskeie tekortkominge het, soos dat dit tydrowend en duur is en dat etiese goedkeuring verkry moet word. Fisies-chemiese karakterisering en *in silico*-metodes is die maklikste en goedkoopste, maar besit nie die fisiese interaksies wat by die membraaninterfase plaasvind nie en het dus nie die betroubaarheid en noue korrelasie met die *in vivo*-situasie nie. *In vivo*-metodes wat intestinale weefsel gebruik verskaf 'n kompromie tussen die fisies-chemiese karakterisering en *in vivo*-toetsing omdat dit maklik is om te doen en die *in vivo*-membraan naboots.

Doel: Die doel van hierdie studie was om die permeabiliteit van geselekteerde geneesmiddels te bepaal deur rotderm te gebruik wat in Sweetana-Grass-diffusiekamers gemonteer is, en om die waargenome permeabiliteit te vergelyk met die absorpsie van dieselfde geneesmiddels in mense deur die F_a-parameters te gebruik ten einde die geskiktheid van die *in vitro*-metode vir die voorspelling van *in vivo*-permeabiliteit te bepaal. Twee verskillende metodes vir die voorbereiding van intestinale weefsel is ook ondersoek om die metode verder te optimaliseer.

Metodes: Validering van die verskillende analitiese metodes gebruik om die geselekteerde geneesmiddels kwantitatief te bepaal, is gedoen. Jejenumweefsel is op een van twee metodes voorberei. Dit is óf gebruik net soos dit is (ongestroop) óf die sereuse spierlaag is verwyder (gestroop). Die transport van kaffeïen, furosemied, verapamiel, ketoprofeen, propranolol, karbaamasepien, prometasien, parasetamol, asiklovir en ranitidien is bepaal deur 'n vertikale diffusiekamerstelsel te gebruik waarin óf gestroopte óf ongestroopte weefsel gemonteer is. Al die studies is in die apikale na basolaterale rigting gedoen. Permeabiliteit is uitgedruk deur berekening van die skynbare permeabiliteitskoëffisiënt (P_{app}).

Resultate: Die permeabiliteit van alle bestudeerde geneesmiddels was laer as wat in vorige studies met Caco-2-selle gevind is. Dit is te verwagte vanweë die verskil tussen die rotderm en die enkellaag Caco-2-selle asook die langer padlengte wat die geneesmiddels tydens die absorpsieproses deur die jejenumweefsel moet aflê. Die data toon 'n nielineêre verwantskap tussen F_a en P_{app}. Met gestroopte weefsel is 'n r^2-waarde van 0.0579 verkry wat 'n swak korrelasie aantoon. Met ongestroopte weefsel was die r^2-waarde 0.2877 wat 'n swak korrelasie, maar beter as met gestroopte weefsel aantoon. Grafieke wat gebruik is om die korrelasie tussen F_a en P_{app} aan te toon, het gewys dat asiklovir 'n uitskieter is. Nadat hierdie middel uit die groep verwyder is, is 'n r^2-waarde van 0.4013 vir die gestroopte weefsel en 0.4623 vir die ongestroopte weefsel verkry wat 'n baie beter korrelasie tussen transport oor die rotderm en die geabsorbeerde fraksie in mense aantoon.

Gevolgtrekking: Dit was moontlik om die P_{app}-waardes vir tien geselekteerde geneesmiddels in hierdie studie te bepaal deur die *in vitro* Sweetana-Grass-diffusiemetode te gebruik. Hierdie P_{app}-waardes het 'n nielineêre verwantskap tussen F_a en P_{app} in die rotjejunum aangetoon soos wat in ander studies waargeneem is. Vergelyking van die twee metodes vir die voorbereiding van die weefsel toon dat ongestroopte weefsel 'n beter korrelasie tussen P_{app} en F_a gee (r^2 = 0.0579 vergeleke met r^2 = 0.2877). As die uitskieter asiklovir weggelaat word, verbeter die korrelasie beduidend (gestroopte r^2 = 0.4013, ongestroopte r^2 = 0.4623). 'n Moontlike rede vir die hoë permeabiliteit van asiklovir waargeneem in hierdie studie kan die feit wees dat die transport daarvan van die konsentrasie afhanklik is. Die absorpsie van asiklovir wissel ook tussen spesies wat dit nie die ideale middel vir die vergelyking van transport oor die rotjejunum met absorpsie in die mens maak nie. Hierdie metode vir die voorspelling van intestinale transport toon 'n korrelasie met die fraksie geabsorbeer in mense, maar 'n groter aantal geneesmiddels met 'n wyer verspreiding van F_a-waardes moet beoordeel word voordat dit gebruik kan word om *in vivo*-absorpsie met vertroue te kan voorspel.

INTRODUCTION AND STATEMENT OF THE PROBLEM

Of all drug delivery routes, the oral route still remains the most preferred. This is due to its convenience, low cost and high patient compliance. However, drugs intended for oral use need to be able to cross the intestinal membrane in order to reach their site of action. With the growth in the technology used for the synthesis of new compounds, the rate determining step in the development of new drugs is no longer the discovery of new chemical entities (NCE), but rather the screening of these compounds for suitable biopharmaceutical properties (Balimane *et al.,* 2000:301).

With the high cost associated with the development of drugs, it is essential that drug permeability be established as soon as possible in order to eliminate NCEs which will be of no use as they possess insufficient intestinal permeability and thus won't be bioavailable. This has provided great impetus within the pharmaceutical industry to implement appropriate screening models which have capacity, are cost-effective and are highly predictive of *in vivo* permeability (Balimane *et al.,* 2000:301).

These screening methods can also be used to classify drugs according to the biopharmaceutical classification system (BCS). This system classifies drugs according to their aqueous solubility and intestinal permeability. The classification scheme provides a basis for establishing *in vitro–in vivo* correlations and for estimating the absorption of drugs based on the fundamental dissolution and permeability properties of physiologic importance (Amidon *et al.,* 1995:413). The BCS classification of a drug can be used to apply for a waiver of *in vivo* bioavailability and bioequivalence studies for immediate release solid oral dosage forms. The criteria for such a waiver and the methods for determining permeability and solubility are given in 'Guidance for industry: Waiver of *in vivo* bioavailability and bioequivalence studies for immediate-release solid oral dosage forms based on a biopharmaceutics classification system' (FDA, 2000:1).

Thus, the rapid and accurate prediction of intestinal permeability is not only extremely useful in the development of new chemical entities, but also in die registration of bioequivalent rapid release dosage forms.

The permeability of drugs and drug substances can be predicted by one of five methods, each with their own advantages and disadvantages. These include physicochemical characterisation and *in silico*, *in vitro*, *in situ* and *in vivo* methods. Of these methods physicochemical characterisation and *in silico* determination are closely linked and provide the fastest route for permeability screening. These methods, however, depend on large databases and lack the direct interaction with the membrane that is found with other methods (Hämäläinen & Frostell-Karlsson, 2004:400).

While *in situ* and *in vivo* methods are ideal for testing of drugs, they are not suitable for screening of compounds, as the methods are laborious and require specialised surgical procedures and large amounts of laboratory animals, making them expensive and therefore they are not used often (Habucky, 1995:30, Grass, 1997:203).

In vitro methods are the most popular method of prediction at the moment, with transport studies in Caco-2 cells being used most frequently (Mälkiä *et al.*, 2004:24). A method which is becoming increasingly popular is the use of excised intestinal tissue mounted in Ussing or Ussing-like chambers. In this method, the morphology of the membrane more closely resembles the *in vivo* situation, however, the tissue has limited viability and shows a higher degree of variation when compared to Caco-2 cells. The effect of the stripping of the muscle and serosa layers on the transport of chemical entities across intestinal mucosa has not previously been investigated (Hämäläinen & Frostell-Karlsson, 2004:399).

The aims of this study were to:

- determine the apparent permeability coefficient of ten selected drugs with diverse absorption characteristics in humans using the Sweetana-Grass diffusion technique,

- compare the effect of stripping of the serosal layer from the jejenum tissue membranes on the apparent permeability coefficients of the various selected drugs with the apparent permeability coefficient observed with the unstripped jejunum tissue membranes and

- correlate the apparent permeability coefficients obtained *in vitro* with the fraction absorbed in man (F_a) as found in literature.

CHAPTER 1
PREDICTION OF INTESTINAL PERMEABILITY

1.1 Introduction

Despite tremendous innovations in drug delivery methods in the last few decades, the oral route still remains the most preferred route of administration for most new chemical entities (NCE). The oral route is preferred by virtue of its convenience, low cost, and high patient compliance compared to other routes. However, compounds intended for oral administration must have adequate aqueous solubility as well as intestinal permeability in order to achieve therapeutic concentrations in the blood. With the explosive growth in the field of genomics and combinatorial chemistry coupled with technological innovations in the last few years, synthesising a large number of potential drug candidates is no longer a bottleneck in the drug discovery process. Instead, the task of screening compounds simultaneously for biological activity and biopharmaceutical properties (e.g. solubility, permeability/absorption, stability, etc.) has become the major challenge (Balimane *et al.,* 2000: 301).

The ability of a drug to cross biological membranes shapes its pharmacokinetic profile in the body, affecting absorption, distribution and elimination and thus the duration of its clinical action. The importance of this ability can be seen in the fact that according to the Centre for Medicines Research (UK), up to 39% of drugs fail during development due to poor pharmacokinetic properties (Mälkiä *et al.,* 2004:13, Kennedy, 1997:442).

In today's cost-constrained pharmaceutical environment, it is essential that researchers have an accurate means of predicting the *in vivo* permeability of potential drugs as early as possible. This can help medicinal chemists to optimise absorption characteristics and avoid wasting valuable resources on developing drugs that are ultimately destined to fail (Hämäläinen & Frostell-Karlsson, 2004:397). This has provided a great impetus within the pharmaceutical industry to implement appropriate screening models that have high capacity, are cost-effective and are highly predictive

of *in vivo* permeability and absorption (Balimane *et al.*, 2000: 301).

1.2 The biopharmaceutics classification system

To this end the biopharmaceutics classification system (BCS) has been established. This system divides drugs into high/low solubility-permeability classes and the expectations regarding *in vitro-in vivo* correlations are more clearly stated (Amidon *et al.*, 1995: 417). There are four classes in this system and they are discussed below.

Class 1: High solubility-high permeability drugs. This is the case where the drug is well absorbed (though its systemic availability may be low due to first pass extraction/metabolism) and the rate limiting step to drug absorption is drug dissolution or gastric emptying if dissolution is very rapid. In this case the dissolution profile must be well defined and reproducible to ensure bioavailability. For immediate release dosage forms that dissolve very rapidly, the absorption rate will be controlled by the gastric emptying rate and no correlation with dissolution rate is expected (Amidon *et al.*, 1995: 417).

Class 2: Low solubility-high permeability drugs. This is the class of drugs for which the dissolution profile must be most clearly defined and reproducible. More precisely this is the case where absorption is high, while dissolution is low. Drug dissolution *in vivo* is then the rate controlling step in drug absorption and absorption is usually slower than for class 1 (Amidon *et al.*, 1995: 417).

Class 3: High solubility-low permeability drugs. For this class of drug, permeability is the rate limiting step in drug absorption. While the dissolution profile must be well defined, the simplification in dissolution specification as in class 1 is applicable for immediate release dosage forms where drug input to the intestine is gastric emptying rate controlled. Both the rate and extent of drug absorption may be highly variable for this class of drugs, but if dissolution is fast, i.e. 85% dissolved in less than 15 min, this variation will be due to the variable gastrointestinal transit, luminal contents and membrane permeability rather than dosage form factors (Amidon *et al.*, 1995: 417).

Class 4: Low solubility-low permeability drugs. This class of drug presents significant problems for effective oral delivery. The number of drugs that fall into this class will depend on the precise limits used for the permeability and solubility classification (Amidon *et al.,* 1995: 417).

1.2.1 Uses for the BCS

The BCS is a scientific framework for classifying drug substances based on their aqueous solubility and intestinal permeability. When combined with the dissolution of the drug product, the BCS takes into account three major factors that govern the rate and extent of drug absorption from immediate release (IR) solid dosage forms: dissolution, solubility and intestinal permeability (Amidon *et al.,* 1995:413). In addition, IR solid dosage forms are categorised as having rapid or slow dissolution. Within this framework, when certain criteria are met, the BCS can be used as a drug development tool to help sponsors justify requests for waivers of *in vivo* bioavailability (BA) and bioequivalence (BE) studies (FDA, 2000:1).

Observed *in vivo* differences in the rate and extent of absorption of a drug (bioavailability) from two pharmaceutically equivalent solid oral products may be due to differences in drug dissolution *in vivo*. However, when the *in vivo* dissolution of an IR solid oral dosage form is rapid in relation to gastric emptying and the drug has high permeability, the rate and extent of drug absorption is unlikely to be dependent on drug dissolution and/or gastrointestinal transit time. Under such circumstances, demonstration of *in vivo* BA or BE may not be necessary for drug products containing Class 1 drug substances, as long as the inactive ingredients used in the dosage form do not significantly affect absorption of the active ingredients (FDA, 2000:2).

1.3 Permeability

Before orally delivered drugs can exert their effects, they must dissolve and pass through the lipid bilayer of epithelial cells lining the intestinal wall. Several mechanisms of transport are possible (Figure 1.1). Passive diffusion, where molecules move through or between cells (transcellular and paracellular,

respectively), is by far the most common route for pharmaceutical compounds. Polar or hydrophilic compounds tend to be transported paracellularly but this pathway is limited to molecules of less than 200 Da because of small pore sizes at the tight junctions between cells. Most compounds are larger than this and take the transcellular route (Hämäläinen & Frostell-Karlsson, 2004:398).

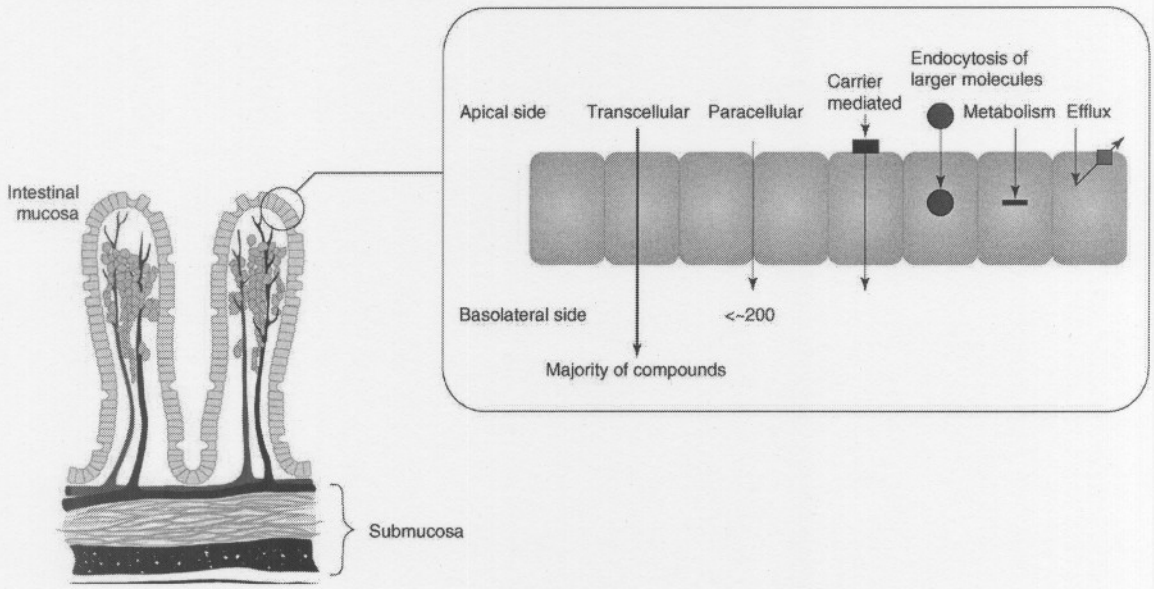


Figure 1.1: A schematic representation of the intestinal wall, with insert showing different routes of drug entry from the intestine into the blood stream (Hämäläinen & Frostell-Karlsson, 2004:399).

Gastrointestinal permeability is an estimate of the selective ability of intestinal epithelium to provide a barrier to absorption of drugs (Chaturvedi *et al.,* 2001:453). The brush border of the intestine is the barrier that must be traversed by nutrients, water, electrolytes and drugs on the way to the blood or lymph. The plasma membrane of the enterocyte is considered the only factor restricting the free movement of substances from the gut lumen into the blood or lymph. However, transmural movement actually takes place over a complex pathway. This is conveyed schematically in Figure 1.2 and includes an unstirred layer of fluid, the glycocalyx covering the microvilli, the cell membrane, the cytoplasm of the enterocyte, the basal or lateral cell membrane, the intercellular space, the basement membrane and the membrane of the capillary or lymph vessel (Johnson, 1997:114).

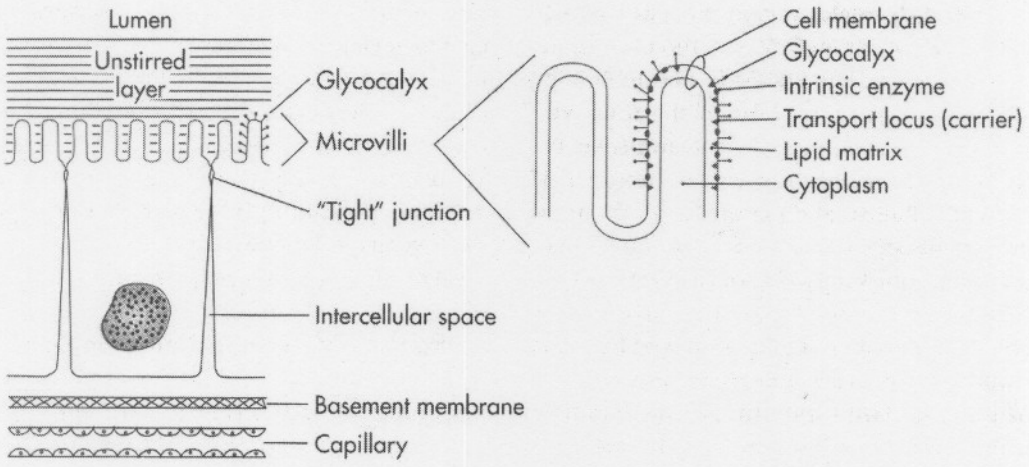


Figure 1.2: Pathway for drug absorption (Johnson, 1997:114).

Absorption of drugs is also affected by formulation and the stability of the dosage form of the drug, contents of the gastrointestinal tract, residence time in the intestine, aqueous solubility of the drug, intestinal metabolism, carrier-mediated influx *via* active transporter mechanisms and active transporters such as p-glycoprotein (p-gp) or the multidrug resistance protein (MRP) families. These various factors can confound one another, i.e. the individual effect of each factor is difficult or even impossible to determine because they occur simultaneously. For example, a molecule's poor absorption could be due to a strong affinity for the p-gp transporter, despite the molecule actually being passively permeable, or a molecule could be highly soluble but also too hydrophilic to pass through the cell membrane (Egan & Lauri, 2002:274).

1.3.1 Methods for determining permeability

The permeability of drugs and drug substances can be predicted/determined by one of five methods: physicochemical characterisation, *in silico*, *in vitro*, *in situ* and *in vivo* (Lipinsky *et al.,* 1997:3; Hidalgo, 2001:385). Each of these methods contains several sub categories. The research scientist thus has several choices as to which method(s) to use, and the choice depends on what the laboratory has available and what kind of data the scientist wishes to generate.

The FDA lists the following methods for the determination of drug permeability for classification in the BCS:

- *in vivo* intestinal perfusion studies in humans,
- *in vivo* or *in situ* intestinal perfusion studies using suitable animal models,
- *in vitro* permeation studies using excised human or animal intestinal tissues or
- *in vitro* permeation studies across a monolayer of cultured epithelial cells (FDA, 2000:4).

1.3.1.1 Physicochemical characterisation

The physicochemical characteristics of a drug such as molecular weight, pKa, solubility and lipophilicity will influence the way the drug partitions from the aqueous phase into membranes and will thus influence its ability to cross cellular barriers, such as the lining of the gastrointestinal tract (Youdim *et al.*, 2003:997). Physicochemical parameter-based estimation methods are attractive because of their high throughput capacity, efficiency and reproducibility to predict passive drug transport. These methods are also suited because of their ability to predict permeability values with minimum usage of resources and manpower. However, using these methods, the complex but extremely significant drug-membrane interactions are completely unaccounted for (Balimane *et al.*, 2000:302).

The molecular weight of a molecule is easy to calculate. Conventional wisdom holds that smaller molecules will be less problematic drugs. In particular, molecular size will impact on transport through biological membranes, with a relationship that is inversely proportional to the transport rate (Chan & Stewart, 1996:463). However, molecular weight on its own may not be sufficient, as it contains no information about the actual three-dimensional shape of the molecules (van de Waterbeemd, 2000:38). Molecular surface properties have potential interest as predictors of drug absorption. Using molecular mechanics calculations to assess the three-dimensional shape, various dynamic surface properties such as polarity and size can be calculated. Palm *et al.*, (1997:32) used this concept to establish a correlation between polar van der Waals' surface area and intestinal drug absorption using a series of β-adrenoreceptor

antagonists as model compounds. Excellent correlations were obtained between the dynamic polar surface area of the drugs and their permeability coefficients in Caco-2 monolayers and excised rat intestinal segments (Palm *et al.,* 1997:32).

For several decades, the n-octanol-water partitioning coefficients (log P) dominated absorption prediction (Krämer, 1999:373). No general rules are applicable across the vastly diverse drug molecules, but each co-generic series for a drug backbone usually demonstrates its own optimal log P. The low absorption observed for compounds with a high log P value can be attributed to the poor aqueous solubility of these compounds. Alternatively, very polar compounds are unable to penetrate membrane barriers (Navia & Chaturvedi, 1996:180). However, there is little or no correlation between log P and oral bioavailability in man. Among the reasons for this lack of correlation are the presence of multiple pathways for intestinal absorption (Figure 1.1), carrier mediated transport processes and the efflux effects of p-glycoprotein (Lee *et al.,* 1997:49).

Physicochemical parameter based estimation methods are attractive because of their high throughput capacity, reproducibility and because they do not involve cumbersome cell cultivation. Some of the limitations associated with these estimations include their inability to predict active transport, the influence of efflux transporters, as well as to catalyse enzymatic degradation of drugs (Balon *et al.,* 1999:882).

Figure 1.3 gives a summary of the major physicochemical parameters used in absorption prediction models. Prediction complexity increases as the factors are added from the basic physicochemical parameters to drug absorption. The arrows indicate the predictability of the different processes from less complex parameters (Krämer, 1999:379).

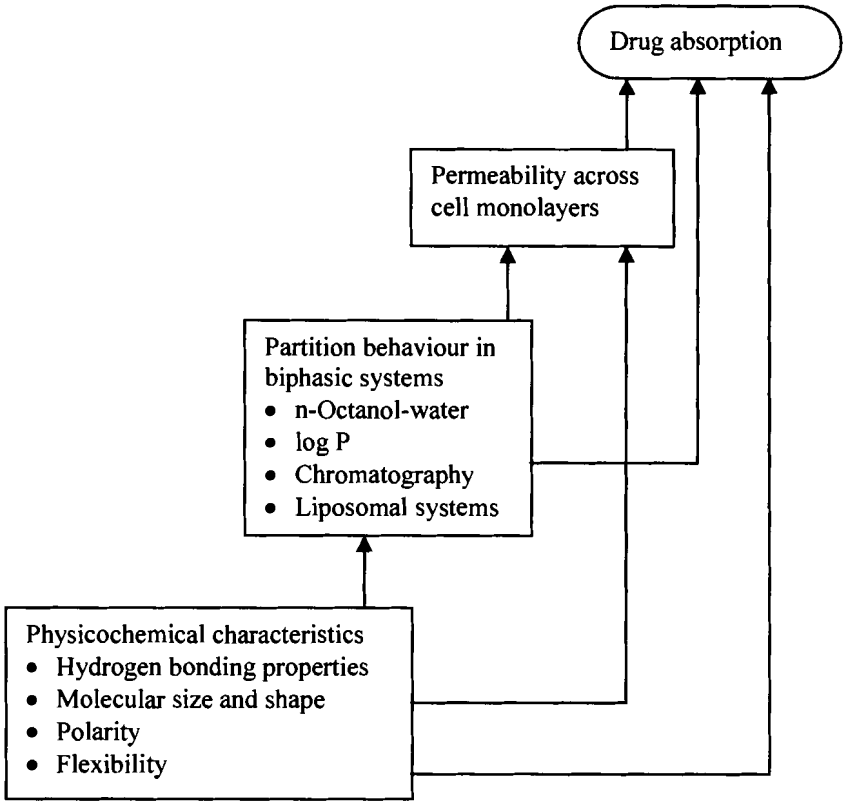


Figure 1.3: The major physicochemical parameters for predicting drug absorption (Krämer, 1999:379)

1.3.1.2 *In silico* permeability prediction

Computational or virtual screening has received much attention in the last few years. *In silico* models that can accurately predict the membrane permeability of test drugs based on lipophilicity, H-bonding capacity, molecular size, polar surface area and quantum properties has the potential to specifically direct the chemical synthesis and therefore, revolutionise the drug discovery process. Such *in silico* models would minimise extremely time consuming steps of syntheses as well as experimental studies of thousands of test compounds (Balimane *et al.*, 2000:309).

In silico predictive tools can be divided into filters, models and simulation tool. Filters can be a set of rules which must be met for absorption to take place or flags, which identify toxic compounds. Filters are generally used at very early stages of drug development, such as the design of virtual libraries. Models become useful during lead optimisation when lower throughput is required. Simulation tools are

valuable in the selection of a clinical candidate. A whole range of input data is required to make full use of the predictive power of the simulations. These data are normally only available at later stages of drug development (Dickins & van de Waterbeemd, 2004:39).

These *in silico* methods are an extension of the physicochemical characterisation process, as the computer programs analyse physicochemical characteristics and predict the degree of permeability. To this end, Lipinski *et al.,* (1997:9) developed the 'Rule of 5', which states that poor absorption or permeation is more likely when:

- There are more than 5 H-bond donors (expressed as sum of OH's and NH's);
- The molecular weight is over 500;
- The log P is over 5;
- There are more than 10 H-bond acceptors (expressed as sum of N's and O's) and
- Compound classes that are substrates for biological transporters are exceptions to the rule.

These rules are used by researchers as a guide, and if two parameters are out of range, the chances of poor absorption or permeability are increased. However, this is only a guide and not all chemical entities outside the parameters are discarded (Lipinski *et al.,* 1997:9).

The 'Rule of 5' is based on calculated properties of thousands of drugs and by definition some drugs will fall outside the parameter cut-offs in the rule. The orally active drugs that fall outside the 'Rule of 5' are antibiotics, antifungals, vitamins and cardiac glycosides. It is believed that these few therapeutic classes contain orally active drugs which have structural features that allow the drugs to act as substrates for naturally occurring transporters. When the 'Rule of 5' is modified to exclude these drug categories only a small number of exceptions can be found (Lipinski *et al.,* 1997:9).

Another *in silico* prediction model is proposed by Dressman *et al* (As quoted by Yu *et al.*, 1996:361) where the absorption potential (AP) of a drug is predicted from the partition coefficient (P), the fraction ionised at pH 6.5 (F_{un}) and the dose number of the drug (D_0). This model is best illustrated with the following equation:

$$AP = \log(\frac{PF_{un}}{D_0})$$

The dose number is obtained by calculating the ratio of the dose concentration to solubility, as illustrated in the following equation:

$$D_0 = \frac{D/V_0}{S}$$

where S is the physiological solubility, D is the dose and V_0 is the volume of water taken with the dose, that is generally set at 250ml (Yu *et al.*, 1996:361).

Several drugs were selected to evaluate the accuracy of the absorption potential concept. These drugs had a wide range of absorption characteristics, from poorly absorbed compounds, to those with virtually complete absorption and a wide range of physicochemical characteristics. A good correlation was found between the AP predicted and the fraction dose absorbed. An advantage of this method is the simplicity, as it is solely based on the physicochemical properties of the drugs (Yu *et al.*, 1996:361).

Another method used to simulate oral absorption is the mixing tank model. This approach considers the small intestine as a series of serial mixing tanks from which the drug is absorbed by linear transfer kinetics. The number of tanks used in the model varies, but the model using seven tanks has given the best correlation between calculated values and actual values. This model is known as the compartmental absorption and transit model (CAT). The CAT model is described by a set of differential equations that considers simultaneous movement of a drug in solution through the GI tract and absorption of the dissolved material from each compartment into the portal vein. A good correlation has been found between fraction dose

absorbed and the effective permeability of ten drugs covering a wide range of absorption characteristics when the effects of drug dissolution and dosage form could be neglected (Agoram *et al.*, 2001:S44); Yu *et al.*, 1996:361).

The accuracy of these methods is highly dependant on the accuracy and reliability of database information. Dose dependency and inter-individual variability in drug response, together with inter-laboratory variation in experimental protocols can lead to widely varying results and this can compromise the reliability of the conclusions reached (Hämäläinen & Frostell-Karlsson, 2004:400).

1.3.1.3 *In vitro* permeability prediction

The use of whole animal or human studies have two setbacks: firstly, they are unsuitable for screening large numbers of compounds at an experimental stage. Secondly, they pose ethical difficulties if the pharmacological effects and side effects are insufficiently well defined. As a result, a great variety of alternative methods have been developed to assess the permeation characteristics of new drugs (Tukker, 2000:51).

The use of *in vitro* methods in the drug discovery process is commonplace. Compared to *in vivo* studies, evaluation of intestinal permeation by *in vitro* studies require less compound, are relatively easier to perform, is more rapid and has the potential to limit the amount of animals used, as a number of variables can be evaluated in one experiment. Further, these methods are analytically simpler as the compounds being analysed are in aqueous buffer as opposed to whole blood or plasma (Smith, 1996:13).

One drawback of all *in vitro* studies is that the effect of physiological factors such as gastric emptying rate, gastrointestinal transit time, gastrointestinal pH, etc. cannot be incorporated in the data interpretation. Each *in vitro* method has its own advantages and drawbacks. Based on the goal of the investigator, one or more of these methods can be used as a screening tool in early drug discovery. The success of an *in vitro* method to predict intestinal permeability depends on how closely the method mimics

in vivo gastrointestinal conditions (Balimane *et al.*, 2000:305). The low permeability of some drug substances in humans could be caused by efflux transporters such as permease glycoproteins (p-gp). When the efflux transporters are absent in these models, or their degree of expression is low compared to that in humans, there may be a greater likelihood of misclassification of permeability class for a drug subject to efflux compared to a drug transported passively (FDA, 2000:5).

Expression of known transporters in selected study systems should be characterised. Functional expression of efflux systems (such as p-gp) can be demonstrated with techniques such as bidirectional transport studies, demonstrating a higher rate of transport in the basolateral-to-apical direction using selected model drugs or chemicals at concentrations that do not saturate the efflux system (e.g. cyclosporine A, vinblastine, rhodamine 123) (FDA, 2000:5).

The complexity of the intestinal mucosa with a continuously renewing epithelium and the possibility of extensive interactions among different epithelial and mesenchymal cell types has always presented formidable challenges to the development of representative *in vitro* model systems (Quaroni & Hochman, 1996:37).

The observed low permeability of some drug substances in humans could be caused by efflux of drugs *via* membrane transporters such as p-gp.

There are several barriers that may be used in *in vitro* transport experiments; these include artificial membranes, cell culture monolayers [e.g. human adenocarcinoma colon cells (Caco-2 cells), Madin Darby canine kidney cells (MDCK)], isolated mucosal cells and intact tissue techniques (e.g. rat intestine, rabbit intestine, human intestine). When selecting an *in vitro* or *in situ* absorption model, the following criteria should be considered:

- Simplicity
- Reproducibility
- Rapid turnaround time
- Predictability of *in vivo* absorption in humans (Habucky, 1995:22).

1.3.1.3.1 Artificial membranes

Avoiding biological systems completely, artificial membranes do not incorporate transporters, paracellular pathways, enzymes or other cell-associated absorption processes. They only measure passive diffusion. The advantages include results that are more reproducible and that can be used to study permeability-related structure-activity relationships more efficiently. There are several types of artificial membrane systems available including immobilised artificial membrane (IAM) columns and the parallel artificial membrane permeation assay (PAMPA) (Hämäläinen & Frostell-Karlsson, 2004:400).

It has been proposed by Kansy *et al.*, (1998:1007) that PAMPA be used as a high throughput alternative to Caco-2 cell monolayer in studies for the prediction of passive drug absorption. In the PAMPA approach, a filter plate is prepared by depositing a small amount of phospholipid in the immobilising filter material, which forms bilayer structures in the filter pores. This filter separates the aqueous donor and acceptor phases. The solute concentrations in the acceptor phase are determined by UV spectrophotometry. The entire experiment is carried out in 96-well microtiter plates and analysis is done by a 96-well microplate photometer. It was found that the PAMPA flux could be successfully used to classify compounds of low, intermediate and high human intestinal absorption (Kansy *et al.*, 1998:1007).

PAMPA is a remarkable 'open-system' approach where scientists can formulate their own lipid barriers for any number of different applications, not all focused on permeability screening. The method can be a low-cost, very fast and a particularly helpful add-on to cellular permeability assays, such as Caco-2. Future areas of PAMPA can be expected to include early preformulation screening to identify excipients suitable for oral formulations of low-solubility compounds (Kansy *et al.*, 2004:353).

There are, however, problems with these membranes, as it has been shown that time dependant thinning of the micromembranes takes place, although this can be reduced by the addition of cholesterol and Ca^{2+} (Ikematsu *et al.*, 1996:66).

1.3.1.3.2 Cell culture monolayers

Cell culture models offer many features which complement and minimise whole animal studies. Due to the intermediate complexity of these systems these models provide a bridge between whole animal studies and isolated enzymes or membrane factions. From a basic research perspective, cell culture models permit mechanistic analysis of transport and metabolism by allowing manipulation and precise control of experimental conditions. Drug concentrations can be precisely controlled, experimental conditions such as pH can be modulated and the effects of inhibitors and metabolic poisons can be studied. Moreover, using genetic manipulation of cell populations or by selecting clonal cell populations it is possible to modulate the level of expression of specific transport proteins or metabolic enzymes allowing more detailed mechanistic analysis (Quaroni & Hochman, 1996:4).

Much progress has been made in recent years, and there are now at least three well established or promising cell culture systems. Each of them has advantages and limitations, making them useful for different applications. The IEC-type (cultured intestinal epithelial) cells remain the only 'normal' intestinal epithelial cell lines obtained to date: they are likely derived from stem cells and their main limitation rests with our inability to induce their full differentiation *in vitro*; they appear best suited to the study of growth regulation and other functions of crypt cells. Human tumour cell lines have received intense scrutiny and established themselves as excellent *in vitro* models for a variety of intestinal activities and functions; with reference to drug transport and metabolism studies, the Caco-2 cells and the mucus producing sublines of HT-29 (human colon goblet cell line) cells have proven their worth and in spite of their acknowledged limitations, must be considered the benchmark standard against which any new models will have to be compared (Quaroni & Hochman, 1996:37).

The most frequently used cell cultures for studies of passive drug transport are the Caco-2 cell cultures. The Caco-2 cells can be cultivated to spontaneously differentiate to form monolayers of polarised cells, with functions similar to intestinal enterocytes. The monolayers are grown on filter supports and drug passage from the donor to the acceptor compartment is measured. An advantage studying biological permeation with cell monolayers is that they measure the transport of the drug across the cell membrane, instead of just its interaction with the lipid bilayer (Mälkiä *et al.*, 2004:24).

Yee (1997:766) found an excellent correlation between *in vivo* absorption and the *in vitro* apparent permeability coefficient (P_{app}) for a variety of compounds encompassing transcellular, paracellular and carrier-mediated mechanisms. Therefore, the Caco-2 cells can be used as a predictive as well as a screening tool, provided dissolution and gastrointestinal metabolism are not limiting the portal availability. For compounds that are substrates of p-gp, use of inhibitors gave a better estimate of absorption in humans (Yee, 1997:766).

Caco-2 monolayers consist of a cell layer on a supporting membrane, which does not limit the absorption of low molecular weight compounds. A drug that crosses the cell layer is quickly detected on the serosal side. The process of *in vitro* permeation is essentially the same as *in vitro* absorption. In this respect, the Caco-2 monolayer system mimics and therefore predicts *in vivo* drug absorption better than isolated intestinal membranes (Yamashita *et al.*, 1997:490). These cells are, however, neither normal nor derived from the small intestine and this can be seen as one of their main limitations (Quaroni & Hochman, 1996:27).

MDCK cells can be used in a similar manner to Caco-2 cells and have a shorter culture time (approximately five days), but have the disadvantage in that they are 'non-human' in nature (Saunders, 2004:373).

Advantages of the *in vitro* cell absorption models include a rapid turnaround of information, the potential to decrease the number of animal studies and the ability to, in some cases, use human tissue in the transport studies (Habucky, 1995:22). They

can be used to determine both cellular uptake and transepithelial transport and they remain viable for long periods (Hidalgo, 1996:48).

Despite their undeniable benefit as model membranes, cell cultures come with certain disadvantages. The method is rather laborious (aseptic techniques need to be applied to culture and maintain the cells) and time consuming as cells have to be cultured for approximately three weeks prior to use. From a physiological point of view cell cultures have the following shortcomings: (1) the tissue in intestinal villi contain more than one type of cell, (2) most cell lines do not produce the mucus layer found in normal intestine and (3) not all metabolising enzymes found in the enterocytes are present in cell lines. (Mälkiä *et al.,* 2004:24; Habucky, 1995:25). Further, the thickness of the layers and the density of cells may vary between batches which may cause variation in rates of absorption of drugs when compared with each other or with other methods. Another problem is that various batches of cell cultures may contain differences in the concentrations of transport proteins, although this phenomenon may also be present when excised membranes are used.

1.3.1.3.3 Isolated mucosal cells

Procedures for isolating mucosal cells include mechanical agitation, scraping, hydrolytic collagenase enzymes or chelating agents such as ethelynediaminotetraacetate (EDTA). Such isolated cells may be used to investigate enzyme activity, drug transport and cellular metabolism. However, these cells have several disadvantages. Absorption and transport studies are limited as rapid autolysis occurs. The isolation technique also opens the tight junctions, destroying cell polarity. Also, if separation is incomplete, the epithelial cell will be contaminated with other cell types. Given the limitations, the mucosal cell model falls short of meeting the selection criteria of a model for the study and prediction of drug transport (Habucky, 1995:26).

1.3.1.3.4 Excised tissue models

In excised tissue models a compound in solution is applied to one side of a small piece of excised intestinal tissue, typically in an Ussing or Ussing-type chamber (an environmentally controlled chamber used for epithelial cell membrane studies). Permeability is monitored by measuring the disappearance of compound from the donor side and/or its appearance on the acceptor side. Various parts of the gut can be used to compare absorption in different regions of the intestine. Everted intestinal sacs, intestinal segments and muscle stripped mucosa have all been used as model systems. The advantage of this assay is that gut architecture is preserved and closely mimics the *in vivo* situation. The sum of all absorption processes can be measured in one assay (Hämäläinen & Frostell-Karlsson, 2004:399).

1.3.1.3.4.1 Everted gut sacs

The everted gut sac is a simple and useful *in vitro* model to study drug transport. The system provides information of drug absorption mechanisms through testing the drug content in the intestinal sac. The everted sac has been used to study the uptake of lipid vesicles (Rowland & Woodley, 1981:221), proteins, and macromolecules with oral drug delivery potential, bioadhesive lectins and synthetic nondegradable polymers. It provides quantitative information on the uptake and absorption of the tested drug (Guo *et al.,* 2004:416).

This technique consists of everting a freshly excised section of small intestine (rat or rabbit), filling it with oxygenated buffer of tissue culture medium at 37°C, and dividing it into sacs approximately 25 mm long. Each sac is secured using braided silk and is then placed in a suitable container, once again containing oxygenated buffer or tissue culture medium at 37°C. This medium also contains the chemical entity which is being studied. It has been shown that tissue culture medium ensures excellent tissue viability and metabolic activity. The sacs are then incubated at 37°C in an oscillating water bath. At the appropriate time points, sacs are removed, washed four times with 0.9% sodium chloride solution and blotted dry. The sacs are cut open and the serosal fluid drained into small tubes. The amount of drug in the serosal fluid,

as well as in the tissue is then analysed by appropriate means. To test the viability of the tissue, glucose is measured in the incubation medium and in the sac contents. As glucose is actively transported by small intestine, healthy metabolically active sacs that are not leaking will accumulate glucose in the serosal medium (Barthe *et al.*, 1998:256).

In a study done by Lacombe *et al.*, (2004:390) it was found that drug transport was higher in the jejunum than in the ileum or the duodenum. They also found that transport of digoxin (p-gp substrate) decreased along the length of the intestine, indicating an increase in p-gp expression along the length of the intestine. This shows that the everted gut sac method is versatile and can be used for a number of different transport studies (Lacombe *et al.*, 2004:390).

The everted technique is simple, inexpensive, eliminates variations due to blood flow and permits sampling from mucosal and serosal sides. However, there are several disadvantages associated with everted sacs, including inaccurate data due to experimental variables such as method of oxygenation, method of serosal sampling, fluid loss and damaging of tissue during removal (Habucky, 1995:28). The kinetics of drug absorption using this method may be unrealistically slow, since this technique measures drug transport through the epithelial layers and the associated longitudinal and circular muscle layers. Since the mesenteric capillaries reside between the epithelial cells and serosal muscle layers, these muscle layers do not represent a barrier to drug transport in the *in vivo* situation. The fluid inside the sac is also stagnant and this is also not physiologically correct.

1.3.1.3.4.2 Intestinal segments

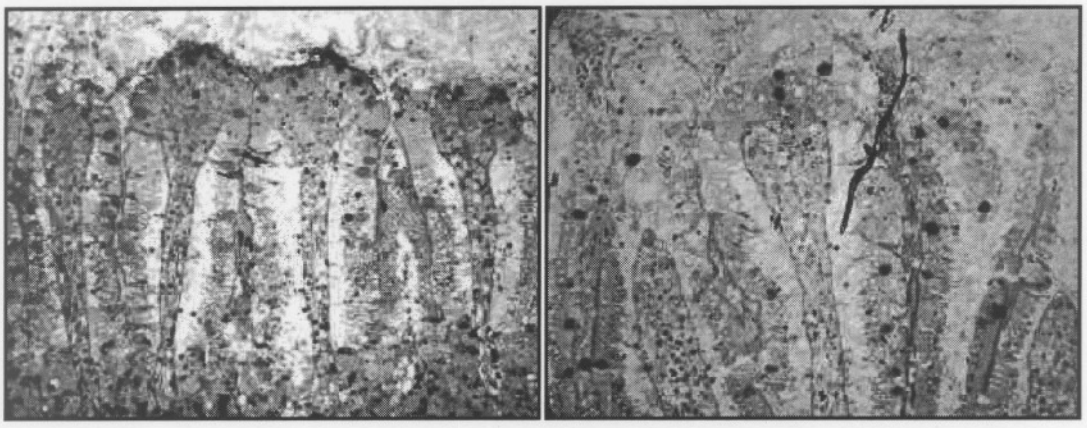
A segment of the intestine (usually jejunum) is removed from the animal after anaesthesia by an appropriate method. The tissue can then be used as is, or the outer serosa and muscle layers may be removed. For studies designed to determine the mechanisms and rates of drug transport and metabolism, stripped tissues are preferable because they more closely resemble the *in vivo* situation (drug absorption into the intestinal vasculature does not involve permeation through the intestinal smooth muscle). This stripping, however, may result in damage to the mucosal

membrane as physical pressure is applied to the tissue. Once the tissue is ready, it is cut open along the mesenteric border and mounted in Ussing or Ussing-type chambers. Buffers of the appropriate pH are added to both sides and the tissue is left for 15-30 minutes to acclimatise. Carbogen (95% O_2 / 5% CO_2) is bubbled through the buffer (15–20 ml/min) for the duration of the experiment. The chambers are kept at 37 C throughout the experiment by heating blocks (Habucky, 1995:29; Smith 1996:17).

In 1988 Sweetana & Grass designed a new type of diffusion chamber for the study of drug transport across rabbit intestine. This chamber system has advantages over the classic Ussing chamber setup. The chambers are made of the same acrylic material and there is no connecting tubing, as in normal Ussing chambers. The fluid flow in these chambers is also parallel to the tissue surface, as it is *in vivo*. The volume of these chambers has been reduced (5–7 ml) and special small volume chambers are also available. The surface area of the exposed tissue has been increased and the use of an oblong opening means that tissue from different species, as well as from different sections of the intestine can be used. The temperature of the chambers are also easier to maintain as all six chambers are kept in the same heating block and the area of the chamber exposed to the heating block is greater (Grass & Sweetana, 1988:375).

The experiment is started by die addition of the drug being studied. At defined time intervals samples are removed and the amount removed is replaced with an equivalent amount of fresh buffer to maintain a constant volume (Smith, 1996:20). The experiment can last as long as the investigator needs, although it has been found that the tissue is only viable for 120 minutes. After this time the epithelium on the villi tips start to disintegrate (Figure 1.4). This disintegration is more pronounced in tissue where the serosa and muscle layers have been removed (Hattingh, 2002:83).

There are a number of chambers that can be used. They vary in size, volume and area of exposed tissue. In this way, if small amounts of drug are available, small volume chambers can be used. Chambers with small tissue area can be used for smaller animals. The type of buffer used can be varied according to need, with pH varying to mimic the different parts of the intestine.



a b

Figure 1.4: Villi of rat intestine (unstripped [a] and stripped [b]) after 120 min mounted in Sweetana-Grass diffusion chambers (Hattingh, 2002:58)

This method provides a means to compare intestinal epithelial permeability of molecules. Molecules with sufficient intestinal permeability may not be sufficiently bioavailable due to first-pass metabolism or instability in the gastro-intestinal tract. Molecules that do not have sufficient membrane permeability using this screening procedure will not be orally bioavailable. The evaluation of intestinal permeability by this method provides a means for selecting drug candidates for further testing *in vivo*, reducing the time and resources needed to identify an oral development candidate (Smith, 1996:29).

1.3.1.4 *In situ* permeability prediction

Regardless of the type of model selected (e.g. closed loop, single pass or recirculating), *in situ* absorption models permit the study of individual organ processes and site specific absorption. The tissue is maintained intact with blood flow to the organ. Samples of drug solution from *in situ* loop experiments can be obtained to measure drug disappearance and metabolite formation (Habucky, 1995:30).

1.3.1.4.1 Closed loop studies

Two types of closed loop studies have been described: anaesthesia recovery and cannulated closed loop studies. In anaesthesia recovery, the animal is anaesthetised,

ligatures are formed in the desired intestinal region and drug is introduced into the desired section. The abdominal area is sutured and the animal is allowed to recover. Blood samples can then be taken at the appropriate times. After a specified time the animal is sacrificed and the amount of drug remaining in the loop can be analysed. With this method the effect of anaesthesia is limited. Limitations of the technique include volume fluctuations and the fact that only one data point can be obtained from each animal's luminal content (Habucky, 1995:30).

1.3.1.4.2 Perfused loop studies

Accurate determinations of effective intestinal permeability (P_{eff}) for drugs and nutrients is difficult to study *in vivo* in humans, but different single-pass perfusion techniques have developed over the years. The basic principle of perfusion experiments is that the absorption is calculated from the disappearance rate of the drug from the perfused segment. The absorption rate can be calculated in many ways but it has been found that the best description is given by the intestinal P_{eff}. Calculation of P_{eff} is dependant on the hydrodynamics within the segment, which in turn is determined by the perfusion technique, perfusion rate and the degree of intestinal motility (Lennenäs, 1998:403).

Three perfusion methods are used; the open, semi-open and closed loop systems. For open and semi-open systems, the concentration of the drug will decrease exponentially along the segment as the drug solution enters proximally and exits distally and absorption occurs along the intestinal segment. In the closed loop system, the segment is closed off by two balloons; the drug solution enters the segment via a central port and exits via a whole at either end of the segment. This means that the solution goes in two directions, which is similar to the movement of fluid back and forth over a short region during physiological intestinal contractions (Lennenäs, 1998:403).

This method more closely resembles the *in vivo* situation. It can be adapted to allow recycling of the drug solution and the venous blood supply can be collected to determine the amount of drug (Habucky, 1995:31).

Isolated gut loops represent undisrupted tissues with relatively undisturbed morphology. The use of these methods allows investigation of absorption processes without interference from gastric emptying, gastrointestinal motility, bile acids or hormones and provides flexible and viable experimental conditions. However, the preparations are not simple and considerable resources are required to set up, run and validate such studies. They are therefore best reserved for the answering of specific questions when a problem has been encountered in *in vivo* experiments (Griffith *et al.*, 1996:82).

1.3.1.5 *In vivo* permeability studies in animals

The results obtained from *in vivo* studies in animals depend upon the species selected and little consistency is observed in the study of specific compounds in different species. No single laboratory species has been defined as a reliable model of human drug absorption so that species selection for early studies often results from such physiologically irrelevant characteristics as ease of handling and cost (Grass, 1997:203).

Although whole animal studies have been routinely used as predictors of oral drug absorption in humans, the data generated from such studies can be of little predictive value and in some cases may be so much in error that they may be misleading. Whole animal studies are also not suitable for conducting large numbers of screening studies needed in the drug discovery process. Each animal must be dosed and plasma samples taken and analysed. It is difficult to imagine this technique easily adapted to the requirement of screening hundreds or thousands of compounds. The general problem with whole animal studies is that when a specific species is selected, all the characteristics of that species are selected and a correlation must be drawn to human characteristics. For example, when rats are selected, the scientists' attempts to force a correlation for all the characteristics of GI transit, pH, bile secretion, etc. from rats to humans. This is not always successful, as can be seen in the case of ganciclovir. Early on in development bioavailability was examined in several species. Results obtained in dogs showed the most promise, with 100% bioavailability at the dose

intended for humans. Unfortunately, after significant development effort it was found that the bioavailability in humans was only 10–20% (Grass, 1997: 203).

Despite these drawbacks, an advantage of whole animal studies is that the species used in absorption studies could be the same one used in pharmacological and/or toxicological evaluations. They also can be used to evaluate complex formulations which would be very difficult to test *in vitro*. Some further disadvantages of studies with whole animals include the need for relatively large amounts of material, the complexity of the analytical methods needed for plasma analysis, the time-consuming and labour-intensive nature of experiments and the fact that they provide little mechanistic information on drug absorption (Hidalgo, 2001:389).

1.3.2 Drugs used in validating permeability prediction

The permeability class boundary is based indirectly on the extent of absorption (fraction absorbed, not systemic BA) of a drug substance in humans and directly on measurements of the rate of mass transfer across human intestinal membrane. Alternatively, nonhuman systems capable of predicting the extent of drug absorption in humans can be used (e.g. *in vitro* epithelial cell culture methods). In the absence of evidence suggesting instability in the gastrointestinal tract, a drug substance is considered to be highly permeable when the extent of absorption in humans is determined to be 90% or more of an administered dose based on a mass balance determination of in comparison to an intravenous reference dose (FDA, 2000:2).

In order to demonstrate suitability of a permeability method, a rank order relationship between test permeability values and the extent of drug absorption data in human subjects should be established using a sufficient number of model drugs. Model drugs should represent a range of permeability values (FDA, 2000:6).

For the establishment of suitability of method for permeability prediction, the FDA recommends the model drugs given in Table 1.1.

Table 1.1: Drugs suggested for use in establishing suitability of a permeability method for classification in the BCS (FDA, 2000:13)

Drug	Permeability class
Antipyrine	High
Caffeine	High
Carbamazepine	High
Fluvastatin	High
Ketoprofen	High
Metoprolol	High
Naproxen	High
Propranolol	High
Theophylline	High
Verapamil	High
Amoxicillin	Low
Atenolol	Low
Furosemide	Low
Hydrochlorthiazide	Low
Mannitol	Low
Methyldopa	Low
Polyethylene glycol (400)	Low
Polyethylene glycol (1000)	Low
Polyethylene glycol (4000)	Low
Ranitidine	Low

For this study ten compounds were chosen that have published F_a values (Table 2.1), were readily available and easily analysed by HPLC. Of these ten, seven are recommended by the FDA (Table 1.1). The other three (promethazine, paracetamol and aciclovir) were chosen as they were available in the lab and have diverse F_a values. As all three drugs have been used previously in various studies for the comparison of *in vitro*, *in situ* and *in silico* methods with F_a (Irvine *et al.*, 1999:30; Sugama *et al.*, 2002:247; Turner *et al.*, 2004:71; Yee *et al.*, 1997:764) their inclusion did not seem to be a problem.

CHAPTER 2
EXPERIMENTAL PROCEDURES

2.1 Introduction

The transport of ten compounds with diverse absorption characteristics across rat jejunum was investigated using a vertical diffusion chamber system, comprising six Sweetana-Grass diffusion chambers, one heating block and one gas manifold (Corning Costar Corporation, Cambridge, USA) (Figure 2.1).

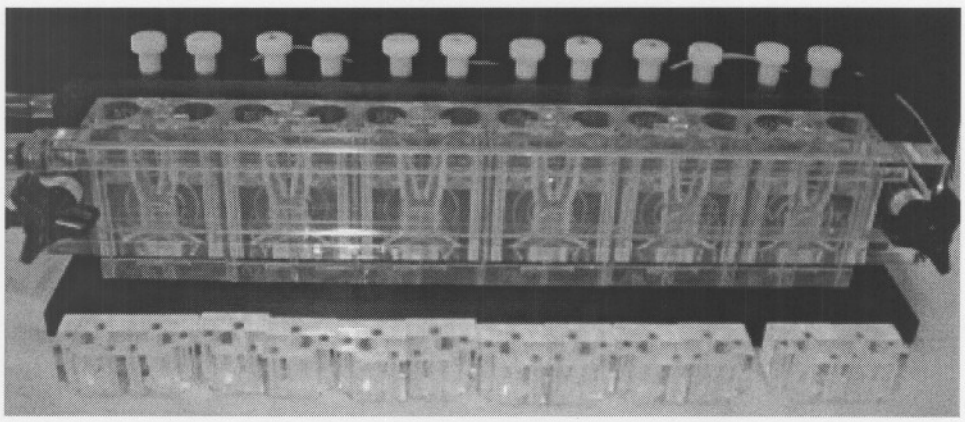


Figure 2.1: Sweetana-Grass diffusion chambers, heating block and gas manifold

2.2 Materials

Krebs-Ringer bicarbonate buffer, verapamil, ketoprofen, carbamazepine, promethazine, acyclovir and ranitidine (Sigma Chemical Company Ltd., St. Louis, Missouri, USA) were obtained from Sigma-Aldrich (Pty) Ltd, Johannesburg. Caffeine, potassium dihydrogen orthophosphate, sodium bicarbonate, ethylenediamine, absolute ethanol, acetonitrile for HPLC, methanol for HPLC, THF for HPLC, glacial acetic acid were obtained from Merck (Pty) Ltd, Germiston. Furosemide was obtained from Adcock Ingram, Wadeville, South Africa. Propranolol was obtained from Kothari Phytochemicals international, Tamilnadu, India. Paracetamol was obtained from Fine chemicals corporation, Cape Town, South Africa.

2.3 Tissue Preparation

Tissue was prepared in one of two ways. It was either used as is (unstripped) or the serosal muscle layer was removed. This was done as little or no investigation has taken place to determine the effect of stripping on the transport of chemical entities.

Unfasted adult male Sprague-Dawley rats (350-450 g) (obtained from the Laboratory Animal Centre at the Potchefstroom campus of the North-West University, South Africa; Ethics Committee approval number 04D14) were anaesthetised by halothane inhalation. An abdominal incision was made and starting 10 cm from the stomach a 20-30 cm strip of intestinal tissue (jejunum) was excised, rinsed with ice cold Krebs-Ringer bicarbonate buffer (KR) through which 95% O_2 / 5% CO_2 had been bubbled for 10 minutes (Figure 2.2 (a)) and then pulled onto a glass rod (Figure 2.2 (b)).

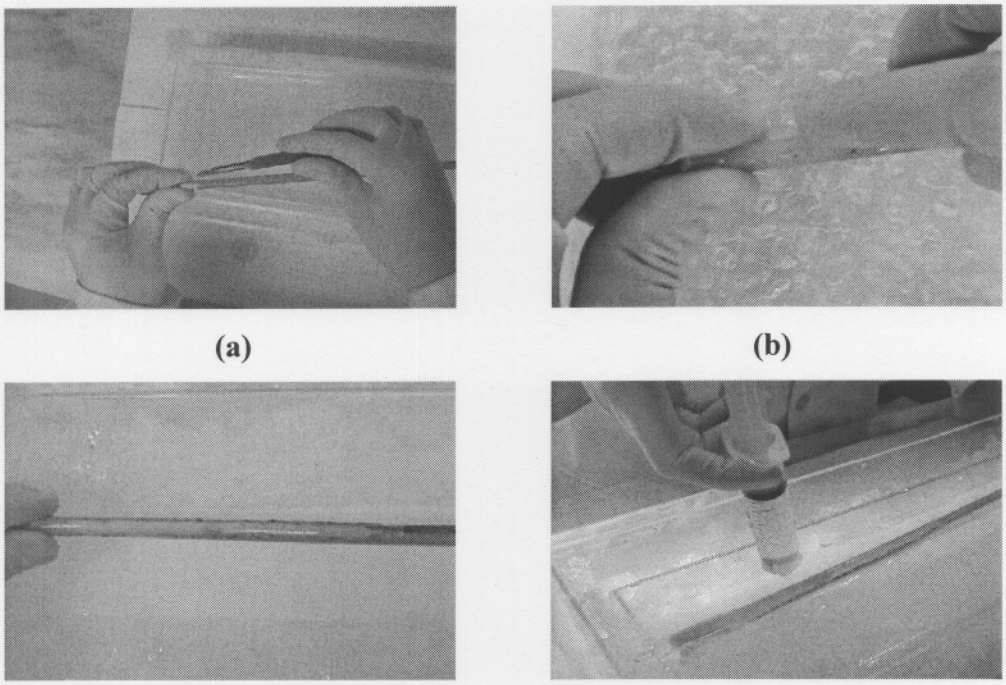
(a) **(b)**

Figure 2.2: Preparation of tissue by (a) flushing out of intestinal contents and (b) pulling onto a glass rod

In the experiment where stripped tissues were used, the excised tissue was then gently scoured along the mesenteric border with the back of a scalpel (Figure 2.3 (a)). The serosal muscle layer was removed by gentle rubbing along the mesenteric border with the forefinger (Figure 2.3 (b)). Throughout the procedure, the tissue was immersed in ice cold KR which was kept in an ice bath.

The excised strip was then cut along the mesenteric border (Figure 2.3 (c)) and

washed off the glass rod with KR onto a strip of filter paper (Figure 2.3 (d)).

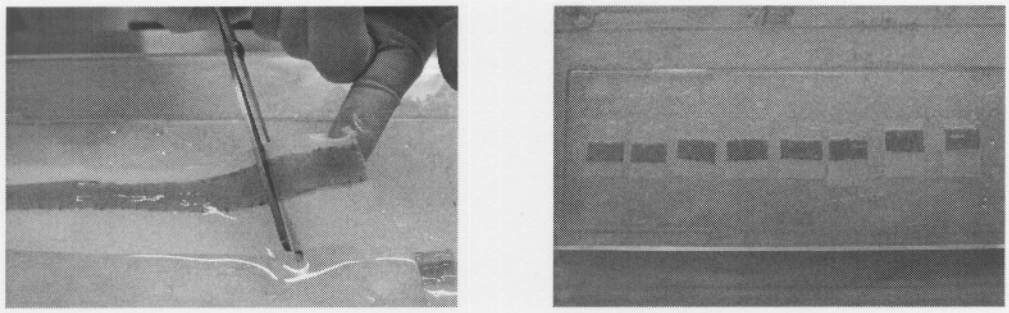


(a) **(b)**

(c) **(d)**

Figure 2.3: Tissue preparation by (a) scouring along the mesenteric border with the back of a scalpel, (b) removal of the serosal muscle layer by gentling rubbing with the forefinger (c) cutting open along the mesenteric border, (d) washing the tissue of the glass rod onto a strip of filter paper

The strip was then cut into lengths approximately 3 cm long (Figure 2.4 (a)). The segments were kept on ice and were kept moist with ice cold KR (Figure 2.4 (b)). Care was taken to avoid segments containing Payer's patches (Figure 2.5), as these lymph-like tissues would probably cause greater variation in the rates of transport because of altered morphology and thickness of the epithelial layer.



(a) (b)

Figure 2.4: Cutting of the intestinal strip into segments approximately 3 cm long

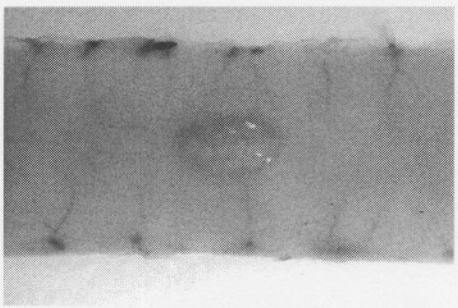
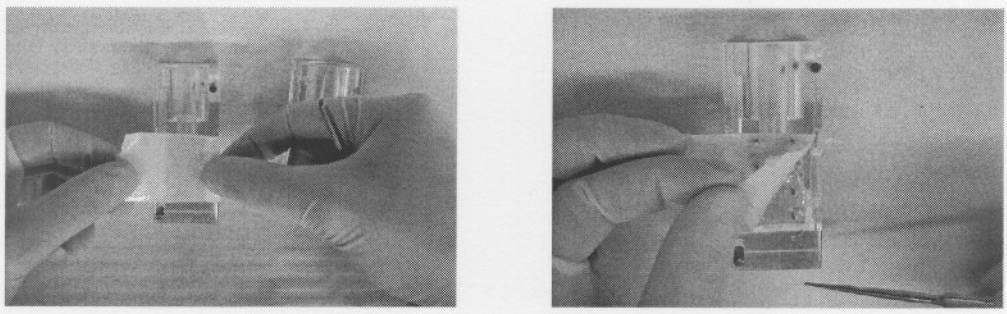


Figure 2.5: A segment of intestinal tissue containing a Payer's patch

2.3.1 Mounting of tissue

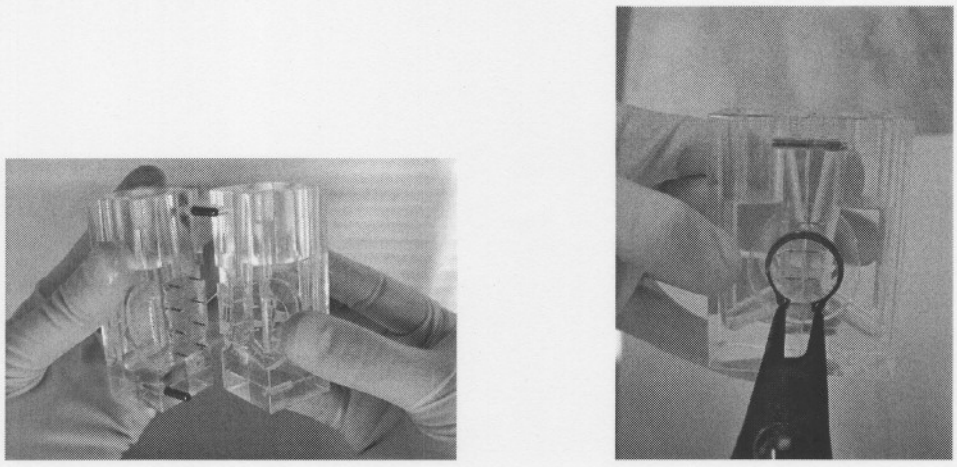
The segments were then carefully mounted onto the half cells (preheated to 37 C) containing pins (Figure 2.6 (a) & (b)).



(a) (b)

Figure 2.6: Mounting of tissue onto the pins of the half cell

The matching half-cells were then carefully clamped together without damaging the jejunal membrane (Figure 2.7 (a) & (b)).



(a) (b)

Figure 2.7: Clamping together of the half cells

The assembled chamber was then placed in the heating block (37 C) and 5 ml KR preheated to 37°C was added (Figure 2.8).

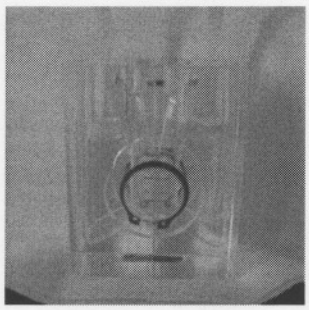


Figure 2.8: Filling of half chambers with KR

Circulation of the buffer was maintained by a gas-lift using 95% O_2 / 5% CO_2 at a flow rate of 15-20 ml/min. All six chambers were assembled and placed in the heating block (Figure 2.9). The entire procedure from removal of the intestine from the rat until all six chambers were mounted took approximately 10 minutes for unstripped tissue and 18 minutes for stripped tissue. The tissue was acclimatised for 15 minutes before a transport study was started by adding the various compounds under investigation to the donor cell. The cells were kept in the heating block at 37 C for the duration of the experiment.

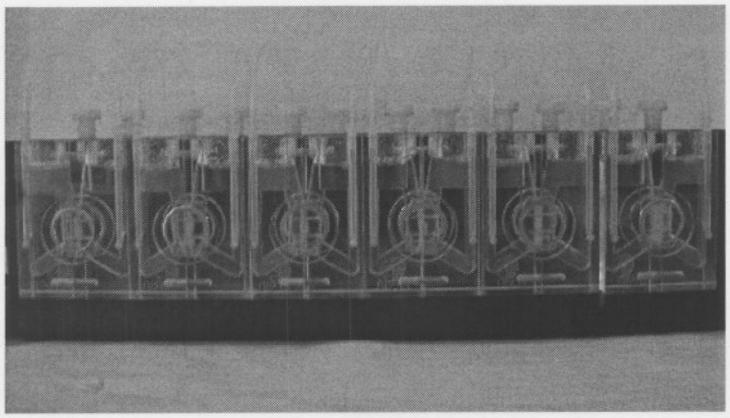


Figure 2.9: All assembled chambers placed in the heating block

2.4 Procedures used during transport studies

The transport of caffeine, furosemide, verapamil, ketoprofen, propranolol, carbemazepine, promethazine, paracetamol, acyclovir and ranitidine were measured across rat jejunum. A sufficient amount of compound was added to the apical side in 2 ml of KR to give the concentration given in Table 2.1. At the same time 2 ml KR was added to the basolateral side.

Table 2.1: List of compounds investigated in the transport studies

Compound	F_a [a,b]	Molecular weight	Aqueous solubility (mg/ml)	Maximum dose (mg)[c]	Dose in 250ml water (µg/ml)	Concentration used (µg/ml)
Caffeine	1.00	194.19	20	-	-	50
Carbemazepine	1.00	236.27	0.1	800	3200	2.5
Ketoprofen	1.00	254.28	0.5	100	400	400
Verapamil	1.00	491.06	83	160	640	160
Propranolol	0.90	295.80	50	80	320	320
Promethazine	0.85	320.88	1667	100	400	100
Paracetamol	0.80	151.16	14.3	1000	4000	4000
Furosemide	0.60	330.74	0.18	250	1000	150
Ranitidine	0.55	350.56	1.8	300	1200	430
Acyclovir	0.21	225.20	0.7	800	3200	200

[a] Zhu, *et al.,* 2002:402, [b] Ingels, *et al.,* 2004:223, [c] Gibbon, 2003:35

The total volume in each half chamber after the final additions was 7 ml. On the mucosal side, this consisted of KR (pH 7.4) and the added drug and on the serosal side KR (pH 7.4) alone. The total exposed tissue surface area was 1.78 cm^2. Aliquots (250 µl) were taken from the basolateral side at 30, 60, 90, and 120 min after the addition of the compound, and replaced with an equivalent amount of fresh KR. It was previously shown (Hattingh, 2002:83) that the maximum period to perform transport studies was approximately 120 min before structural damage to the epithelium occurred. The aliquots were analysed by HPLC.

Carbemazepine and promethazine were dissolved in absolute ethanol to create stock solutions. All other compounds were dissolved in KR. The final ethanol concentration in the various sample solutions was kept ≤1%, a concentration proven to not alter cell viability or permeability (Soldner *et al.*, 1999: 479). The concentrations used were similar to those used previously in literature (FDA, 2000:5; Makhey *et al.*, 1998:1161; Watanabe *et al.*, 2004:661; Zakelj *et al.*, 2004:175).

The average apparent permeability coefficient (P_{app}) was calculated according to the following equation:

$$P_{app} = \frac{dQ/dt}{60 \times A \times C_0}$$

where dQ/dT is the transport rate, C_0 is the initial concentration of the drug investigated (100%) and A is the area of exposed tissue (1.78cm^2). An example of the calculations performed is presented in Appendix A.

2.5 HPLC Analysis

The samples were analysed by high performance liquid chromatography (HPLC) using the apparatus and conditions given below (Table 2.2):

Apparatus:	Pump:	Spectra Physics SP 8810
	Autosampler:	Spectra Physics AS 3000

Detector:	Spectra Physics UV 1000 and FL 2000
Integrator:	Computerised integration system, with Chromquest chromatographic database for Windows® NT as software
Guard column:	SecurityGuard cartridges, C18, 4 x 3 mm
Column:	Luna 5μ C18(2) reverse phase 250mm

All mobile phases were mixed using HPLC grade reagents and Milli Q50 water for HPLC. Mobile phases were filtered through a MN 85/90 glass fibre filter (Machery-Nagel, Germany) prior to use.

Table 2.2: HPLC conditions used for analysis.

Compound	Reference	Injection volume (µl)	Flow rate	Mobile phase		pH	UV Wavelength	Fl wavelengths	
								Ex (nm)	Em (nm)
Caffeine	1	50	1.0	Acetonitrile	5%		274		
				Tetrahydrofuran	3%				
				Glacial acetic acid	0.5%				
				Water	91.5%				
Carbemazepine	2	100	1.5	Acetonitrile	40%		285		
				Ammonium dihydrogen phosphate (0.025M, pH=4.0)	60%				
Ketoprofen	3	20	1.5	Acetonitrile	25%	7.4	260		
				Potassium dihydrogen o-phosphate (0.02M)	74.98%				
				Triethylamine	0.02%				
Verapamil	4	200	1.5	Acetonitrile	30%	3.6		280	314
				Potassium dihydrogen o-phosphate (0.05M)	70%				
Propranolol	5	50	1.0	Acetonitrile	32%		210		
				Potassium dihydrogen o-phosphate (0.05M)	68%				
Promethazine	2	10	1.5	Acetonitrile	40%		250		
				Ammonium dihydrogen phosphate (0.025M, pH=4.0)	60%				
Paracetamol	1	50	1.5	Acetonitrile	5%		230		
				Tetrahydrofuran	3%				
				Glacial acetic acid	0.5%				
				Water	91.5%				
Furosemide	6	50	1.5	Acetonitrile	38%	3		224	390
				Potassium dihydrogen o-phosphate (0.01M)	62%				
Ranitidine	7	50	1.0	Acetonitrile	10%		254		
				Potassium dihydrogen o-phosphate (0.01M, pH=3.0)	90%				
Acyclovir	8	100	1.0	Methanol	10%	7.0	254		
				Potassium dihydrogen o-phosphate (0.05M)	90%				

1. Kamimori *et al.*, 2002:161
2. Owen *et al.*, 2001:574
3. Guadiano *et al.*, 2003:153
4. Özkan *et al.*, 2000:377
5. Panchagnula *et al.*, 2004:278
6. Abou-Auda *et al.*, 1998:123
7. do Nascimento *et al.*, 2005:779.
8. Myburgh, 2003:33

2.6 Statistical analysis

Statsoft® Statistica for Windows (Statsoft Inc., Tulsa, Oklahoma, USA) was used to perform the statistical analyses on the data obtained. A ln-transformation was performed on the P_{app} values. These data were then used to perform a linear regression of fraction absorbed in humans (F_a) on P_{app}. The coefficient of determination (r^2) was calculated. The equation of the linear line for prediction of F_a from ln P_{app} was obtained. 95% confidence bands were drawn on the graphs (Figure 5.2 & Figure 5.3).

CHAPTER 3
VALIDATION OF ANALYTICAL METHODS

3.1 Introduction

Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended analytical applications. The acceptability of analytical data corresponds directly to the criteria used to validate the method. The fundamental parameters for a bioanalytical validation include (1) accuracy, (2) precision, (3) selectivity, (4) sensitivity, (5) reproducibility, and (6) stability. As samples in this study were analysed as soon as they were obtained, stability was omitted (FDA, 2001: 5).

3.2 Selectivity

Selectivity, also known as specificity, is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. For selectivity, analyses of blank samples of the appropriate matrix should be obtained from at least six sources (FDA, 2001:4).

3.2.1 Method

For each method used, six different blank samples of KR were injected to test for interfering peaks.

3.2.2 Results and conclusion

No interfering peaks were obtained for any of the methods, showing that all methods were specific for the analyte examined.

3.3 Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of analyte in the sample. For the establishment of linearity five standard solutions for each analyte should be injected three times each (Sarrio & Salvestri, 1996:3).

The correlation coefficient and the y-intercept are used to judge the acceptability of linearity data. A correlation coefficient of greater than 0.997 is taken as acceptable, while the y-intercept should be less than a few percent of the response obtained for the analyte at the target level (Bruce *et al.*, 1998:94).

3.3.1 Method

For each analyte, a concentration series consisting of five concentrations was made up in KR, as it would be done for the transport studies. Each concentration was then injected three times. The average for the three analyses of each concentration was calculated and the data were used to plot concentration against response (AUC). From the resulting straight line the slope, intercept and correlation coefficient were obtained.

3.3.2 Results and conclusion

The results obtained during the linearity study are given in Table 3.1.

Table 3.1: Concentration range used during linearity study as well as slope, intercept, y-intercept as percentage of response and correlation coefficient obtained during study.

Compound	Range (µg/ml)		Slope	Intercept	Y-intercept percentage of response (%)	Correlation coefficient
	Low	High				
Caffeine	0.300	5.0	1803	493649	0.007	0.99969
Carbemazepine	0.048	0.3	117487	551	2.169	0.99931
Ketoprofen	0.500	14.0	43615	-1240	0.357	0.99989
Verapamil	0.500	20.0	15500	-5021	1.618	0.99927
Propranolol	1.000	14.0	181332	-134953	5.446	0.99751
Promethazine	0.500	3.5	5865	-866	4.329	0.99719
Paracetamol	1.400	85.7	72909	17786	0.841	0.99999
Furosemide	0.300	20.0	274	4504	0.010	0.99995
Ranitidine	1.000	14.0	34666	-10640	2.218	0.99890
Acyclovir	0.500	14.0	13331	311100	0.845	0.99994

The correlation coefficient for all methods is above 0.997, showing that the methods are linear. The y-intercept values are not more than 5.5 % of the response (AUC), indicating that they are within acceptable limits.

3.4 Accuracy

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value of the analyte. Accuracy is determined at three concentrations, with at least five determinations per concentration. These concentrations should represent a low, medium and high concentration in the range where studies will be taking place. The mean value should be within 15% of the actual value, except at the limit of quantification, where is should not deviate by more than 20%. The deviation of the mean from the true value serves as the measure of accuracy (FDA 2001:5).

3.4.1 Method

For each analyte under investigation, low, medium and high concentrations were prepared, as it would be done during the transport studies. Each concentration was analysed six times, and the average of the response calculated. This average and the linearity data were then used to calculate the concentration of the analyte. This calculated concentration was then expressed as a percentage of the actual concentration (Table 3.2).

3.4.2 Results and conclusion

Table 3.2: Concentrations used during accuracy study (Theo), calculated concentrations (Calc) and the average accuracy calculated.

Compound	Concentration (µg/ml)		Average accuracy (%)	Compound	Concentration (µg/ml)		Average accuracy (%)
	Theo	Calc			Theo	Calc	
Caffeine	0.300	0.288	96.0	**Promethazine**	0.500	0.550	110.0
	1.000	0.997	99.7		1.250	1.190	95.2
	5.000	4.973	99.5		3.500	3.290	94.0
Carbemazepine	0.048	0.049	102.9	**Paracetamol**	1.400	1.305	93.2
	0.120	0.120	100.2		7.100	7.186	101.2
	0.336	0.337	100.2		85.700	85.631	99.9
Ketoprofen	0.500	0.528	105.5	**Furosemide**	0.300	0.255	85.0
	3.000	2.954	98.5		5.000	5.071	101.4
	14.000	14.027	100.2		20.000	20.032	100.2
Verapamil	1.000	1.033	103.3	**Ranitidine**	3.000	2.862	95.4
	5.000	4.730	94.6		9.000	8.730	97.0
	20.000	20.930	104.6		14.000	13.978	99.8
Propranolol	2.000	2.093	104.7	**Acyclovir**	0.500	0.449	89.9
	5.000	4.487	89.7		5.000	5.034	100.7
	14.000	14.165	101.2		14.000	14.005	100.0

All accuracy values deviate less than 15 % above or below 100 %, showing that all values are within acceptable limits.

3.5 Precision

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogenous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15 %. Precision is further subdivided into within-run, intra-batch precision or repeatability and between run or inter-batch precision or repeatability, which measures precision with time and may involve different analysts, equipment, reagents and laboratories (FDA, 2001:5).

3.5.1 Intra-batch precision

3.5.1.1 Method

For the determination of intra-batch precision, three concentrations of each analyte were made up as they would be for the transport studies. Each concentration was analysed six times and the relative standard deviation calculated (Table 3.3).

3.5.1.2 Results and conclusion

Table 3.3: Concentrations used during intra-batch precision study and the calculated standard deviation.

Compound	Concentration (µg/ml)		RSD (%)	Compound	Concentration (µg/ml)		RSD (%)
Caffeine	Low	0.300	1.74	**Promethazine**	Low	0.500	4.68
	Medium	1.000	1.72		Medium	1.250	2.89
	High	5.000	0.89		High	3.500	9.39
Carbemazepine	Low	0.048	2.22	**Paracetamol**	Low	1.400	4.16
	Medium	0.120	3.27		Medium	7.100	0.67
	High	0.336	0.94		High	85.700	0.18
Ketoprofen	Low	0.500	3.36	**Furosemide**	Low	0.300	4.64
	Medium	3.000	2.21		Medium	5.000	0.43
	High	14.000	0.26		High	20.000	0.43
Verapamil	Low	0.500	3.80	**Ranitidine**	Low	1.000	4.38
	Medium	2.000	3.87		Medium	5.000	2.64
	High	20.000	4.65		High	14.000	1.60
Propranolol	Low	1.000	1.95	**Acyclovir**	Low	0.500	4.05
	Medium	5.000	2.25		Medium	5.000	0.62
	High	14.000	2.45		High	14.000	0.65

All RSD values were below 15 %, showing that the methods are precise during intra-batch studies.

3.5.2 Inter-batch precision

3.5.2.1 Method

For the determination of inter-batch precision, experiments were done over three days. On each day three concentrations of each analyte were made up as they would be for the

transport studies. Each concentration was analysed three times and the relative standard deviation over the three days calculated (Table 3.4).

3.5.2.2 Results and conclusion

Table 3.4: Concentrations used during inter-batch precision study and the calculated standard deviation.

Compound	Concentration (µg/ml)		RSD (%)	Compound	Concentration (µg/ml)		RSD (%)
Caffeine	Low	0.300	2.17	**Promethazine**	Low	0.500	9.72
	Medium	1.000	1.54		Medium	1.250	4.38
	High	5.000	1.39		High	3.500	3.69
Carbemazepine	Low	0.048	6.26	**Paracetamol**	Low	1.400	2.14
	Medium	0.120	1.48		Medium	7.100	3.20
	High	0.336	1.03		High	85.700	3.31
Ketoprofen	Low	0.500	5.76	**Furosemide**	Low	0.300	4.70
	Medium	3.000	0.56		Medium	5.000	1.85
	High	14.000	2.00		High	20.000	1.72
Verapamil	Low	0.500	9.51	**Ranitidine**	Low	1.000	2.80
	Medium	2.000	2.98		Medium	5.000	1.78
	High	20.000	5.33		High	14.000	0.96
Propranolol	Low	1.000	1.38	**Acyclovir**	Low	0.500	4.90
	Medium	5.000	2.76		Medium	5.000	0.51
	High	14.000	2.92		High	14.000	0.64

All RSD values were below 15 %, showing that the methods are precise during inter-batch studies.

3.6 Sensitivity

The sensitivity of an analytical method can be measured by determining the limit of quantification (QL) and limit of detection (DL). The limit of quantification of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The detection limit is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value (Sarrio & Salvestri, 1996:3).

3.6.1 Method

These two values can be calculated with the following formulae:

Limit of quantification

$$QL = \frac{10\sigma}{S}$$

Limit of detection

$$DL = \frac{3.3\sigma}{S}$$

where, σ = the standard deviation of the response,

and S = the slope of the calibration curve.

3.6.2 Results and conclusion

The limits of quantification and of detection for all drugs used in this study are given in Table 3.1.

Table 3.5: Limits of quantification (QL) and detection (DL).

Compound	QL (µg/ml)	DL (µg/ml)
Caffeine	0.238	0.078
Carbemazepine	0.026	0.009
Ketoprofen	0.212	0.070
Verapamil	0.448	0.148
Propranolol	0.393	0.130
Promethazine	0.286	0.094
Paracetamol	0.949	0.313
Furosemide	0.218	0.072
Ranitidine	0.541	0.178
Acyclovir	0.416	0.137

The limits of quantification were low enough to determine all drug concentrations in this study.

CHAPTER 4
INFLUENCE OF TISSUE PREPARATION ON DRUG PERMEABILITY

4.1 Introduction

The transport of 10 compounds across rat jejunum was studied in the basolateral to apical direction. For each compound, three experiments consisting of six chambers each were done to give a total of 18 transport values (P_{app}). This was done for stripped tissue and unstripped tissue. This is more than has been previously used (4-10 transport values [Collet *et al.*, 1999:173; Lennernäs *et al.*, 1997:668; Varna & Panchagnula, 2005:449]). The P_{app} (all P_{app} values are in $cm.s^{-1}$) for each chamber in each experiment was calculated and the average taken. This was then correlated to the fraction absorbed in humans (F_a) as obtained from literature. An example of the calculations performed is given in appendix A and the individual cumulative transport and P_{app} values in appendix B.

4.2 Transport across rat intestine

4.2.1 Transport of individual drugs

4.2.1.1 Caffeine transport

Figure 4.1 gives the cumulative transport of caffeine across rat intestine. As can be seen from the cumulative transport graph of caffeine, the preparation method of the tissue has little effect on the transport. The average P_{app} for caffeine in this study was 4.533×10^{-6} for the stripped tissue and 4.704×10^{-6} for the unstripped tissue. This is lower than has been seen previously in Caco-2 cells (50.5×10^{-6}, 30.8×10^{-6}) and PAMPA (10.8×10^{-6}) but this can be expected as the rat intestine is much more complex than the Caco-2 monolayer (Yee, 1997:764; Yazdanian *et al.*, 1998:1492).

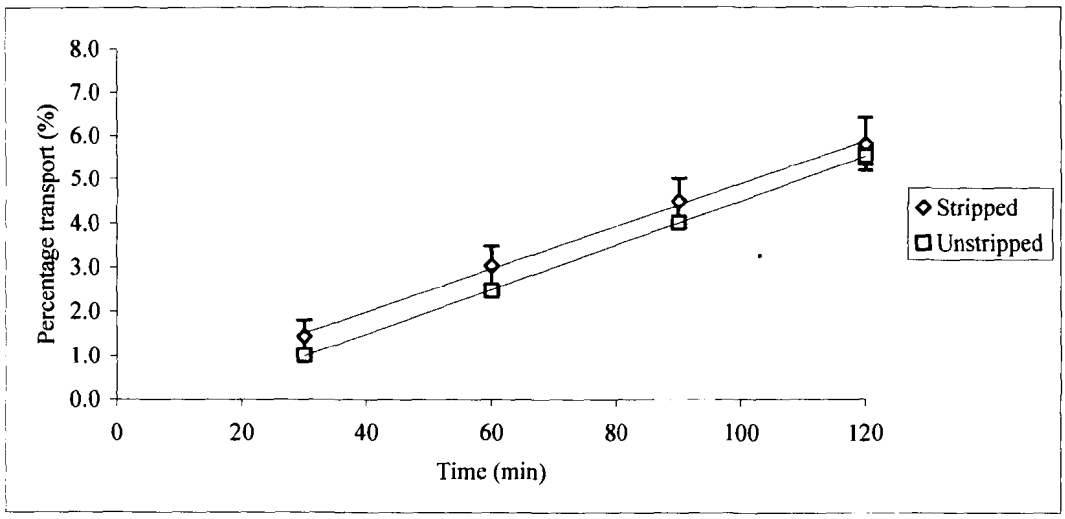


Figure 4.1: Cumulative transport of caffeine.

4.2.1.2 Carbamazepine transport

Figure 4.2 gives the cumulative transport of carbamazepine across rat intestine.

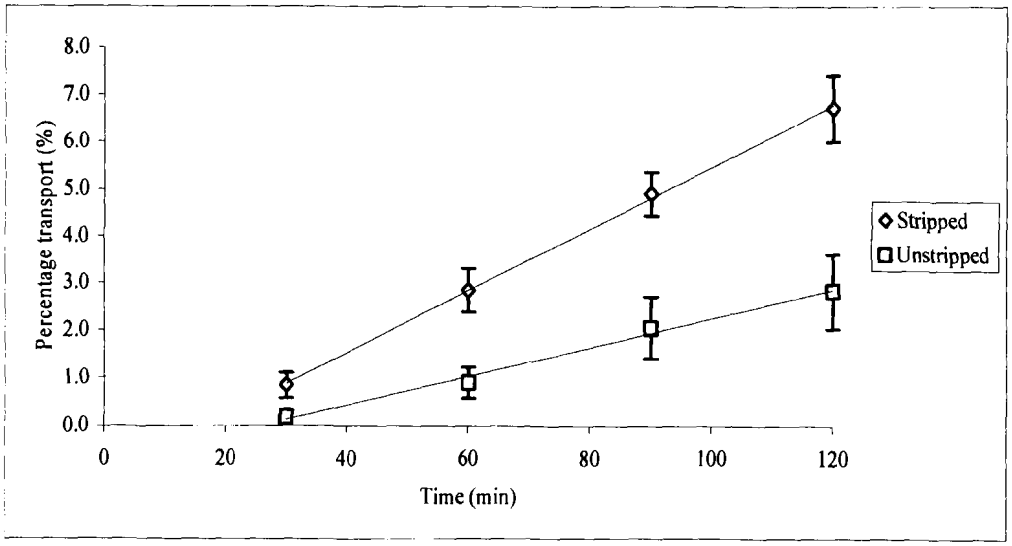


Figure 4.2: Cumulative transport of carbamazepine.

As can be seen from the cumulative transport graph of carbamazepine, the preparation method of the tissue has an effect on the transport. The average P_{app} for carbamazepine in this study was 6.129×10^{-6} for the stripped tissue and 2.828×10^{-6}

for the unstripped tissue. This is lower than has been seen in MDRI-MDCKII cells (60.2×10^{-6}) (Varma *et al.*, 2004:17).

4.2.1.3 Ketoprofen transport

Figure 4.3 gives the cumulative transport of ketoprofen across rat intestine.

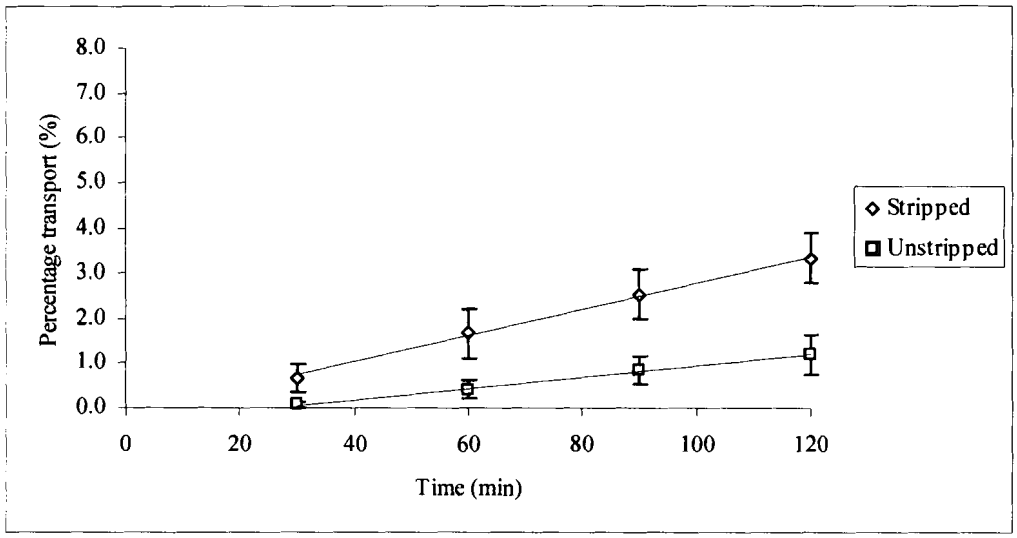


Figure 4.3: Cumulative transport of ketoprofen.

As can be seen from the cumulative transport graph of ketoprofen, the preparation method of the tissue has some effect on the transport. The average P_{app} for ketoprofen in this study was 2.755×10^{-6} for the stripped tissue and 1.177×10^{-6} for the unstripped tissue. This is lower than has been seen in Caco-2 cells (93.0×10^{-6}) and MDCK cells (20.0×10^{-6}) (Irvine *et al.*, 1999:30).

4.2.1.4 Verapamil transport

Figure 4.4 gives the cumulative transport of verapamil across rat intestine.

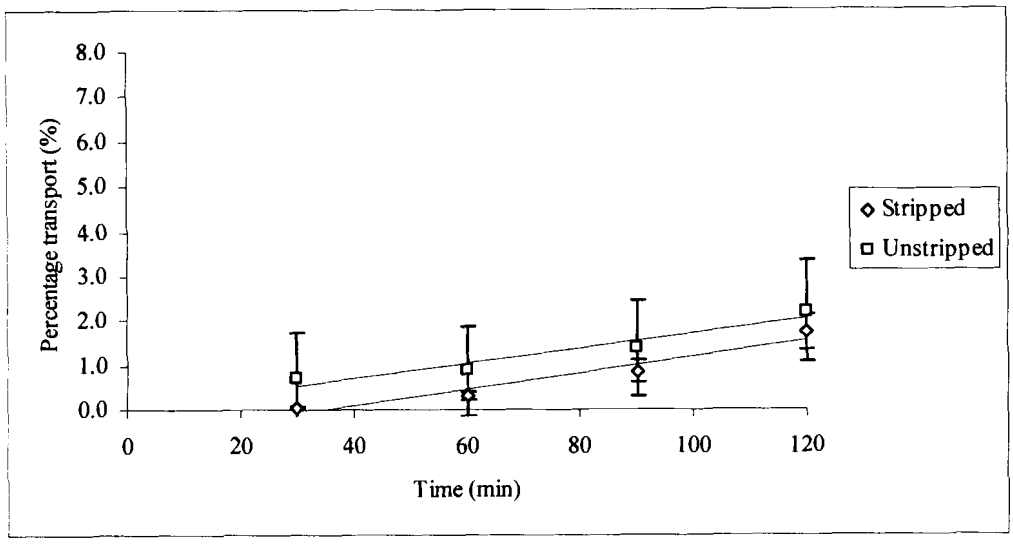


Figure 4.4: Cumulative transport of verapamil.

As can be seen from the cumulative transport graph of verapamil, the preparation method of the tissue has little effect on the transport. The average P_{app} for verapamil in this study was 1.727×10^{-6} for the stripped tissue and 1.545×10^{-6} for the unstripped tissue. This is lower than has been seen in Caco-2 cells (29.60×10^{-6}) and MDRI-MDCKII (multidrug resistance transfected MDCK type II) cells (41.5×10^{-6}) (Ingels *et al.*, 2004:225; Varma *et al.*, 2004:17).

4.2.1.5 Propranolol transport

Figure 4.5 gives the cumulative transport of propranolol across rat intestine.

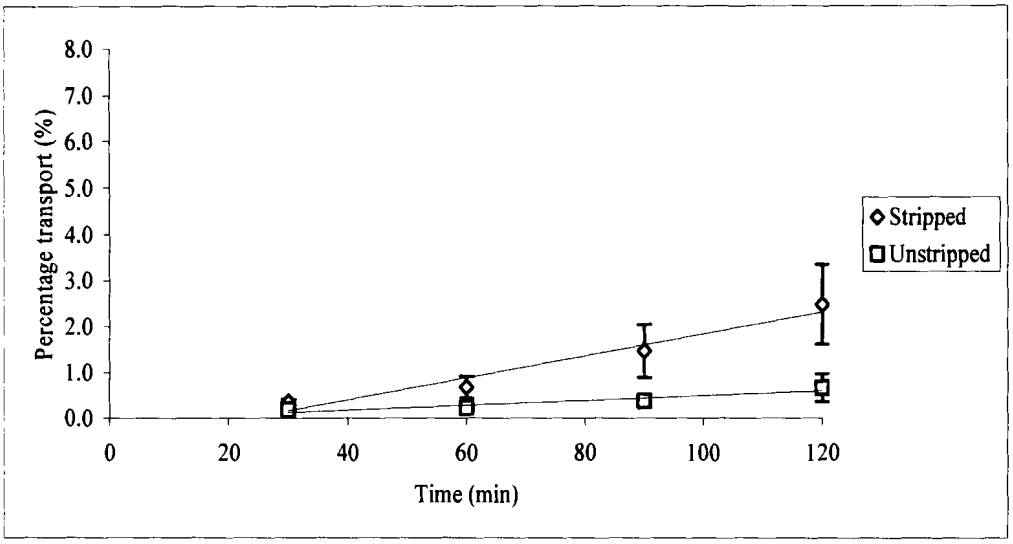


Figure 4.5: Cumulative transport of propranolol.

As can be seen from the cumulative transport graph of propranolol, the preparation method of the tissue has an effect on the transport. The average P_{app} for propranolol in this study was 2.245 x 10^{-6} for the stripped tissue and 0.497 x 10^{-6} for the unstripped tissue. This is lower than has been seen in Caco-2 cells (30.1 x 10^{-6}, 41.9 x 10^{-6}, 27.5 x 10^{-6}, 110.0 x 10^{-6}, 21.8 x 10^{-6}) and MDCK cells (170.0 x 10^{-6}) (Artursson & Karlsson, 1991:882; Irvine *et al.*, 1999:30; Pade & Stavchansky, 1998:1605; Yazdanian *et al.*, 1998:1492; Yee, 1997:764).

4.2.1.6 Promethazine transport

Figure 4.6 gives the cumulative transport of promethazine across rat intestine.

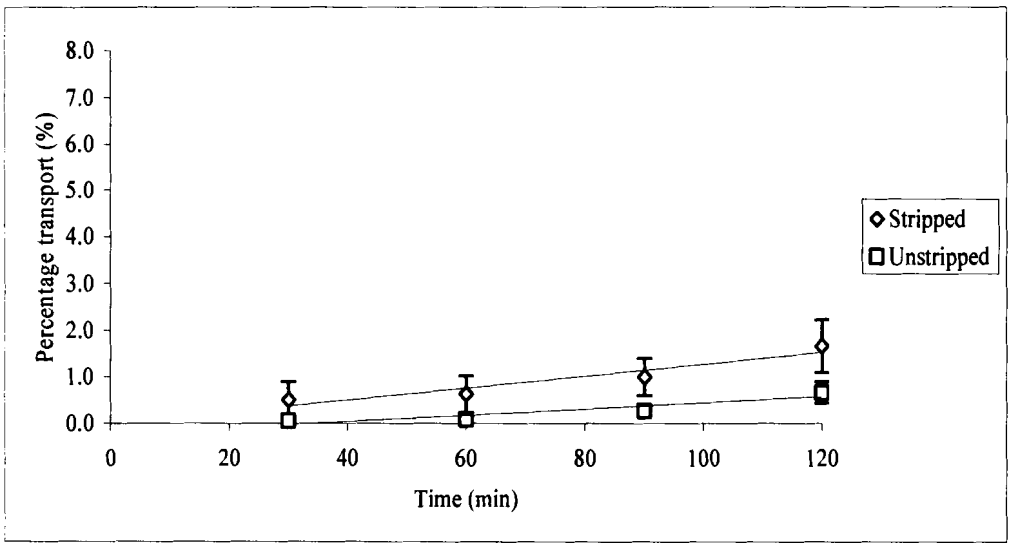


Figure 4.6: Cumulative transport of promethazine.

As can be seen from the cumulative transport graph of promethazine, the preparation method of the tissue has little effect on the transport. The average P_{app} for promethazine in this study was 1.199×10^{-6} for the stripped tissue and 0.630×10^{-6} for the unstripped tissue. This is lower than has been seen in MDRI-MDCKII cells (43.0×10^{-6}) (Varma *et al.*, 2004:16).

4.2.1.7 Paracetamol transport

Figure 4.7 gives the cumulative transport of paracetamol across rat intestine.

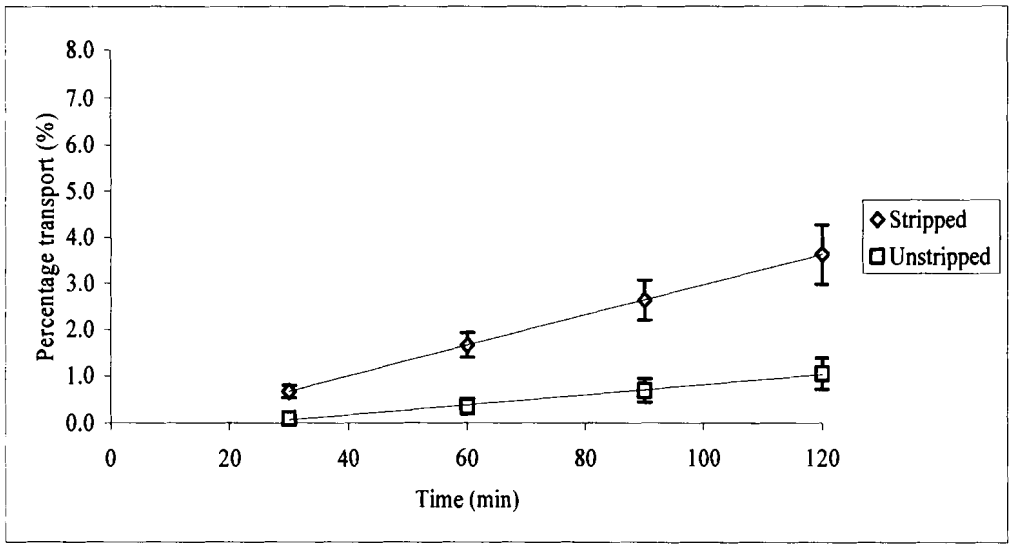


Figure 4.7: Cumulative transport of paracetamol.

As can be seen from the cumulative transport graph of paracetamol, the preparation method of the tissue has some effect on the transport. The average P_{app} for pracetamol in this study was 3.061×10^{-6} for the stripped tissue and 1.006×10^{-6} for the unstripped tissue. This is lower than has been seen in Caco-2 cells (23.4×10^{-6}; 100×10^{-6}) and MDCK cells (35×10^{-6}) (Irvine *et al.*, 1999:30; Yamashita *et al.*, 2000:197).

4.2.1.8 Furosemide transport

Figure 4.8 gives the cumulative transport of furosemide across rat intestine.

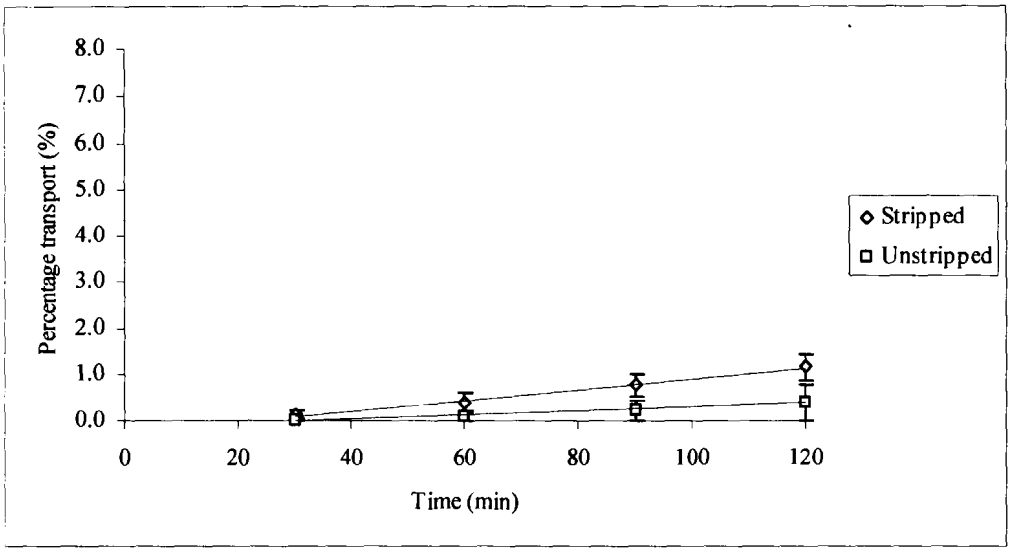


Figure 4.8: Cumulative transport of furosemide.

As can be seen from the cumulative transport graph of furosemide, the preparation method of the tissue has some effect on the transport, with stripped tissue giving a greater amount and tempo of transport. The average P_{app} for furosemide in this study was 1.073×10^{-6} for the stripped tissue and 0.399×10^{-6} for the unstripped tissue. This is higher than has been seen in Caco-2 cells (0.12×10^{-6}, 0.045×10^{-6}, 0.14×10^{-6}). Compared to transport in MDCK cells (0.62×10^{-6}), the stripped tissue gives a higher P_{app}, and the unstripped tissue a lower P_{app}. This lower value may be due to efflux by unknown efflux carriers in the Caco-2 cells (Basolateral-Apical transport 56.5 times higher than Apical-Basolateral transport in study) (Ingels *et al.*, 2004:225; Irvine *et al.*, 1999:30; Yamashita *et al.*, 2000:197).

4.2.1.9 Ranitidine transport

Figure 4.9 gives the cumulative transport of ranitidine across rat intestine.

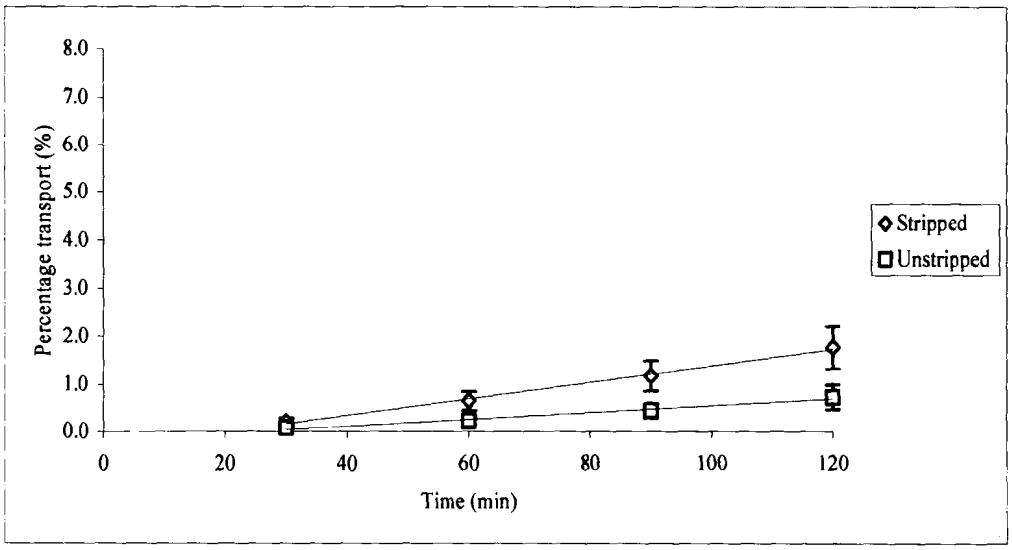


Figure 4.9: Cumulative transport of ranitidine.

As can be seen from the cumulative transport graph of ranitidine, the preparation method of the tissue has some effect on the transport. The average P_{app} for ranitidine in this study was 1.625 x 10^{-6} for the stripped tissue and 0.676 x 10^{-6} for the unstripped tissue. This is higher than has been seen two studies utilising Caco-2 cells (0.49 x 10^{-6}; Insufficient transport) (Irvine *et al.*, 1997:30; Yazdanian 1998:1492). In another study done with Caco-2 cells, a Papp of 6.78 x 10^{-6} was obtained (Masungi *et al.*, 2004:2517). These values were all lower than values obtained in a study done in rats by the everted gut sac method, which showed a P_{app} of 39.5 x 10^{-6} (Lacombe *et al*, 2004:389).

4.2.1.10 Aciclovir transport

Figure 4.10 gives the cumulative transport of aciclovir across rat intestine.

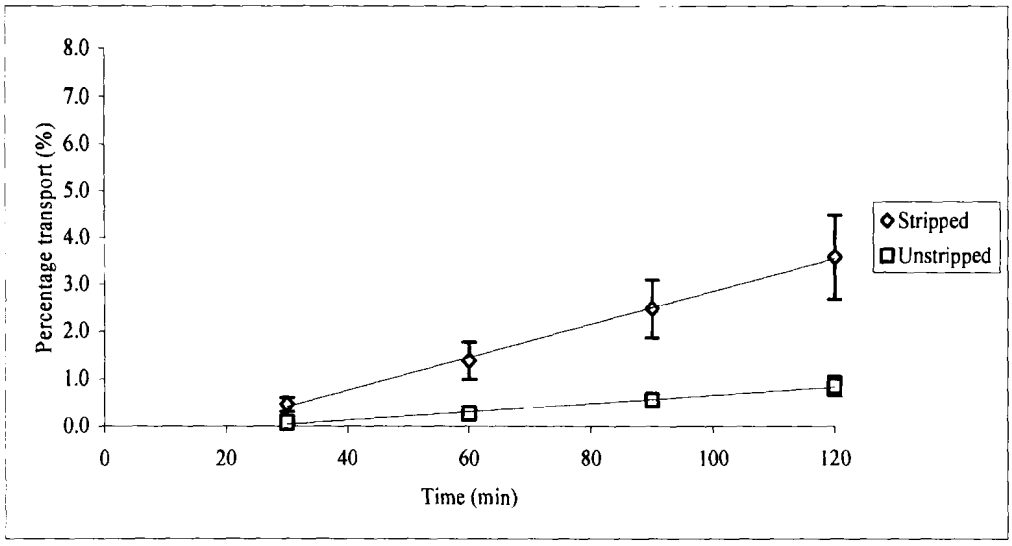


Figure 4.10: Cumulative transport of aciclovir.

As can be seen from the cumulative transport graph of aciclovir, the preparation method of the tissue has some effect on the transport. The average P_{app} for aciclovir in this study was 3.258 x 10^{-6} for the stripped tissue and 0.825 x 10^{-6} for the unstripped tissue. This is higher than has been seen in one study in Caco-2 cells (Insufficient transport) (Irvine *et al.*, 1999:30). In other studies (Masungi *et al.*, 2004:2517; Yee, 1997:764) the Papp values were 1.7 x 10^{-6} and 2.0 x 10^{-6}. This is lower than the stripped tissue, but higher than the unstripped tissue.

4.2.1.11 Discussion

The average P_{app} values obtained for the various drugs using stripped as well as unstripped jejunum are presented in Table 4.1. The values obtained in this study are lower than the values obtained in studies conducted in cultures cell studies. This can be expected as the drugs have a much longer route to complete using rat intestine which is much more complex compared to cultured cell monolayers (Irvine *et al.*, 1999:30; Varma *et al.*, 2004:17; Yazdanian *et al.*, 1998:1492; Yee, 1997:764).

4.2.2 Influence of tissue preparation of P_{app}

4.2.2.1 Introduction

There is no standardised method for the preparation of tissue for use in Ussing type chambers. Some scientists use the tissue unstripped, while others first remove the serosa and muscle layers (Berggren *et al.*, 2004:555).

There are advantages and disadvantages to both methods of tissue preparation. If the tissue is used as is, there is no additional trauma to the tissue other than the removal and rinsing of the intestine before mounting. Legen *et al.*, (2005:185) states that the serosa and overlaying longitudinal and circular muscle layers should not be stripped off because these layers are too thin to be properly removed without damaging the mucosal layer. This unstripped model, however, does not modulate the *in vivo* situation as crossing of the muscle layers and serosa by drugs is not part of the normal absorption process (Figure 4.11). The normal pathway for absorption is simply over the epithelium of the villi and into the blood vessels. In the unstripped model, however, the drug also needs to cross the submucosa which presents a significant increase in the path length for permeation. This increase in path length may lead to an decrease in the rate of drug transport (Legen *et al.*, 2005:185; Žakelj *et al.*, 2004:175).

The stripped method of tissue preparation provides an *in vitro* model which more closely resembles the *in vivo* situation as the villi, microvilli and mucous layer stay intact. The additional trauma the tissue is subjected to could lead to damage to the tissue and a decline in tissue viability. In spite of this, there are still many scientists who prefer this method of tissue preparation for Ussing and Ussing-type chamber intestinal transport studies (Berggren *et al.*, 2004: 555; Collet *et al.*, 1999:172; Lennernäs *et al.*, 1997:667).

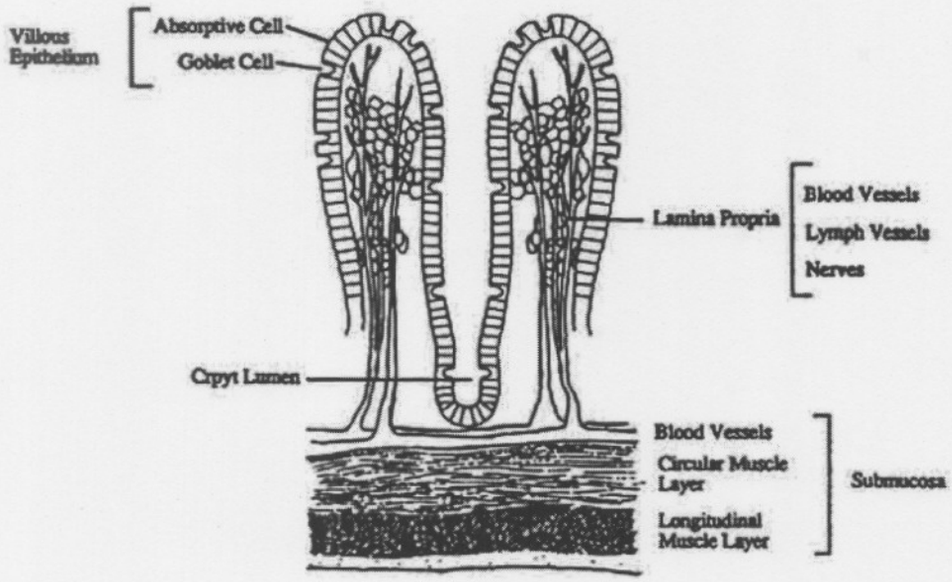


Figure 4.11: Structure of the intestinal mucosa (Hidalgo, 2001:386)

4.2.2.2 Effect of tissue preparation on drug transport

The stripping of the serosa and muscle layers has little effect on transport of caffeine, verapamil and promethazine. These are the substances with the highest aqueous solubility (Table 4.1). The other drug with high aqueous solubility in the group is propranolol, but the P_{app} for the stripped method of preparation was 4.52 times higher than for the unstripped method (Table 4.1). From the structures of these four drugs it can be seen that caffeine, verapamil and promethazine each contain at least one N-Methyl group, while propranolol has no such group (Figure 4.12). Molecular weight and logP give no further insight into the problem. When looking at the number of hydrogen bond donors (as expressed by number of hydrogens attached to oxygen and nitrogen [Krämer, 1999:378]), it can be seen that all three of these molecules contain no hydrogen bond donors. It was found by Raevsky & Schaper (1998:802), that a decrease in the number of hydrogen donor groups led to an increase in intestinal absorption. The other six drugs show a profile as would be expected, as the P_{app} across unstripped tissue is much higher than across stripped tissue. The higher P_{app} is expected as the drugs need to cross the muscle and serosal layers, which are an additional barrier to permeation.

Table 4.2 gives the rank order for maximal permeability as would be expected from molecular weight, aqueous solubility and logP. The rank order were obtained according to the following reasoning. (i) For molecular weight: the lower molecular weight, the higher should be intestinal permeability. (ii) For aqueous solubility: as the intestinal membrane is mainly lipoid in nature, it will be more easily penetrated by lipophilic molecules than by hydrophilic molecules (Lund, 1994:70). (iii) For logP: the higher logP, the greater is fraction absorbed in humans (Krämer, 1999:376). As can be seen from Table 4.2, there does not seem to be any pattern and the physicochemical properties do not explain the permeability as observed in the transport experiments. This may be due to the fact that the physicochemical properties do not affect absorption individually, but their combined effects give the permeability observed. Another reason for this discrepancy may be the influence of active transporters, both influx and efflux, which can have an effect on the absorption of drugs.

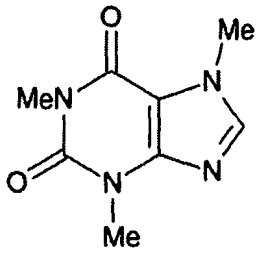
Table 4.1: Physicochemical properties of drug investigated, P_{app} obtained for stripped and unstripped tissue transport and ratio unstripped to stripped P_{app}.

	Fa	Molecular weight	Aqueous solubility (mg/ml)	logP	H Acceptors*	H Donors#	Average P_{app} (10^{-6})		Ratio (S/U)
							Stripped (S)	Unstripped (U)	
Caffeine	1.00	194.19	20	0.00	6	0	4.533	4.704	0.96
Carbemazepine	1.00	236.27	0.1	2.67	3	2	6.129	2.282	2.69
Ketoprofen	1.00	254.28	0.5	0.00	2	1	2.755	1.177	2.34
Verapamil	1.00	491.06	83	4.68	6	0	1.727	1.545	1.12
Propranolol	0.90	295.80	50	1.20	3	2	2.245	0.497	4.52
Promethazine	0.85	320.88	1667	2.90	3	0	1.199	0.630	1.90
Paracetamol	0.80	151.16	14.3	0.34	2	2	3.061	1.006	3.04
Furosemide	0.60	330.74	0.18	1.51	7	4	1.073	0.399	2.69
Ranitidine	0.55	350.56	1.8	2.06	7	2	1.625	0.676	2.40
Acyclovir	0.21	225.20	0.7	0.22	8	4	3.258	0.825	3.95

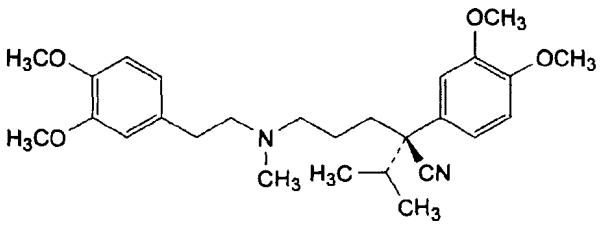
* H – Acceptors as expressed by the number of nitrogen and oxygen atoms in the molecule
# H – Donors as expressed by the number of hydrogen atoms attached to nitrogen and oxygen in the molecule

Table 4.2: Physicochemical properties placed in rank order for maximal permeability.

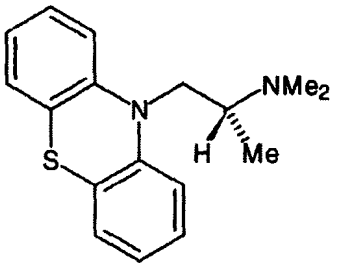
	Fa	Rank order for maximal permeability				
		Molecular weight	Aqeous solubility	LogP	Stripped (S)	Unstripped (U)
Caffeine	1.00	2	7	1	2	1
Carbemazepine	1.00	4	1	8	1	2
Ketoprofen	1.00	5	3	2	5	4
Verapamil	1.00	10	9	10	7	3
Propranolol	0.90	6	8	5	6	9
Promethazine	0.85	7	10	9	9	6
Paracetamol	0.80	1	6	4	4	5
Furosemide	0.60	8	2	6	10	10
Ranitidine	0.55	9	5	7	8	8
Acyclovir	0.21	3	4	3	3	7



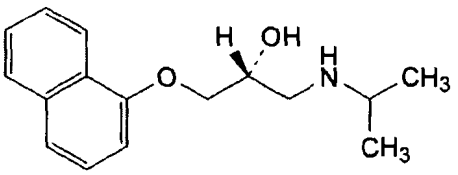
Caffeine



Verapamil



Promethazine



Propranolol

Figure 4.12: Structures of caffeine, verapamil, promethazine and propranolol.

CHAPTER 5
STATISTICAL COMPARISON OF P_{app} TO F_a

5.1 Introduction

The comparison of human fraction absorbed (F_a) to transport across various membranes (normally represented by P_{app}) is a useful tool, as it can be used to show a potential correlation between *in vitro* data and *in vivo* absorption in man. A correlation has been shown in cell culture models, other *in vitro* models such as everted gut sac and *in situ* studies using various animal models. It has been found that this correlation is not linear, but rather logarithmic, with a straight line correlation if the ln (or log) of P_{app} is taken and compared with F_a (Artursson *et al.,* 2001:34; Irvine *et al.,* 1999:31; Pade & Stavchansky, 1998:1606; Watanabe *et al.,* 2004:661; Yee, 1997:766)

5.2 Transport across rat intestine (P_{app}) compared to fraction absorbed in humans (F_a)

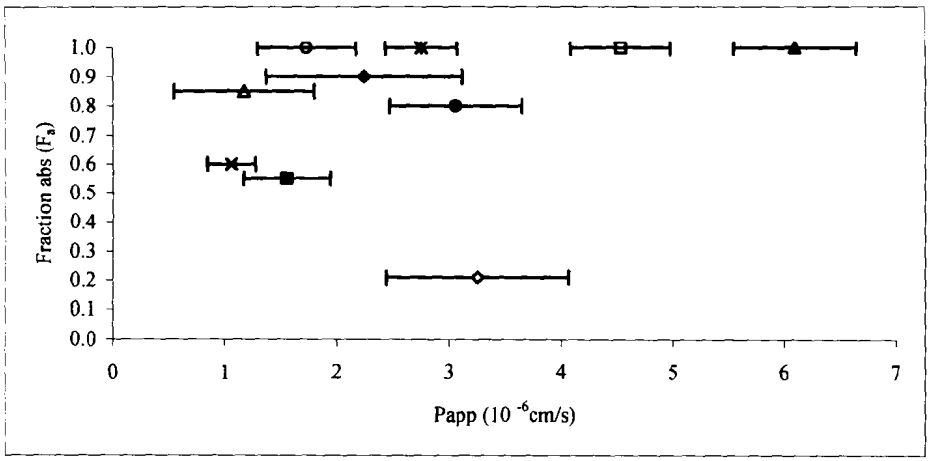
The P_{app} values obtained in the above experiments were compared to fraction absorbed in humans (obtained from literature). The average P_{app} and fraction absorbed for both stripped and unstripped techniques are given in Table 5.1. Figures 5.1 a & b show graphs of F_a plotted against average P_{app} for both stripped and unstripped preparation methods.

Table 5.1: Average P_{app} values for both stripped and unstripped experimental techniques.

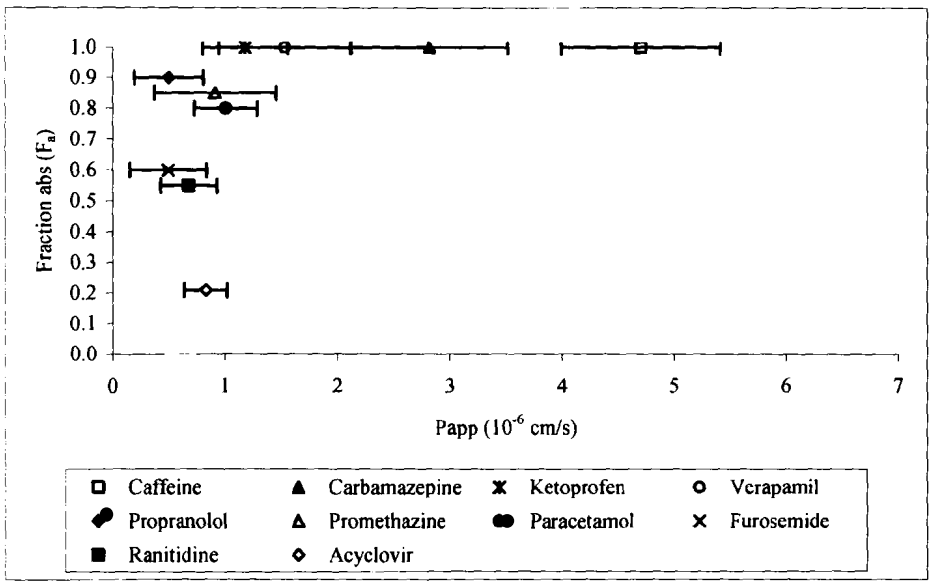
Drug	F_a	Average P_{app} (10^{-6})*	
		Stripped	Unstripped
Caffeine	1.00	4.533 ± 0.445	4.704 ± 0.710
Carbamazepine	1.00	6.129 ± 0.577	2.828 ± 0.697
Ketoprofen	1.00	2.755 ± 0.320	1.177 ± 0.382
Verapamil	1.00	1.727 ± 0.439	1.545 ± 0.600
Propranolol	0.90	2.245 ± 0.875	0.497 ± 0.306
Promethazine	0.85	1.199 ± 0.640	0.630 ± 0.280
Paracetamol	0.80	3.061 ± 0.591	1.006 ± 0.280
Furosemide	0.60	1.073 ± 0.227	0.399 ± 0.371
Ranitidine	0.55	1.625 ± 0.390	0.676 ± 0.252
Aciclovir	0.21	3.258 ± 0.813	0.825 ± 0.190

* Represents value ± standard deviation

From the graphs it can be seen that there is no clear differentiation between drugs with high and low F_a based on the P_{app} values. The data given in the graphs also show that the relationship between F_a and P_{app} is not linear. For this reason a ln-transformation was done on the P_{app} values, to give the graphs shown in Figure 5.2.

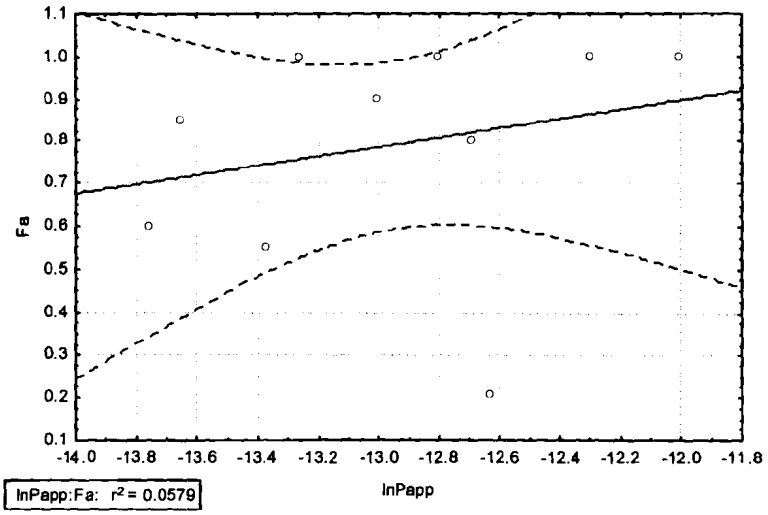


a

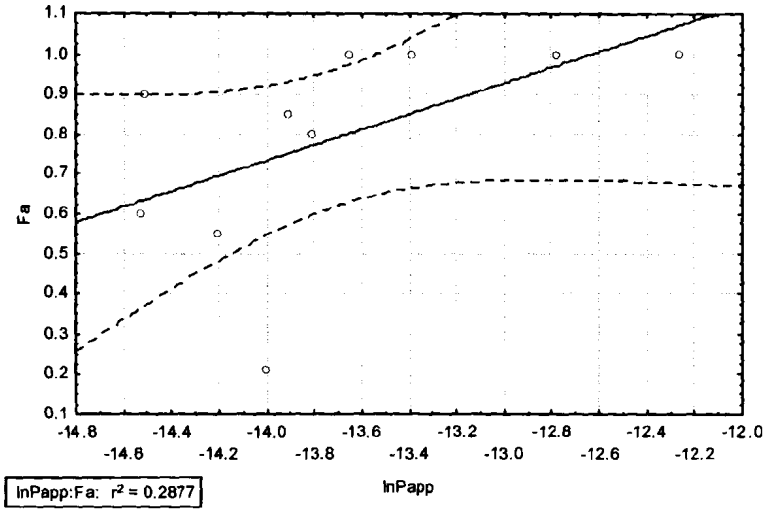


b

Figure 5.1: F_a plotted against average P_{app} for stripped (a) and unstripped (b) tissue.



(a)



(b)

Figure 5.2: Statistical analysis of data obtained in this study: (a) stripped, (b) unstripped.

5.2.1 Stripped method of tissue preparation

For this method the equation obtained after linear regression is $F_a = 0.111\ln P_{app} + 2.2289$. The r^2 value obtained is 0.0579. This shows that with these data points there is a very poor correlation between P_{app} and F_a.

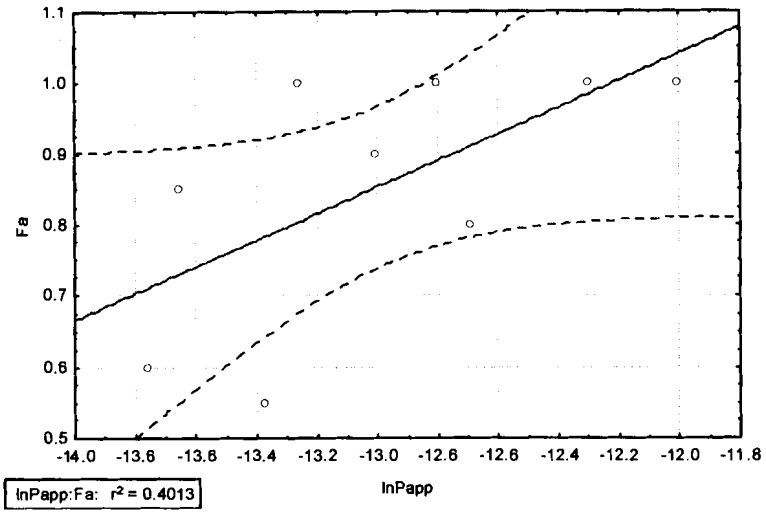
5.2.2 Unstripped method of tissue preparation

For this method the equation obtained after linear regression is $Fa = 0.1941\ln P_{app} + 3.4511$. The r^2 value obtained is 0.2877. This shows that although with these data points give a better correlation, the correlation between P_{app} and F_a is still poor.

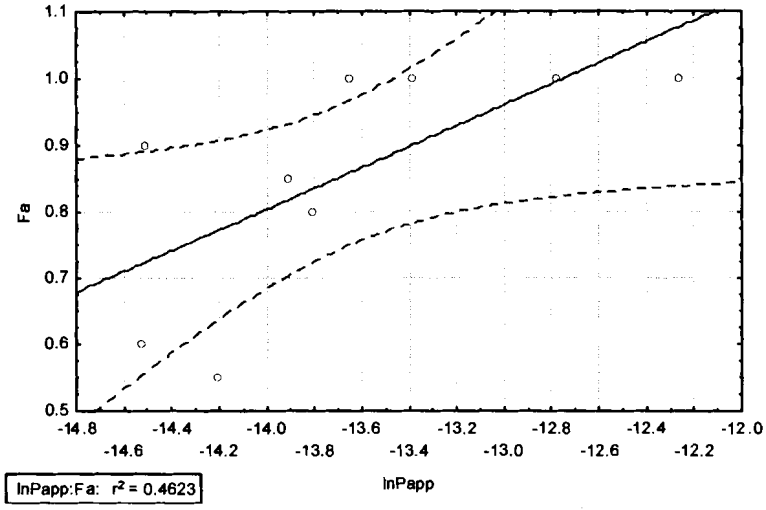
5.2.3 Repeat analysis excluding outlier

As can be seen from the graphs, the data point for aciclovir in both cases is an outlier. Therefore it was decided to repeat the linear regression after removal of this point. The graphs obtained after this removal are given in Figure 5.13.

After the removal of aciclovir, the r^2 of both linear plots improved, the stripped data from 0.0579 to 0.4013 and the unstripped data from 0.2877 to 0.4623. After the removal of aciclovir, the two methods of tissue preparation give very similar profiles. The equations obtained after linear regression on this data set are $F_a = 0.1878\ln P_{app} + 3.2945$ for the stripped data and $F_a = 0.1567\ln P_{app} + 2.9983$ for the unstripped data.



(a)



(b)

Figure 5.3: Statistical analysis of data obtained after the removal of aciclovir: (a) stripped, (b) unstripped.

5.2.4 Predictive error

If the P_{app} values obtained during this study are substituted into the equations obtained during statistical analysis, it is possible to calculate the error made when using this data to predict F_a. The predictive errors for both methods of tissue preparation and with and without the outlier are given in Table 5.2.

Table 5.2: Error made when F_a is predicted with equations obtained from statistical analysis.

Drug	F_a	Predicted F_a Error (%)			
		Stripped		Unstripped	
		All	No outlier	All	No outlier
Caffeine	1.00	13.68	1.62	-16.01	-7.60
Carbamazepine	1.00	10.34	-4.04	-6.13	0.37
Ketoprofen	1.00	19.21	10.97	10.88	14.10
Verapamil	1.00	24.40	19.74	5.60	9.84
Propranolol	0.90	12.76	5.36	19.57	19.57
Promethazine	0.85	15.82	13.64	9.43	10.47
Paracetamol	0.80	-2.45	-13.76	-7.58	-4.29
Furosemide	0.60	-17.21	-18.88	-13.52	-14.90
Ranitidine	0.55	-36.23	-43.83	-42.46	-40.37
Aciclovir	0.21	-293.56		-291.55	
Average (Absolute value)		44.56	13.18	42.27	12.15

As can be seen from Table 5.2, aciclovir is the drug which gives the greatest error. This can be seen from the plots of F_a against P_{app}. The reason for this deviation could be that the transport of aciclovir is dose dependant (Zhao *et al.*, 2001:774). It has been reported. According to de Miranda (As quoted by Lee *et al.*, 1997:55) it has been shown that the oral bioavailability of acyclovir is highly variable and species dependant. The existence of a saturable, carrier mediated process in the oral absorption of acyclovir by mice, rats and dogs has been proposed based on a decline in the fraction of dose absorbed with increasing doses. The presence of these carrier mediated processes could result in a higher rate of absorption in rats compared to humans (Lee *et al.*, 1997:56).

The drug which gives the second greatest error is ranitidine. The discrepancy of ranitidine from F_a may be explained by Collet *et al.*, (1999:174) who found that, although ranitidine is a substrate for p-gp, the extrusion of ranitidine is concentration dependant. It is possible that the concentration of ranitidine (1.23 mM) in this experiment is sufficient to saturate p-gp in the rat intestine. Collet *et al.*, (1999:174) found that p-gp had an effect on absorption at a concentration of 0.1 mM and no effect on concentration at a concentration of 10 mM. If the concentration in the human intestine is insufficient to saturate p-gp, then the permeability across the rat intestine will be higher than that seen in humans (Collet *et al.*, 1999:174).

CHAPTER 6
CONCLUSION AND RECOMMENDATIONS

The prediction of intestinal permeability has become an important part of not only drug development but also drug registration. This prediction makes it possible to exclude new chemical entities that would have insufficient bioavailability and would thus be a waste of money. Prediction also makes it possible to apply for a waiver of *in vivo* bioavailability and bioequivalence studies. This makes the registration of generic medicines much easier and cheaper (Balimane *et al.,* 2000:301; FDA, 2000:2).

The use of *in vivo* methods for the prediction of intestinal permeability in both animals and humans is impractical, as these methods are laborious, expensive and have ethical repercussions. Other methods can, however, never completely mimic the *in vivo* situation. Thus, a method must be chosen which is simple, reproducible, has a rapid turnaround time and can predict *in vivo* absorption in humans (Habucky, 1995:22).

The methods which best mimic the *in vivo* situation and are rapid and reproducible are *in vitro* methods utilising cultured epithelial monolayers or intestinal tissue sheets. These methods are able to predict the intestinal permeability of molecules. Molecules with sufficient intestinal permeability may not be sufficiently bioavailable due to first-pass metabolism or instability in the gastro-intestinal tract, but molecules that do not have sufficient membrane permeability by these screening methods will not be orally bioavailable (Mälkiä *et al.,* 2004:24, Smith, 1995:29).

Despite their undeniable benefit as model membranes, cell cultures come with certain disadvantages. The method is rather laborious (aseptic techniques need to be applied to culture and maintain the cells) and time consuming as cells have to be cultured for approximately three weeks prior to use. From a physiological point of view cell cultures have the following shortcomings: (1) the tissue in intestinal villi contain more than one type of cell, (2) most cell lines do not produce the mucus layer found in normal intestine and (3) not all metabolising enzymes found in the enterocytes are

present in cell lines. (Mälkiä *et al.*, 2004:24, Habucky, 1995:25). Also, there have been many studies done to compare the transport across Caco-2 cells with absorption in humans, while very few studies have been done to compare transport across rat jejunum with absorption in humans (Yee, 1997: 764, Zhu *et al.*, 2002:402). For these reasons it was decided to test the validity of the Sweetana-Grass diffusion chamber technique as a method for prediction intestinal absorption in humans. Two methods of tissue preparation were also chosen, as there has been little or no studies on the effect of this variable on the transport of drugs across rat intestinal tissue.

The final conclusions of this study are as follows:

- It was possible to determine the P_{app} values for the ten drugs in this study by using the Sweetana-Grass diffusion method. These P_{app} values showed a non-linear relationship between F_a and P_{app} in rat jejunum, as has been seen in other studies.

- When comparing the methods of tissue preparation, the unstripped method of tissue preparation gave a better correlation between P_{app} and F_a ($r^2 = 0.0579$ compared to unstripped $r^2 = 0.2877$).

- If the outlier aciclovir is excluded from statistical analysis, the correlation improves significantly and the correlation for the two methods of tissue preparation are much closer together (stripped $r^2 = 0.4013$ unstripped $r^2 = 0.4623$).

- A possible reason for the high permeability of aciclovir observed in this study may be the fact that its transport is concentration dependant. Further, the bioavailability of acyclovir is highly species dependant.

- This method of intestinal prediction does show a direct correlation between $\ln(P_{app})$ and fraction absorbed in humans.

Recommendations:

- The study should be expanded to a larger number of drugs, with more drugs having a low F_a, using only the unstripped method.

- The effect of concentration on the rate of transport should be investigated.

- The study should be done bidirectionally to determine the effect of active transport on the investigated compounds.

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APPENDIX A

Table A.1: Example of the values and calculations done to determine the apparent permeability coefficient (P_{app})

Refrence Concentration[a] (μg/mL)	Time (min)	Peak Area (mAU)	Transport corrected for dilution[b] (mAU)	Concentration[c] (ng/mL)	Relative transport[d] (%)	Slope of relative transport against time[e]	P_{app}[f] ($x10^{-6}$ cm/s)	Mean P_{app} ($x10^{-6}$ cm/s)
153	**Chamber 1**							
	30	847	847	0.16	0.108	0.01251	1.171	1.139
	60	3382	3412	0.68	0.447			
	90	6341	6463	1.30	0.850			
	120	9068	9299	1.87	1.224			
	Chamber 2							
	30	802	802	0.16	0.102	0.01016	0.951	
	60	2886	2915	0.58	0.381			
	90	5261	5365	1.08	0.705			
	120	7483	7675	1.54	1.010			
	Chamber 3							
	30	1092	1092	0.21	0.140	0.01034	0.968	
	60	2453	2492	0.50	0.325			
	90	5612	5701	1.15	0.749			
	120	7642	7846	1.58	1.033			
	Chamber 4							
	30	2285	2285	0.46	0.298	0.01589	1.488	
	60	6123	6205	1.25	0.816			
	90	9729	9951	2.00	1.311			
	120	12709	13064	2.63	1.722			
	Chamber 5							
	30	2605	2605	0.52	0.340	0.01234	1.155	
	60	5322	5415	1.09	0.711			
	90	8097	8290	1.67	1.091			
	120	10688	10984	2.21	1.447			
	Chamber 6							
	30	2306	2306	0.46	0.301	0.01176	1.101	
	60	5641	5723	1.15	0.752			
	90	7902	8106	1.63	1.067			
	120	10124	10414	2.10	1.372			

Table A.2: Values used to obtain standard curve

Concentration (µg/mL)	Peak Area (mAU)				Slope (m)	Y-Intercept (c)
	1	2	3	Mean		
134.5	916	877	936	910	4952	30
269.0	2957	2883	2849	2896		
403.5	58*6	5705	5730	5718		
538.0	13191	13442	13104	13246		

Concentration of furosemide in 7 mL

10.7 mg weighed and dissolved in 20 mL KR

10.7 mg x 1000 / 20 mL = 535 µg/mL

2 mL is added to the chamber to give 7 mL

2 x 535 µg/mL / 7 mL = 153 µg/mL

It is the transport corrected for dilution at one time interval divided by 28.

This value is then added to the value at the next time interval.

Example: 847/28 + 3382 = 3412

The value of 28 is obtained by dividing the volume of the chamber (7000 µL) by the volume of the replaced buffer (250 µL) (7000 µL/250 µL = 28)

By using the standard curve generated for each experiment, the peak area is converted to a concentration by using the standard equation for a straight line (y = mx + c).

Slope (m): 4952

y-intercept (c): 30 µg/mL

Thus $x = (y - c)/m$

$x = (847 - 30 \text{ µg/mL})/4952$

$x = 0.16$ µg/mL

Value calculated by dividing the concentration at each time by the 100% concentration and expressing it as a percentage.

Example: 0.16/153 x 100 = 0.108 %

Slope of line obtained by plotting relative transport against time (Standard curve).

Calculated by the equation given in the experimental procedure chapter

$$P_{app} = \frac{dQ/dt}{60 \times A \times C_0}$$

$$= \frac{0.01251}{60 \times 1.78 \times 100}$$

dQ/dt = Slope (Obtained in (e))

$= 1.171 \times 10^{-6}$ cm/s

APPENDIX B

Table B.1: Cumulative transport and P_{app} values.

Drug	Time	Experiment 1						Experiment 2						Experiment 3						Average (%)	RSD (%)
		1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6		
Caffeine (Stripped)	30	1.167	1.284	1.191	1.370	1.031	1.242	1.989	1.772	1.906	1.987	2.002	1.873	1.419	1.259	1.050	0.936	1.303	1.374	1.453	25.11
	60	2.577	2.739	2.595	2.852	2.272	2.655	3.687	3.303	3.543	3.685	3.725	3.330	3.107	3.133	2.740	2.521	3.117	3.147	3.040	14.66
	90	3.950	4.120	3.902	4.154	3.453	3.869	5.150	4.757	5.008	5.181	5.212	4.623	4.719	4.760	4.515	4.200	4.743	4.718	4.502	11.38
	120	5.156	5.348	5.099	5.344	4.590	5.012	6.508	6.113	6.373	6.538	6.569	5.859	6.142	6.363	5.862	5.478	6.114	6.069	5.808	10.38
	P_{app} (10^{-6})	4.163	4.235	4.068	4.127	3.701	3.910	4.688	4.518	4.640	4.728	4.740	4.136	4.925	5.287	5.059	4.777	5.011	4.886	4.533	9.83
Caffeine (Unstripped)	30	0.806	0.915	0.914	0.830	0.770	1.086	0.847	0.761	0.976	1.442	1.110	1.432	1.034	0.910	1.109	1.027	1.173	1.260	1.022	20.23
	60	1.895	2.099	2.117	2.182	2.163	-	2.149	1.954	2.465	3.242	2.772	3.419	2.490	2.206	2.624	2.523	2.896	3.068	2.486	18.43
	90	3.472	3.656	3.492	3.694	3.905	4.175	3.413	3.186	4.006	4.992	4.419	5.365	4.174	3.266	3.962	3.997	4.635	4.465	4.015	14.80
	120	4.408	4.727	5.179	5.033	4.944	5.633	5.018	4.750	5.942	7.307	6.530	7.387	5.192	4.427	5.539	5.392	5.684	6.564	5.537	16.22
	P_{app} (10^{-6})	3.865	4.056	4.423	4.407	4.452	4.743	4.300	4.120	5.131	6.038	5.589	6.184	4.419	3.624	4.566	4.547	4.767	5.403	4.704	15.10
Furosemide (Stripped)	30	0.084	0.057	0.044	0.064	0.050	0.064	0.119	0.051	0.067	0.077	0.099	0.158	0.108	0.102	0.140	0.298	0.340	0.301	0.124	75.22
	60	0.295	0.242	0.159	0.263	0.215	0.252	0.452	0.211	0.302	0.302	0.349	0.620	0.443	0.381	0.325	0.816	0.711	0.752	0.394	50.70
	90	0.687	0.560	0.389	0.586	0.477	0.572	0.864	0.492	0.676	0.636	0.703	1.150	0.850	0.705	0.749	1.310	1.091	1.067	0.754	33.84
	120	1.224	0.952	0.693	1.053	0.770	0.966	1.371	0.885	1.149	1.062	1.109	1.665	1.224	1.009	1.032	1.721	1.446	1.371	1.150	24.38
	P_{app} (10^{-6})	1.190	0.937	0.680	1.026	0.755	0.944	1.301	0.868	1.131	1.026	1.056	1.577	1.172	0.951	0.967	1.487	1.154	1.100	1.073	21.13
Furosemide (Untripped)	30	0.000	0.000	0.008	0.000	0.002	0.000	0.002	0.002	0.002	0.013	0.019	0.018	0.012	0.035	0.004	0.002	0.025	0.104	0.014	179.27
	60	0.017	0.017	0.047	0.012	0.015	0.055	0.011	0.009	0.021	0.051	0.083	0.058	0.127	0.200	0.088	0.122	0.185	0.435	0.086	121.98
	90	0.056	0.066	0.159	0.059	0.078	0.177	0.021	0.029	0.037	0.123	0.230	0.129	0.329	0.440	0.245	0.351	0.448	0.952	0.218	105.11
	120	0.148	0.149	0.313	0.143	0.215	0.375	0.057	0.060	0.067	0.225	0.417	0.245	0.559	0.678	0.446	0.600	0.820	1.608	0.396	95.20
	P_{app} (10^{-6})	0.151	0.155	0.320	0.148	0.219	0.390	0.055	0.061	0.066	0.221	0.418	0.235	0.575	0.677	0.462	0.631	0.827	1.569	0.399	93.08

Table B.1 continued.

Drug	Time	Experiment 1						Experiment 2						Experiment 3						Average (%)	RSD (%)
		1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6		
Verapamil (Stripped)	30	0.072	0.060	0.046	0.052	0.063	0.068	0.056	0.037	0.044	0.041	0.060	0.045	0.105	0.092	0.091	0.083	0.088	0.097	0.067	31.99
	60	0.262	0.295	0.298	0.232	0.404	0.399	0.293	0.255	0.299	0.334	0.469	0.327	0.387	0.276	0.247	0.147	0.277	0.320	0.307	24.20
	90	0.787	-	0.908	0.752	1.182	1.222	0.886	0.834	0.903	1.014	1.312	0.939	0.929	0.652	0.591	0.374	0.639	0.725	0.862	27.75
	120	1.766	1.916	1.933	1.605	2.437	2.256	1.688	1.745	1.681	1.892	2.383	1.739	1.793	1.368	1.182	0.836	1.407	1.449	1.726	23.27
	P_{app} (10^{-6})	1.750	2.016	1.957	1.617	2.466	2.306	1.712	1.780	1.721	1.945	2.437	1.777	1.749	1.312	1.129	0.776	1.348	1.393	1.727	25.42
Verapamil (Unstripped)	30	0.025	0.025	0.025	0.025	0.054	0.025	0.054	0.054	0.054	0.054	0.068	0.078	1.318	2.130	2.887	1.784	2.120	2.203	0.721	141.90
	60	0.117	0.154	0.211	0.231	0.384	0.282	0.101	0.083	0.077	0.146	0.253	0.290	1.582	2.225	2.922	1.976	2.260	2.395	0.872	116.79
	90	0.493	0.579	0.787	0.807	1.226	0.919	-	0.188	0.187	0.414	0.763	0.923	2.184	2.608	3.341	2.460	2.730	2.910	1.384	76.75
	120	1.361	1.547	1.922	1.893	2.799	2.077	0.886	0.474	0.519	1.157	1.681	1.999	3.176	3.361	4.324	3.340	3.521	3.579	2.201	52.12
	P_{app} (10^{-6})	1.368	1.558	1.956	1.929	2.833	2.120	0.918	0.426	0.470	1.117	1.669	1.996	1.928	1.272	1.477	1.608	1.458	1.449	1.545	37.75
Ketoprofen (Stripped)	30	0.374	0.524	0.662	0.498	0.522	0.684	0.957	0.894	0.869	1.011	1.367	1.276	0.389	0.492	0.357	0.415	0.471	0.510	0.682	45.55
	60	1.052	1.264	1.586	1.129	1.211	1.555	2.193	2.102	2.026	2.223	2.763	2.753	1.277	1.469	1.130	1.319	1.435	1.506	1.666	32.61
	90	1.876	2.213	2.479	1.817	1.947	2.336	3.137	2.939	2.890	3.112	3.608	3.608	2.154	2.408	1.932	2.364	2.511	2.387	2.540	22.00
	120	2.736	3.279	3.460	2.476	2.642	3.031	3.947	3.699	3.614	3.812	4.258	4.268	2.879	3.303	2.653	3.314	3.521	3.098	3.333	16.25
	P_{app} (10^{-6})	2.469	2.876	2.899	2.067	2.215	2.442	3.094	2.888	2.840	2.900	2.970	3.068	2.604	2.925	2.400	3.041	3.192	2.699	2.755	11.61
Ketoprofen (Unstripped)	30	0.099	0.075	0.020	0.152	0.185	0.290	0.000	0.000	0.007	0.024	0.066	0.037	0.064	0.058	0.045	0.065	0.112	0.087	0.077	94.82
	60	0.507	0.386	0.241	0.607	0.729	0.934	0.128	0.163	0.202	0.325	0.400	0.296	0.428	0.390	0.299	0.421	0.561	0.539	0.420	48.63
	90	0.950	0.738	0.833	1.083	1.380	1.614	0.306	0.411	0.490	0.787	0.740	0.599	0.836	0.822	0.597	0.852	0.979	0.920	0.830	38.32
	120	1.337	1.062	1.171	1.516	2.075	2.289	0.595	0.653	0.743	1.206	1.028	0.883	1.203	1.151	0.901	1.230	1.229	1.283	1.197	36.19
	P_{app} (10^{-6})	1.297	1.034	1.263	1.426	1.973	2.084	0.613	0.689	0.779	1.250	1.007	0.886	1.194	1.159	0.894	1.225	1.176	1.239	1.177	32.45

Table B.1 continued.

Drug	Time	Experiment 1						Experiment 2						Experiment 3						Average (%)	RSD (%)
		1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6		
Propranolol (Stripped)	30	0.380	0.424	0.464	0.414	0.412	0.413	0.359	0.356	0.354	0.362	0.370	0.412	0.267	0.271	0.272	0.278	0.275	0.301	0.355	17.80
	60	0.852	0.957	0.762	0.979	1.061	1.067	0.475	0.456	0.516	0.558	0.655	0.943	0.394	0.424	0.447	0.516	0.494	0.708	0.681	34.98
	90	2.010	2.055	1.709	2.254	2.411	2.371	0.840	0.797	1.006	1.131	1.358	1.920	0.824	0.853	0.976	1.167	1.194	1.620	1.472	39.09
	120	2.795	3.403	2.989	3.693	3.903	3.917	1.416	1.362	1.785	1.996	2.340	3.136	1.571	1.564	1.880	2.151	2.105	2.788	2.489	34.72
	P_{app} (10^{-6})	2.622	3.131	2.660	3.468	3.690	3.688	1.104	1.048	1.493	1.709	2.063	2.855	1.355	1.345	1.671	1.957	1.932	2.613	2.245	38.97
Propranolol (Unstripped)	30	0.206	0.210	0.209	0.216	0.233	0.216	0.157	0.149	0.148	0.171	0.148	0.164	0.189	0.179	0.190	0.194	0.190	0.192	0.187	13.97
	60	0.247	0.224	0.242	0.254	0.368	0.328	0.157	0.151	0.172	0.165	0.183	0.212	0.212	0.208	0.222	0.225	0.208	0.270	0.225	25.07
	90	0.362	0.312	0.399	0.463	0.734	0.747	0.246	0.204	0.295	0.242	0.261	0.382	0.298	0.300	0.367	0.379	0.305	0.496	0.377	40.35
	120	0.596	0.523	0.733	0.882	1.364	1.467	0.439	0.328	0.556	0.374	0.423	0.651	0.485	0.501	0.636	0.671	0.507	0.867	0.667	46.79
	P_{app} (10^{-6})	0.401	0.320	0.539	0.689	1.173	1.303	0.291	0.184	0.420	0.215	0.282	0.508	0.304	0.330	0.463	0.495	0.328	0.703	0.497	61.61
Carbemazepine (Stripped)	30	1.165	1.480	0.956	1.057	1.166	1.177	0.609	0.552	0.723	0.652	0.755	0.874	0.482	0.824	0.891	0.526	0.618	0.823	0.852	32.05
	60	3.073	3.849	3.208	2.926	3.887	2.789	3.017	2.427	2.521	2.865	2.316	2.897	2.127	2.678	2.588	2.571	2.619	2.897	2.847	16.20
	90	4.934	5.767	5.495	5.187	5.318	4.719	5.051	5.322	5.009	4.935	4.449	5.148	3.785	4.490	4.517	4.832	4.503	4.794	4.903	9.39
	120	7.306	8.211	7.353	7.290	7.249	6.732	6.761	6.707	6.524	6.812	6.378	6.395	4.787	6.473	6.505	7.012	5.993	6.330	6.712	10.50
	P_{app} (10^{-6})	6.331	6.900	6.703	6.541	6.142	5.803	6.395	6.667	6.208	6.414	5.931	5.873	4.548	5.855	5.858	6.778	5.620	5.749	6.129	9.08
Carbemazepine (Unstripped)	30	0.000	0.000	0.072	0.277	0.240	0.147	0.047	0.129	0.448	0.311	0.130	0.134	0.000	0.190	0.188	0.240	0.447	0.437	0.191	77.87
	60	0.409	0.414	0.617	0.750	1.190	0.968	0.537	0.611	1.533	1.105	0.830	0.819	0.725	1.037	1.305	1.102	1.268	1.221	0.913	35.90
	90	0.992	-	-	-	2.254	2.051	1.211	2.790	2.845	2.184	1.679	1.577	1.163	1.834	2.276	2.389	2.637	3.075	2.064	31.24
	120	1.541	1.731	2.428	2.497	3.439	3.013	1.928	2.323	4.195	3.279	2.515	2.309	2.365	3.033	3.384	3.008	3.521	4.386	2.828	27.86
	P_{app} (10^{-6})	1.624	1.837	2.505	2.370	3.327	3.022	1.972	2.734	3.918	3.116	2.498	2.274	2.352	2.911	3.295	2.993	3.305	4.276	2.828	24.63

Table B.1 continued.

Drug	Time	Experiment 1						Experiment 2						Experiment 3						Average (%)	RSD (%)
		1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6		
Promethazine (Stripped)	30	0.886	0.889	0.908	0.895	0.868	0.881	0.633	0.643	0.635	0.624	0.611	0.650	0.000	0.000	0.000	0.000	0.000	0.000	0.507	75.80
	60	0.951	0.940	1.122	1.047	0.974	1.057	0.687	0.680	0.703	0.719	0.795	0.899	0.210	0.000	0.000	0.075	0.308	0.246	0.634	61.26
	90	1.221	1.109	1.480	1.413	1.276	1.363	0.835	0.914	0.873	1.056	1.281	1.609	0.586	0.428	0.325	0.235	1.142	0.932	1.004	39.95
	120	1.634	1.363	1.986	2.092	1.770	2.019	1.216	1.403	1.265	1.631	1.930	2.490	1.505	1.145	0.843	0.558	2.735	2.372	1.664	34.30
	P_{app} (10^{-6})	0.784	0.497	1.122	1.235	0.938	1.161	0.592	0.784	0.643	1.048	1.387	1.945	1.526	1.206	0.890	0.573	2.821	2.435	1.199	53.35
Promethazine (Unstripped)	30	0.120	0.149	0.096	0.096	0.119	0.153	0.000	0.000	0.000	0.000	0.000	0.000	0.071	0.078	0.091	0.002	0.026	0.038	0.058	97.79
	60	0.119	0.153	0.161	0.178	0.172	0.146	0.000	0.000	0.000	0.000	0.000	0.000	0.082	0.053	0.081	0.161	0.135	0.119	0.087	81.68
	90	0.149	0.152	0.217	0.248	0.257	0.395	0.219	0.242	0.244	0.284	0.309	0.287	0.121	0.280	0.315	0.372	0.385	0.518	0.277	35.23
	120	0.262	0.273	0.445	0.492	0.574	0.792	0.599	0.640	0.674	0.772	0.858	0.665	0.476	0.737	0.777	0.833	0.947	1.192	0.667	34.71
	P_{app} (10^{-6})	0.142	0.115	0.344	0.393	0.453	0.676	0.629	0.675	0.707	0.811	0.900	0.712	0.391	0.688	0.715	0.844	0.940	1.204	0.630	44.46
Paracetamol (Stripped)	30	0.599	0.532	0.579	0.431	0.633	0.688	0.783	0.823	0.571	0.666	0.585	0.810	0.729	0.608	0.676	0.719	0.735	1.014	0.677	19.49
	60	1.619	1.440	1.595	1.225	1.530	1.558	1.994	1.789	1.322	1.494	1.348	1.798	1.719	1.629	1.806	2.012	1.777	2.277	1.663	15.99
	90	2.712	2.375	2.632	2.043	2.290	2.233	3.322	2.723	2.076	2.290	2.065	2.747	2.754	2.604	3.003	3.260	2.886	3.425	2.636	16.41
	120	3.880	3.285	3.662	2.905	3.064	2.854	4.706	3.638	2.787	3.031	2.806	3.619	3.776	3.623	4.258	4.551	4.202	4.546	3.622	17.68
	P_{app} (10^{-6})	3.414	2.869	3.210	2.572	2.514	2.239	4.088	2.927	2.310	2.463	2.304	2.927	3.176	3.127	3.728	3.978	3.593	3.666	3.061	19.30
Paracetamol (Unstripped)	30	0.053	0.046	0.058	0.040	0.093	0.061	0.103	0.089	0.057	0.099	0.136	0.277	0.031	0.038	0.045	0.076	0.166	0.172	0.091	68.84
	60	0.262	0.221	0.284	0.206	0.414	0.295	0.442	0.385	0.247	0.400	0.552	0.807	0.155	0.179	0.237	0.314	0.565	0.554	0.362	47.15
	90	0.553	0.488	0.589	0.463	0.821	0.624	0.830	0.707	0.492	0.743	1.008	1.321	0.371	0.409	0.539	0.633	1.014	0.988	0.700	36.40
	120	0.886	0.775	0.926	0.770	1.242	0.966	1.215	1.061	0.716	1.058	1.485	1.828	0.623	0.672	0.879	0.963	1.456	1.432	1.053	31.31
	P_{app} (10^{-6})	0.871	0.766	0.908	0.763	1.203	0.950	1.162	1.011	0.693	1.005	1.405	1.613	0.622	0.665	0.875	0.930	1.349	1.315	1.006	27.86

Table B.1 continued.

Drug	Time	Experiment 1						Experiment 2						Experiment 3						Average	RSD
		1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6	(%)	(%)
Acyclovir (Stripped)	30	0.304	0.275	0.336	0.372	0.241	0.603	0.374	0.548	0.681	0.540	0.605	0.514	0.309	0.500	0.727	0.504	0.409	0.590	0.468	31.08
	60	1.168	0.932	1.104	1.161	0.731	1.985	0.998	1.544	1.709	1.867	1.528	1.249	1.021	1.490	2.198	1.515	1.198	1.564	1.387	28.25
	90	2.373	1.824	2.131	2.324	1.842	3.467	1.710	2.675	2.823	3.002	2.653	2.086	1.962	2.788	4.026	2.628	1.911	2.542	2.487	24.44
	120	3.551	3.201	3.092	3.340	2.171	4.751	2.389	3.825	3.901	4.085	4.183	2.817	3.047	4.050	5.952	3.783	2.641	3.674	3.581	25.02
	P_{app} (10^{-6})	3.417	3.018	2.901	3.142	2.154	4.347	2.109	3.422	3.363	3.674	3.701	2.417	2.857	3.729	5.463	3.418	2.312	3.193	3.258	24.95
Acyclovir (Unstripped)	30	0.042	0.057	0.054	0.072	0.069	0.089	0.058	0.059	0.063	0.074	0.112	0.112	0.035	0.046	0.122	0.056	0.039	0.084	0.069	37.28
	60	0.226	0.211	0.191	0.235	0.240	0.291	0.347	0.379	0.391	0.257	0.302	0.297	0.187	0.217	0.427	0.163	0.238	0.321	0.273	27.79
	90	0.426	0.403	0.381	0.469	0.482	0.577	0.669	0.787	0.773	0.511	0.593	0.586	0.434	0.451	0.801	0.497	0.543	0.611	0.555	23.71
	120	0.728	0.760	0.592	0.755	0.781	0.892	1.074	1.135	1.090	0.804	0.923	0.903	0.664	0.571	1.268	0.619	0.873	0.986	0.856	23.07
	P_{app} (10^{-6})	0.705	0.719	0.564	0.712	0.742	0.841	1.051	1.136	1.081	0.763	0.851	0.831	0.665	0.564	1.190	0.631	0.876	0.935	0.825	22.96
Ranitidine (Stripped)	30	0.218	0.283	0.334	0.284	0.151	0.165	0.117	0.230	0.315	0.225	0.127	0.137	0.117	0.230	0.315	0.225	0.127	0.137	0.208	36.36
	60	0.705	0.821	0.919	0.891	0.509	0.551	0.379	0.721	0.906	0.700	0.417	0.465	0.379	0.721	0.906	0.700	0.417	0.465	0.643	30.68
	90	1.311	1.445	1.597	1.596	0.986	1.080	0.765	1.264	1.583	1.267	0.767	0.882	0.765	1.264	1.583	1.267	0.767	0.882	1.171	27.00
	120	2.056	2.162	2.258	2.407	1.547	1.705	1.251	1.903	2.381	1.817	1.148	1.332	1.251	1.903	2.381	1.817	1.148	1.332	1.767	25.37
	P_{app} (10^{-6})	1.911	1.954	2.013	2.209	1.456	1.608	1.182	1.736	2.146	1.668	1.065	1.249	1.182	1.736	2.146	1.668	1.065	1.249	1.625	23.99
Ranitidine (Unstripped)	30	0.134	0.104	0.089	0.086	0.105	0.086	0.068	0.075	0.091	0.054	0.089	0.106	0.058	0.048	0.057	0.049	0.052	0.058	0.078	31.19
	60	0.406	0.253	0.178	0.171	0.277	0.203	0.217	0.227	0.284	0.138	0.262	0.316	0.159	0.135	0.169	0.156	0.179	0.175	0.217	32.84
	90	0.851	0.495	0.325	0.320	0.533	0.395	0.438	0.475	0.612	0.290	0.544	0.662	0.326	0.288	0.365	0.304	0.310	0.375	0.439	35.15
	120	1.443	0.841	0.493	0.504	0.853	0.644	0.696	0.760	0.987	0.495	0.877	1.134	0.535	0.473	0.634	0.541	0.523	0.638	0.726	35.97
	P_{app} (10^{-6})	1.365	0.766	0.423	0.438	0.781	0.582	0.657	0.719	0.941	0.460	0.826	1.071	0.499	0.445	0.601	0.507	0.481	0.605	0.676	37.33