

**DETERMINATION OF THE PERMEABILITY OF
BIOLOGICAL MEMBRANES TO VARIOUS CHEMICAL
MARKERS, INCLUDING ANTI-HIV DRUGS**

by

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Declaration

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Summary

Due to modern high-throughput technologies, large numbers of compounds are produced by parallel synthesis and combinatorial chemistry. The pharmaceutical industry therefore requires rapid and accurate methods to screen new drugs leads for membrane permeability potential in the early stages of drug discovery. Around 50 % of all investigational new drugs fail in pre-clinical and clinical phases of development due to inadequate absorption/permeation, distribution, metabolism, excretion and/or unacceptable toxicity. This may be decreased by applying *in vitro* screening methods early in the discovery process. Reliable *in vitro* models can be applied to determine permeation of the test compounds, which will help avoid the wasting of valuable resources for the development of drugs that are destined to fail in preclinical and clinical phases due to insufficient permeability properties. It is important to decide as early as possible on the most promising compound and physical formulation for the intended route of administration.

With awareness of the increasing importance of *in vitro* models in the investigations of the permeability properties of drug compounds, this research project was specifically devoted to determine the suitability of our *in vitro* model to evaluate and predict drug permeability. A continuous flow-through diffusion system was employed to evaluate the permeability of nine different compounds/drugs with different chemical properties, across three biological membranes. The biological membranes chosen for the present study were human vaginal mucosa, human skin tissue and human small intestine mucosa. The continuous flow-through diffusion system was furthermore utilised to investigate the effects of de-epithelialisation of mucosal surfaces, chemical enhancers, temperature, permeant concentration and formulation on the permeability of the test compounds/drugs. The *in vitro* permeability information and data from the flow-through diffusion model were compared to *in vitro* and *in vivo* literature studies and drug profile. An *in vitro* model that is able to reliably predict *in vivo* data will shorten the drug development period, economise resources and may potentially lead to improved product quality.

In this thesis research results are reported on the permeability of the mentioned biological membranes to the various chemical markers, including anti-HIV (human immunodeficiency virus) drugs. The permeability studies will be discussed in three sections: vaginal mucosa, skin tissue, small intestine mucosa.

The results of the vaginal permeability studies showed that the three peptides (MEA-5, MDY-19 and PCI) readily penetrated the vaginal mucosa. MDY-19 had a higher flux rate than MEA-5, commensurate with its smaller molecular size (weight). The surfactant enhanced the flux rate of MDY-19 approximately 1.3 times and decreased the lag time of the peptide. Removal of the vaginal epithelium increased the flux rates of the peptides across the mucosa and may have implications for a more rapid uptake of these and other microbicides *in vivo*. The permeability of 1 mM MDY-19 and PCI at 37 °C were significantly ($p < 0.05$) higher than at 20 °C. At 37 °C the AUCs of the overall mean flux values of MDY-19 and PCI increased with concentration according to well-established diffusion theory.

The experiments on the permeability of different terbinafine hydrochloride formulations through human skin demonstrated that the terbinafine hydrochloride formulations used in this study, readily diffused into the skin tissue. However, no flux values for any of the terbinafine hydrochloride formulations through the skin into the acceptor fluid were found. The mean terbinafine concentrations in the skin after 24 h exposure to the three commercial, terbinafine hydrochloride formulations were 3.589, 1.590 and 4.219 µg/ml respectively. The mean terbinafine concentration in the skin exposed to the 10 mg/ml PBS/Methanol solution was higher than those from the three commercial formulations.

The results of the temperature study demonstrated that an increase of 5 °C caused a significant increase in flux values of tritiated water across skin. The flux values for tritiated water across skin at 37 °C were on average double those at a temperature of 32 °C.

The permeability of excised human small intestine mucosa to different oral dosage drugs was investigated over a 24 h period. The four drugs selected were zidovudine, propranolol hydrochloride, didanosine and enalapril maleate. They were selected as representative model compounds of drug classes 1 (high solubility, high permeability) and 3 (high solubility, low permeability) according to the Biopharmaceutics Classification System. The flux rates of the four chosen test drugs were influenced by the length of the experiment. Between the time periods 2-4 h and 4-6 h, zidovudine's mean flux values across small intestine tissue were respectively 1.8 and 2.0 times higher than didanosine and 2.3 and 2.2 times higher than enalapril. Propranolol's mean flux values were respectively 1.2 and 1.4 times higher than

didanosine and 1.6 higher than enalapril during both the 2-4 and 4-6 h time periods. Between both the time periods 2-4 and 4-6 h AZT's mean flux values were 1.4 times higher than propranolol and didanosine's mean flux values were respectively 1.3 and 1.1 times higher than enalapril during the mentioned time periods. Class 1 drugs showed a significantly higher flux rate across the jejunal mucosa compared to the class 3 drugs and these results are in line with their Biopharmaceutics Classification System classification. The *in vitro* model has proved to be reliable to predict permeability of class 1 and 3 drugs and also showed correlation with human *in vivo* data.

It seems that the *in vitro* flow-through diffusion model used in the present study have the potential to overcome some of the problems and limitations demonstrated by other *in vitro* techniques and may potentially serve as a future tool for pharmaceutical companies to predict the diffusion characteristics of new drugs and different formulations, across different biological membranes. Furthermore, it may serve as a prospective method for assessing the bioequivalence of alternative (generic) vehicles or formulations containing the same drug/compound.

Opsomming

As gevolg van moderne hoë spoed tegnologie kan groot hoeveelhede middels vervaardig word deur ooreenkomende sintese en kombinasieleer chemie. Die farmaseutiese industrie benodig dus vinnige en akkurate metodes om nuwe geneesmiddels te evalueer t.o.v. membraan deurlaatbaarheid. Hierdie evaluasie moet verkieslik so vroeg moontlik in die geneesmiddel se ontwikkelingsproses geskied. Ongeveer 50 % van alle potensiële geneesmiddels misluk in pre-kliniese en kliniese fases van geneesmiddelontwikkeling. Die mislukte pogings kan toegeskryf word aan onvoldoende absorpsie/deurlaatbaarheid, distribusie, metabolisme, ekskresie en/of onaanvaarbare middel toksisiteit. Dit is daarom belangrik om so vroeg moontlik in die geneesmiddelontwikkelingsproses te besluit op die mees belowende middel, asook die geskikte formulasie vir die spesifieke roete van toediening van die middel. Die farmaseutiese industrie benodig tans *in vitro* modelle met die potensiaal om die deurlaatbaarheid van geneesmiddels te bepaal en te voorspel. Betroubare *in vitro* modelle kan aangewend word om die deurlaatbaarheid van potensiële geneesmiddels te toets. Sodoende sal die onnodige uitgawes op die ontwikkeling van geneesmiddels wat in elk geval later gaan faal in pre-kliniese en kliniese fases van geneesmiddelproewe a.g.v. deurlaatbaarheidseienskappe, vermy word.

Hierdie navorsingsprojek was dus spesifiek onderneem om die waarde en toepaslikheid van 'n *in vitro* deurlopende-vloei perfusie model te ondersoek. Die model se potensiaal om geneesmiddels se deurlaatbaarheid en absorpsie te voorspel was geëvalueer. Die deurlopende-vloei perfusie apparaat was gebruik om die deurlaatbaarheidsvloede van drie verskillende biologiese membrane t.o.v. nege chemiese stowwe (MEA-5, MDY-19, PCI, terbinafen hidrochloried, getritieerde water, zidovudien, propranolol, hidrochloried, didanosien, enalapril maleaat) te bepaal. Die drie biologiese membrane wat gebruik was, was vaginale weefsel, vel en klein intestinale weefsel. Al drie weefsel tipes was van menslike oorsprong. Die deurlopende-vloei perfusie apparaat was ook gebruik om die effek wat verwydering van die mukosa se epiteellaag op deurlaatbaarheidsvloede het, te ondersoek. Verder was navorsing gedoen op die effek van temperatuur en die konsentrasie en formulasie van die toetsmiddels op hulle diffusie vloedwaardes. Daar was ook gekyk na die invloed van ander chemiese stowwe op die toetsmiddels se diffusie vloedwaardes. Die *in vitro* deurlaatbaarheidsinformatie en -gegewens was vergelyk

met ander *in vitro* en *in vivo* literatuurstudies en geneesmiddel databasisse. 'n *In vitro* model wat in staat is om *in vivo* resultate betroubaar te voorspel, het die potensiaal om die tyd wat dit neem om geneesmiddels te ontwikkel, te verkort, finansiële uitgawes te besnoei en om geneesmiddelkwaliteit te verseker.

In die tesis word dan die resultate gerapporteer van die deurlaatbaarheidsvloede van die verskillende tipes weefsel ten op sigte van verskeie chemiese stowwe, insluitende anti-MIV (menslike immuuniteitsgebrekswirus) middels. Die deurlaatbaarheidstudies word bespreek in drie afdelings: vaginale mukosa, vel en klein intestinale mukosa.

Die resultate van die deurlaatbaarheidstudies op die vaginale weefsel dui daarop dat die drie peptiede (MEA-5, MDY-19 and PCI) die vaginale mukosa goed penetreer. Soos verwag, het MDY-19 hoër diffusie vloedwaardes as MEA-5 gehad. Dit kan toegeskryf word aan MDY-19 se kleiner molekulere grootte (gewig). Surfactant het die diffusie vloedwaardes van MDY-19 1.3 keer vergroot en het ook die tyd na vaste vlak verminder. Die verwydering van die vaginale epiteel het die diffusie vloedwaardes van die peptiede verhoog en mag dus dui op die vinniger opname van peptiede en moontlike ander mikrobiese *in vivo*, wanneer die belyning van die epiteel onderbreek. Die deurlaatbaarheid van 1 mM MDY-19 en PCI by 37 °C was statisties beduidend ($p < 0.05$) hoër as teem 20 °C. Die area onder die kurwe (AOK) van die gemiddelde vloedwaardes van MDY-19 en PCI by 37 °C, het toegeneem met 'n toename in die konsentrasie van hierdie peptiede. Die toename vloedwaardes ondersteun dus die alombekende diffusie teorie.

Die transdermale diffusie eksperimente van verskillende terbinafen formulasies het getoon dat terbinafen geredelik vrygestel word vanuit hierdie formulasies na die vel. Geen terbinafen vloedwaardes, van enige van die formulasies, was egter gevind in die ontvangselle van die deurlopende-vloei perfusie apparaat nie. Die gemiddelde terbinafen konsentrasies in die vel na 24 h se blootstelling aan drie kommersiële terbinafen hidrochloried formulasies was onderskeidelik 3.589, 1.590 en 4.219 µg/ml. Die gemiddelde terbinafen konsentrasie in die vel wat aan 10 mg/ml PBS/metanol blootgestel was, was hoër as die konsentrasies in die vel wat aan die drie kommersiële formulasies blootgestel was.

Die resultate van die temperatuurstudie op vel het aangetoon dat 'n temperatuur toename van 5 °C 'n statisties beduidende toename in vloedwaardes van getritieerde water oor vel veroorsaak. Die vloedwaardes van die getritieerde water oor vel teen 'n temperatuur van 37 °C was gemiddeld dubbeld so veel as teen 32 °C.

Die deurlaatbaarheidsvloede van klein intestinale mukosa ten opsigte van verskillende geneesmiddels (wat oraal toegedien word) was ondersoek gedurende 'n 24 h eksperiment. Die vier geneesmiddels wat gebruik was, was zidovudine, propranolol hidrochloried, didanosien en enalapril maleaat. Hierdie geneesmiddels is verteenwoordigers van die Biofarmaseutiese Klassifikasie Sisteem se klas 1 (hoë oplosbaarheid, hoë deurlaatbaarheid) en klas 3 (hoë oplosbaarheid, lae deurlaatbaarheid) geneesmiddels. Die vloedwaardes van die vier geneesmiddels het gewissel na aanleiding van die tydsverloop in die eksperiment. Zidovudien se gemiddelde vloedwaardes tussen 2-4 en 4-6 h was onderskeidelik 1.8 en 2.0 keer hoër as didanosien se gemiddelde vloedwaardes vir hierdie tyd periodes en onderskeidelik 2.3 en 2.2 keer hoër as enalapril se gemiddelde vloedwaardes. Tydens hierdie selfde periodes was propranolol se gemiddelde vloedwaardes 1.2 en 1.4 keer hoër as didanosien en vir beide periodes 1.6 keer hoër as enalapril se gemiddelde vloedwaardes. Gedurende beide genoemde tyd periodes was zidovudien se gemiddelde vloedwaardes 1.4 keer hoër as propranolol en didanosien se gemiddelde vloedwaardes was onderskeidelik 1.3 en 1.1 keer hoër as enalapril tydens 2-4 en 4-6 h. Die klas 1 geneesmiddels het statisties beduidende hoër vloedwaardes gehad as die klas 3 geneesmiddels. Hierdie resultate stem ooreen met die geneesmiddels se Biofarmaseutiese Klassifikasie Sisteem klassifikasie. Dit wil dus voorkom asof die *in vitro* model wat gebruik was in die studie, gebruik kan word om die deurlaatbaarheidsvloede van klas 1 en 3 te voorspel. Die resultate van hierdie studie stem ooreen met ander *in vivo* studies.

Dit wil voorkom asof die *in vitro* deurlopende-vloei perfusie apparaat die potensiaal het om sommige van die probleme en tekortkominge van ander *in vitro* modelle te oorkom en dat dit moontlik die potensiaal het om die diffusie-eienskappe van nuwe geneesmiddels en verskillende formulasies oor verskillende biologiese membrane te voorspel. Die model kan verder moontlik dien as 'n potensiele toestel om biogelykbaarheid van alternatiewe (generiese) formulasies, wat dieselfde geneesmiddel/chemiese stof bevat, te bepaal.

Dedication

I dedicate this thesis to my parents, whose endless love, unselfish support and example over many years laid the foundations to complete this work. I am honoured to have you as my parents.

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Abbreviations

A	membrane area exposed (cm ²)
ACE	angiotensin converting enzyme
ANN	artificial neural network
API	active pharmaceutical ingredient
AUC	area under curve
AZT	zidovudine
BA/BE	bioavailability/bioequivalence
BBMV	brush border membrane vesicle
BCS	Biopharmaceutics Classification System
C _{app}	the applied concentration
C _{s,v} and C _{s,m}	the solubility of the drug in the vehicle and in the barrier respectively
C _v /C _{s,v}	represents the degree of saturation of the drug in the formulation
C _{v,i}	the drug concentration dissolved in the vehicle
CV	coefficient of variation
D	the diffusion coefficient of skin
dATP	deoxyadenosine triphosphate
DNA	deoxyribonucleic acid
DPPC	dipalmitoyl-L- α -phosphatidylcholine
EMPRO	European Microbicides Project
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
FITCD	fluorescein isothiocyanate dextrans
FMO	flavin-containing monooxygenase
GI	gastrointestinal
GIT	gastrointestinal tract
H	the diffusional pathlength
HIV	human immunodeficiency virus
HSV	herpes simplex virus
IR	immediate release
J	the flux per unit area
K	the skin-vehicle partition coefficient
k _p	permeability coefficient (= KD/h).
LC/MS	liquid chromatography/mass spectrometry
LLOQ	lower limit of quantification

MALT	mucosal-associated lymphoid tissues
MDCK	Madin–Darby canine kidney
MDY-19	FITC (fluorescein isothiocyanate)-labelled peptide, Mw = 2409.5 Da. It is a transport peptide that has potential applications for transporting therapeutically active compounds into cells.
MEA-5	FITC (fluorescein isothiocyanate)-labelled peptide, Mw = 2911.4 Da. It is an antibacterial peptide that binds to cell surfaces.
MEM	minimum essential medium
MIC	minimum inhibitory concentration
MW	molecular weight
P450	cytochrome P450
PBS	phosphate buffered saline
PCI	FITC (fluorescein isothiocyanate)-labelled peptide, Mw = 2325 Da. It is a transport peptide that has potential applications for transporting therapeutically active compounds into cells.
PG	1,2-dipalmitoyl-L- α -phosphatidylglycerol
pKa	negative logarithm of the acid dissociation constant
PNP	purine nucleoside phosphorylase
Q	quantity of drug crossing membrane (μ g)
QSAR	quantitative structure-permeability relationship
QSPR	quantitative structure-activity relationship
ROF	rule-of-five
RSD	relative standard deviation
RT	retention time
SC	stratum corneum
SD	standard deviation
SEM	standard error of the mean
t	time of exposure (min)
TEER	transepithelium electrical resistance
USP	United States Pharmacopeia
UV	ultraviolet
WHO	World Health Organisation
Δc	concentration difference across the skin

Publications from this thesis

Publications

- European Journal of Inflammation (2007) Vol 5, No. 1
Transvaginal diffusion of the peptides MEA-5, MDY-19 and PCI
Basson E, Van der Bijl P, Van Eyk AD
- European Journal of Inflammation (2008) Vol 6, No. 3
In vitro human skin absorption of different terbinafine hydrochloride formulations
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- AAPS PharmSciTech (2009) Vol 10, No. 1
Permeation of four oral drugs through human intestinal mucosa
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Contents	Page no.
a. Declaration	i
b. Summary	ii
c. Opsomming	v
d. Dedication	ix
e. Acknowledgments	x
f. Abbreviations	xii
g. Publications from this thesis	xiv
 CHAPTER 1: INTRODUCTION OF LITERATURE REVIEW	 6
1.1 <i>In vitro</i> permeability studies for drug testing	8
1.2 Primary aim	13
 CHAPTER 2: VAGINAL MUCOSA	 15
2.1 Summary	15
2.2 Introduction	17
2.2.1 Peptides	17
2.2.1.1 Structure of peptides and physico-chemical properties	19
2.2.1.1.1 Molecular weight and size	19
2.2.1.1.2 Conformation, stereospecificity and immunogenicity	19
2.2.1.1.3 Electrostatic charges	20
2.2.1.1.4 Solubility, hydrophilicity and partition coefficient	20
2.2.1.1.5 Aggregation, self-association and hydrogen bonding	20
2.2.1.2 Peptides as microbicides	21
2.2.1.2.1 Mechanisms of action	23
2.2.2 Vaginal mucosa	23
2.2.2.1 Anatomy of vagina	24
2.2.2.2 Histology of vagina	25
2.2.2.3 Physiology of vagina and drug administration	27
2.2.2.4 Vaginal enzymes	27
2.2.2.5 Epithelium	27
2.2.2.6 Florae	28

2.2.2.7 Immune cells	29
2.2.2.8 PH.....	30
2.2.2.9 Drug delivery systems and physiochemical properties of drugs	30
2.2.2.9.1 Creams and gels	31
2.2.2.9.2 Vaginal rings.....	33
2.2.2.9.3 Suppositories and vaginal tablets	34
2.2.2.9.4 Bioadhesive delivery systems.....	35
2.2.2.9.5 User acceptability	35
2.2.2.9.6 Vaginal immunisation	35
2.2.3 Permeation enhancers	36
2.2.3.1 Chemical enhancers.....	36
2.2.3.1.1 Surfactant.....	37
2.2.3.1.2 Vehicles and adjuvants (co-solvent)	38
2.2.3.2 Enzyme inhibitors	63
2.2.3.3 Lipophilicity modification	38
2.2.3.4 Formulation design	38
2.2.4 Transmembrane diffusion processes.....	38
2.2.5 Aim.....	40
2.3 Materials and Methods.....	42
2.3.1 Human vaginal mucosa	42
2.3.2 Peptides	42
2.3.3 Surfactant.....	44
2.3.4 Permeability Experiments.....	45
2.3.5 Methodology	46
2.3.6 Detection of FITC-labelled peptides.....	47
2.3.7 Calculation of flux values	51
2.3.8 Steady state kinetics and statistical analysis of data.....	51
2.4 Results	52
2.5 Discussion	74
CHAPTER 3: SKIN TISSUE	77
3.1 Summary.....	77
3.2 Introduction	79
3.2.1 Topical administration of drugs.....	79

3.2.2 Skin	80
3.2.2.1 Epidermis	82
3.2.2.2 Dermis.....	84
3.2.2.3 Hypodermis	84
3.2.3 Processes of percutaneous absorption of drugs	85
3.2.4 Predicting skin permeation of compounds	88
3.2.4.1 Mechanistic analysis of skin permeation of drugs	89
3.2.4.1.1 Analysis based on simple diffusion models.....	89
3.2.4.1.1.1 Fick's diffusion law.....	89
3.2.4.1.1.2 Quantitative structure-permeability relationship (QSAR)	90
3.2.4.1.2 Analysis based on heterogeneous diffusion models	91
3.2.4.1.2.1 Development of heterogeneous diffusion models	91
3.2.4.2 Empirical analysis of skin permeation of drugs	91
3.2.4.2.1 Linear empirical modeling for prediction of skin permeability	92
3.2.4.2.2 Neural network modeling for prediction of skin permeability	92
3.2.5 Concentration gradient and supersaturation	92
3.2.6 Methods to breach the skin barrier	93
3.2.6.1 Temperature.....	94
3.2.7 Antifungals	95
3.2.7.1 Terbinafine hydrochloride	96
3.2.8 Tritiated water	99
3.2.9 Aim.....	99
3.3 Materials and Methods.....	102
3.3.1 Skin tissue.....	102
3.3.2 Terbinafine hydrochloride	102
3.3.3 Permeability experiments	104
3.3.4.1 Terbinafine extractions from skin	105
3.3.4.2 LC/MS determination of terbinafine	105
3.3.4.3 Calculation of flux values	110
3.3.4.4 Steady state kinetics and statistical analysis of data.....	110
3.4 Results	111
3.4.1 Terbinafine hydrochloride	111
3.4.2 Tritiated water	113
3.5 Discussion	116

3.5.1 Terbinafine hydrochloride	116
3.5.2 Tritiated water	121
CHAPTER 4: SMALL INTESTINE MUCOSA	123
4.1 Summary.....	123
4.2 Introduction	125
4.2.1 Oral administration of drugs.....	125
4.2.2 Small intestine	125
4.2.2.1 Anatomy	125
4.2.2.2 Histology	126
4.2.3 Functions of the GIT	129
4.2.3.1 Metabolism	130
4.2.3.2 Absorption	130
4.2.3.2.1 Site of absorption.....	130
4.2.3.2.2 Absorption and solubility	131
4.2.4 Different types of permeation routes.....	133
4.2.4.1 Lipophilic and transcellular	133
4.2.4.2 Paracellular and hydrophilic.....	133
4.2.4.3 Active and other transport processes	134
4.2.5 Physicochemical properties of permeants	134
4.2.6 Predicting intestine permeation of compounds	135
4.2.6.1 <i>In vitro</i> methods.....	136
4.2.6.1.1 Animal tissue	136
4.2.6.1.2 Everted gut technique.....	136
4.2.6.1.3 <i>In vitro</i> transport across intestinal segment.....	137
4.2.6.1.4 Isolated membrane vesicles	137
4.2.6.1.5 Artificial membranes	138
4.2.6.1.6 Cell-based	138
4.2.6.2 <i>In situ</i> methods	141
4.2.6.3 <i>In vivo</i> methods	142
4.2.6.4 <i>In Silico</i> methods	142
4.2.6.5 Summary	143
4.2.7 Biopharmaceutics Classification System (BCS)	144
4.2.7.1 Class boundaries.....	145

4.2.7.1.1 High solubility	145
4.2.7.1.2 High permeability	146
4.2.7.1.3 Rapidly dissolving	146
4.2.8 Drugs	147
4.2.8.1 Zidovudine (AZT)	148
4.2.8.2 Propranolol hydrochloride	149
4.2.8.3 Didanosine	150
4.2.8.4 Enalapril Maleate	152
4.2.9 Aim	154
4.3 Materials and Methods	155
4.3.1 Small intestine mucosa	155
4.3.2 Drugs	155
4.3.3 Permeability experiments	156
4.3.4 LC/MS detection of zidovudine, propranolol, didanosine and enalapril	157
4.3.4.1 Zidovudine (AZT)	158
4.3.4.2 Propranolol	159
4.3.4.3 Didanosine	160
4.3.4.4 Enalapril	161
4.3.5 Calculation of flux values	162
4.3.6 Steady state kinetics and statistical analysis of data	162
4.4 Results	163
4.5 Discussion	169
CHAPTER 5: GENERAL DISCUSSION AND CONCLUSIONS	174
CHAPTER 6: FUTURE STUDIES	178
REFERENCES	180

Chapter 1: Introduction of literature review

The traditional drug administration routes used were oral administration for systemic effects and topical for local effects. Drugs could also be self-administered by inhalation, suppository and sometimes injections. The other routes of delivery usually required the intervention of a healthcare provider and the pain, fear and the possibility of infections associated with injections often resulted in low patient compliance and therefore have aided the development of suitable non-parenteral routes of administration. Technology advancement in drug delivery has allowed for a wider choice of sites for drug administration. During the last two decades attention has shifted to alternative drug administration routes, which allowed a single intervention by a healthcare provider to provide sustained therapy (Alexander *et al.*, 2004). Suitable non-parenteral routes include mucosal surfaces of the vagina and skin (Starokadomskyy and Dubey, 2006; Pettit and Gombotz, 1998).

Increasing attention is therefore given to using mucosae or skin as a non-invasive drug administration route for systemic delivery. The high vascularity and accessibility of the mucous membranes, as well as the relative absence of proteolytic enzymes, have made this tissue a potential route for administration of peptides and proteins. The skin has a surface area of approximately 2 m² and receives about a third of the body's blood circulation (MacKie, 1987). Since it is the biggest organ (that of an average adult male weighs 4.5 to 5 kg) and easily accessible, it offers great opportunities for the administration of therapeutic compounds (Williams, 2003).

Unfortunately, all of these approaches have some limitations, particularly in view of the fact that mucosal linings and stratum corneum of the skin have important protective functions in the body. Mucosae protect the deeper lying tissues and organs from mechanical damage, act as barriers against the many potentially invasive organisms colonizing the tissue, prevent fluid loss from underlining tissues and prevent the ingress of many harmful chemical agents. The skin regulates heat and water loss from the body and protects the body from the penetration of harmful chemicals or microorganisms, including agents applied to the skin. The stratum corneum seems to be the main permeability barrier of the skin and provides an incredibly effective barrier to penetration, even though it is merely 15-20 µm thick (Hadgraft, 2001b).

Oral administration still dominates drug therapy and more than 60 % of marketed drugs are oral products (Masaoka *et al.*, 2006). This type of drug administration is preferred due to its convenience, high patient compliance, less stringent production conditions and lower costs. Unfortunately, this traditional drug delivery method has its limitations, due to gastrointestinal permeability, metabolism and elimination of drugs by the liver or gastrointestinal mucosa (first-pass effect). The main drawback of the oral route is that only those compounds that are stable in the gastrointestinal tract can be administered in this way. For this reason, the oral route has been used for mainly non-peptide drugs (Starokadomskyy and Dubey, 2006).

Investigation of the passage of chemical substances across various biological membranes is of high importance in devising systems for optimal drug delivery. Estimates of the permeability across biological membranes provide a valuable tool for determining the factors limiting the potential absorption and bioavailability of drugs (Katneni *et al.*, 2006). Numerous methods for drug administration exist and an increasing amount of research is focused on optimising delivery as a method of improving pharmacotherapy. The availability of effective, highly reproducible, economic and rapid *in vitro* permeation assays for prediction of the drug absorption properties in humans is highly desirable for the preliminary screening of new drug compounds in the early stages of the drug discovery process, thus improving efficiency and the probability of success in the development of efficacious pharmaceutical formulations.

The aim of the presented research project was to assess the suitability of an *in vitro* permeation simulation model based on the use of human tissue. The actual predictive power of the flow-through diffusion system will be tested on a wide range of compounds/drugs with different properties, including the model drugs belonging to two classes of the BCS. Moreover, the accuracy of our method in assessing drug permeation potential and in establishing *in vitro-in vivo* correlations will be compared with that of the other principal *in vitro* methods available at present.

The tissues selected were human vaginal mucosa, skin tissue and small intestine mucosa. A diverse set of 9 test compounds/drugs were selected and the permeability of the different biological membranes to these compounds/drugs were investigated, to ensure a reliable model.

Due to the artificial milieu to which the excised tissues are exposed, it is crucial to ensure tissue viability and integrity during the experiment. Previous studies with the *in vitro* flow-through diffusion system have shown that skin tissue, vaginal and intestinal mucosa can be frozen and banked without their permeability properties to a number of different permeants being changed. In view of the foregoing, the assumption of using frozen/thawed vaginal mucosa, skin tissue and small intestine mucosa for the current permeability study was considered to be a reasonable one (Van der Bijl *et al.*, 2003; Van der Bijl and Van Eyk, 2002; Van der Bijl *et al.*, 1998a; Swarbrick *et al.*, 1982; Franz, 1975).

1.1 *In vitro* permeability studies for drug testing

Penetration of chemicals through vaginal mucosa, skin and gastrointestinal mucosa can be characterised using both *in vivo* and *in vitro* methods. *In vitro* studies are generally conducted using a diffusion cell system with either a static or a flow-through cell. There are several benefits of utilising an *in vitro* flow cell system for the initial testing of drug diffusion in the laboratory, prior to undertaking studies in human volunteers. The environment, specific permeation parameters and variables may be controlled in an attempt to reveal specific factors affecting the kinetic processes of the transmembrane diffusion/penetration.

Different types of diffusion equipment are available for the study of *in vitro* tissue permeability. The different flow cells include the conventional static Franz cells (Fig. 1), the Ussing chamber (Fig. 2), as well as flow-through diffusion cells (Fig. 3) (Hug, 2002; Córdoba-Díaz *et al.*, 2000). All three types of cells contain a donor and acceptor compartment, between which a membrane/cell layer can be sandwiched. Different variations of these designs are also available. Membranes/cell layers that are frequently used for permeability determination of the drug substance include excised human or animal intestinal tissue, monolayer of cultured epithelial cells (e.g. Caco-2 that are commonly used for intestinal permeability studies), artificial membranes and isolated membrane vesicles (Martinez *et al.*, 2002; Ungell *et al.*, 1998; Waclawski and Sinko, 1996; Corti *et al.*, 2006; Press and Di Grandi, 2008).

Since Hans Ussing (Ussing, 1949) described the measurement of ion transport across frog skin held between two half chambers in 1949, numerous modifications of this

apparatus have been proposed for examining the *in vitro* permeability of a variety of tissues. The Ussing chamber may contain an additional amplifier and data acquisition system and can therefore be used for either electrophysiology- and/or diffusion-based studies. One of the disadvantages of the original side-by-side arrangement of the Ussing chambers for measurements of skin permeability was the need to immerse both sides of the tissue in an aqueous environment, leading to hydration of the normally dry skin surface. In 1975, Franz proposed a vertical design that allowed the membrane surface to remain dry and also facilitated the direct application (and removal) of different agents (Franz, 1975).

Even though Franz cells and the Ussing system are regularly used in permeability studies it has certain shortcomings. Both these models are labour intensive, since an accurate volume of sample must be removed at fixed time intervals from the acceptor compartment with a simultaneous media replacement to maintain sink conditions in the cells. The manual sampling requires constant attention and is therefore often limited to the normal laboratory hours which mean a less accurate fitting of the curve. Also, air bubbles are easily formed in the receptor compartments while withdrawing samples, interfering with permeability results.

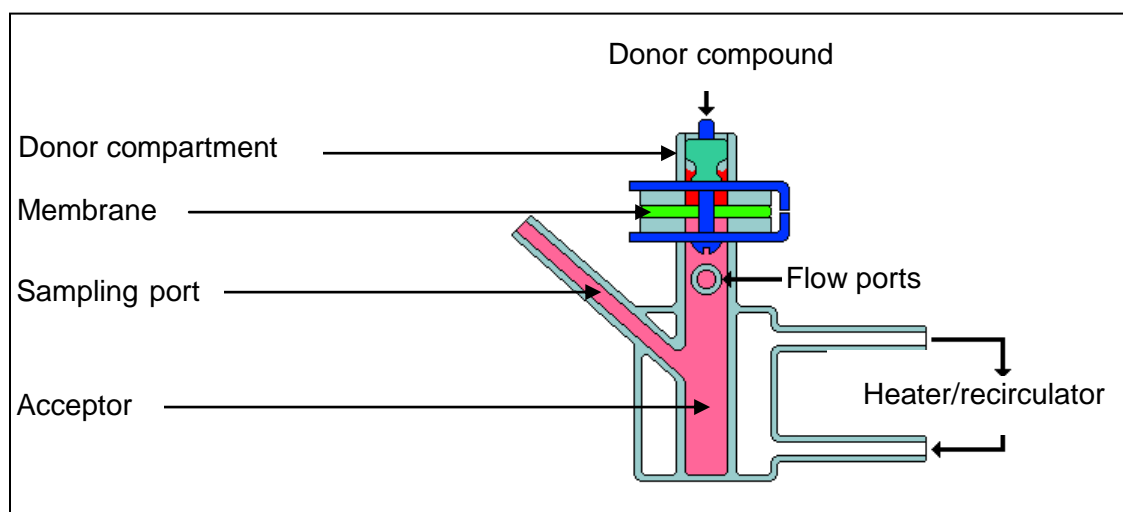


Fig. 1. Schematic drawing of a Franz cell

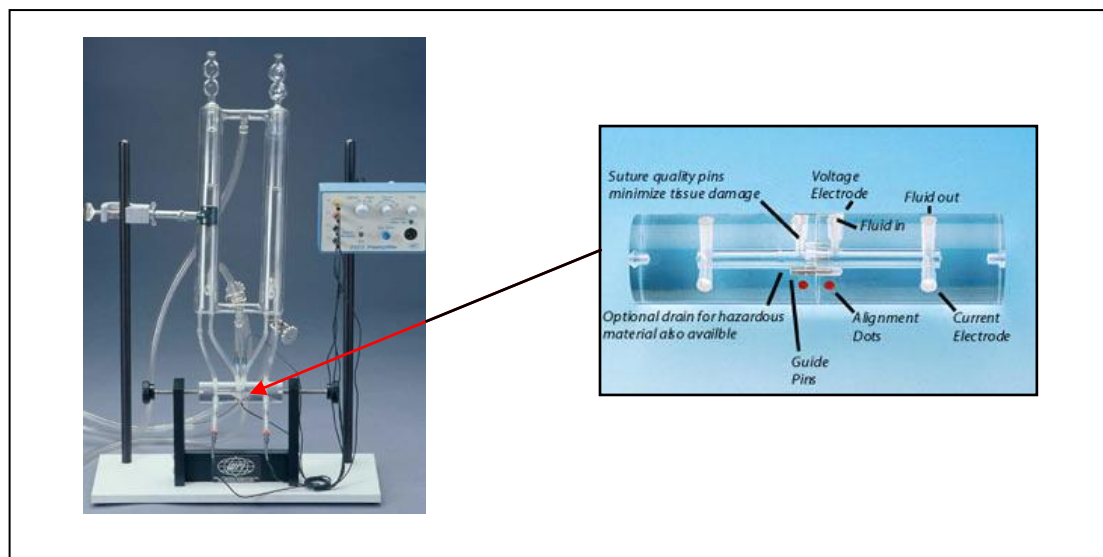


Fig. 2. An Ussing chamber

It is important to select an *in vitro* model design where the diffusion of the test compound is limited by the tissue and not in any stagnant diffusion (non-stirred) layers adjacent to the tissue's surface (Hadgraft and Lane, 2005). A flow-through diffusion apparatus was used in the present study (Figs. 3 and 4). The flow-through diffusion system has a constant flow of buffer and therefore does not have the problem of an unstirred water layer in the acceptor and donor wells, like the Franz and Ussing *in vitro* models, which may influence the permeability results. It contains 7 flow-through diffusion cells. Each cell contains an acceptor chamber through which there is a continuous flow of buffer. Since the flow-through diffusion apparatus has a constant flow (1.5 ml/h) of PBS through the acceptor chamber, any drug absorbed across the biological membrane is immediately carried away, preserving the concentration gradient as driving force for drug transport. It was therefore accepted that sink conditions were maintained (at the completion of each run the concentration of permeant in the acceptor chamber never reached 10 % of that in the donor compartment), since there is not a method to measure concentration in the acceptor chamber. In contrast to the static Franz and Ussing chamber cells, the flow-through diffusion cells offers automation with the addition of a pump that offers an accurate, constant flow rate of buffer. The only other requirement is the addition of a fraction collector (Córdoba-Díaz *et al.*, 2000). A flow-through diffusion cell method, by virtue of continuous replenishment of perfusion medium, helps in maintaining the viability of the skin tissue/mucosa and thus would mimic better a physiological environment than a static cell. The drug is added to the donor

compartment of the flow cell and collected by means of a fraction collector from the acceptor compartment of the flow cell. This is done for the required time period of the experiment, at a constant flow rate. The drug in the effluent may be detected by various means e.g. scintillation counting, UV spectroscopy, fluorospectroscopy, high-performance liquid chromatography or LC/MS (Liquid Chromatography/Mass Spectrometry). The use of small tissue samples (4 mm Ø for mucosa and 1 cm Ø for skin tissue) and the maintenance of a continuous high gradient of permeant across the biological membrane are major benefits of this system. The system has also potential for automatisation on a larger scale.

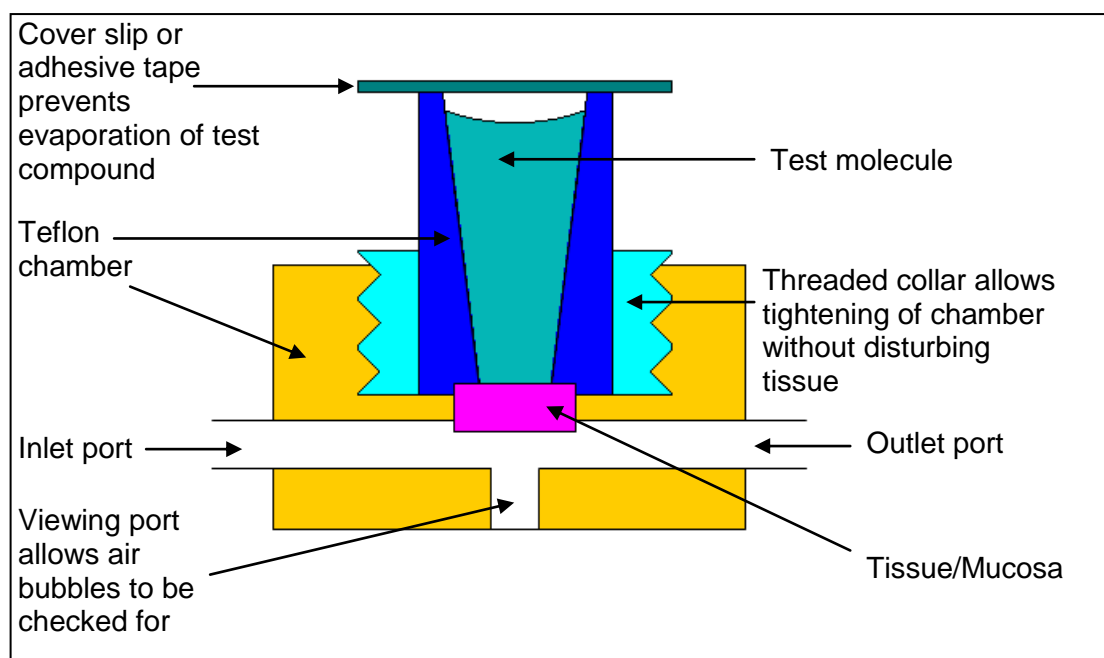


Fig. 3. Schematic drawing of a flow-through diffusion cell

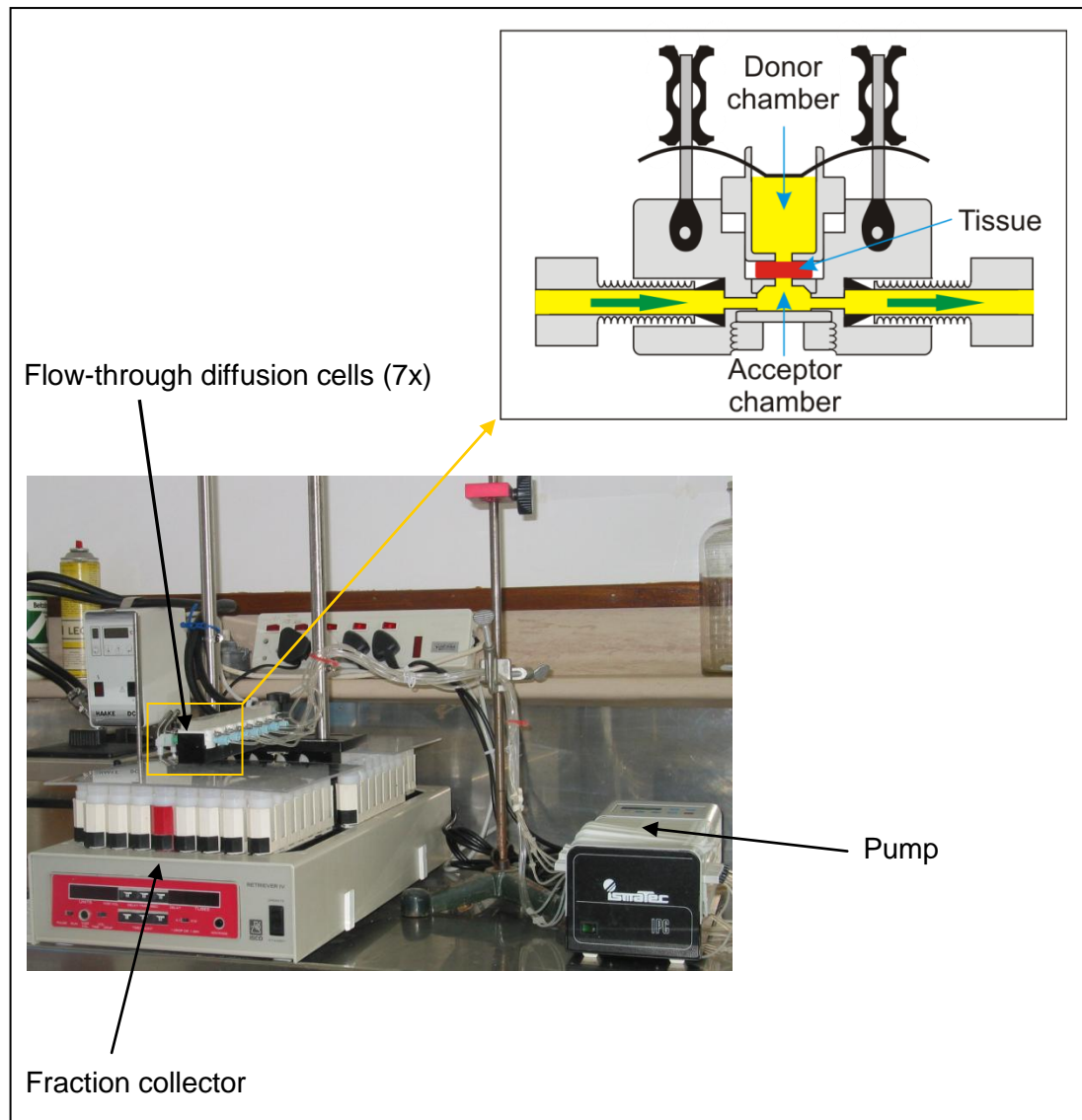


Fig. 4. Flow-through diffusion apparatus used in the present study

1.2 Primary aim

Recent advances made in molecular biology and combinatorial chemistry have changed the way in which pharmaceutical companies conduct drug discovery research. The biggest challenges are in screening a large number of drug candidates (synthesised in very small quantities, 1-10 mg) in a very short period of time. Due to modern high-throughput technologies, large numbers of compounds are produced by parallel synthesis and combinatorial chemistry. The fields of miniaturisation and automation have made remarkable advancements to meet these challenges. In contrast to the successful use of miniaturisation and automation in biological activity screening, intestinal permeability and absorption screening have not made similar progression. One of the most important challenges facing the pharmaceutical industry at present is to develop high-throughput, cost-effective and highly predictive screening models for drug absorption/permeation that can be used during the decision making process early in drug discovery. Even though, physicochemical parameters such as lipophilicity, charge, molecular weight, etc. are often used as initial indicators of absorption, they are not entirely reliable because of their inability to incorporate the *in vivo* conditions. Furthermore, the available experimental methods for assessing the permeation and absorption characteristics of compounds have various disadvantages.

With awareness of the increasing importance of the role played by *in vitro* models in investigations of the permeability properties of drug compounds, this research project's primary aim was to determine the suitability of our *in vitro* flow-through diffusion model to evaluate and predict drug permeability. Towards this primary aim, a flow-through diffusion system was employed to evaluate the permeability of various biological membranes e.g. human vaginal mucosa, skin tissue and small intestine mucosa to a set of 9 different compounds/drugs with different solubility and permeability properties. To serve as a suitable *in vitro* model with versatile applications, different test conditions were applied to evaluate the suitability of the *in vitro* flow-through diffusion system. The different test conditions included the effect of epithelial stripping, chemical enhancers, temperature, concentration and formulation on the permeability of compounds/drugs. Four secondary aims were therefore set to achieve the primary aim. The secondary aims are discussed in more detail in the three sections of the thesis (vaginal mucosa, skin tissue and small intestine mucosa).

Human vaginal tissue was used to test the *in vitro* flow-through diffusion model's suitability to evaluate the permeability of vaginal mucosa to three different peptides and to evaluate its value to examine test conditions such as different temperatures and concentrations, removal of epithelium and the use of a surfactant. The three different peptides were chosen since they were manufactured to serve as potential microbicides or as transporters of microbicides against viruses such as HIV, which portal of entry is often the vaginal mucosa.

Secondly, human skin tissue was utilised to assess the *in vitro* flow-through diffusion model's ability to evaluate the permeability of skin to terbinafine hydrochloride and tritiated water. Terbinafine hydrochloride is often used as a topical drug and the target site of this drug is the skin, therefore the test condition included the use of different formulations and concentrations. Furthermore, the effect of different temperatures on the permeability of skin to tritiated water was investigated.

Thirdly, human small intestine mucosa was employed to assess the *in vitro* flow-through diffusion model's ability to evaluate the permeability of small intestine to 4 oral drugs (zidovudine, propranolol HCl, didanosine, enalapril maleate) and to investigate the system's ability predict of BCS permeability classification.

Fourthly, correlation of the *in vitro* permeability information from the *in vitro* flow-through diffusion apparatus to the *in vivo* drug profile was performed for the three membrane models. A model that reliably predicts *in vivo* data shortens the drug development period, economises resources and leads to improved product quality.

Chapter 2: Vaginal mucosa

2.1 Summary

Due to an absence of an effective prophylactic anti-HIV vaccine or therapy, there has been a current surge in interest at the development of a topical intravaginal formulation to combat the mucosal and perinatal HIV or microbicide transmission (D'Cruz and Uckun, 2004). Mucosal surfaces of the vagina are the portals for heterosexual transmission of HIV and therefore play a fundamental role in the pathogenesis of the primary infection. Topical microbicides, including peptides, are being developed for blocking the transfer of this virus, but little is known about the diffusion of these compounds into and through vaginal mucosal epithelium (Hussain and Ahsan, 2004). Although the vagina has been used since ancient times for drug delivery, it still remains a relatively unexplored route for drug administration. Some studies have suggested that the vagina's molecular weight cut-off point for absorption may be higher than that of other mucosal surfaces. Experience with a variety of compounds demonstrates that the vagina is a highly effective site for drug administration and particularly in women's health (Justin-Temu *et al.*, 2004). Data on human vaginal permeability to drugs with different physiochemical properties is very limited and therefore much research is necessary to establish the effects of physicochemical parameters of drugs on vaginal absorption (Hussain and Ahsan, 2004). It was therefore decided to include vaginal tissue in the present study.

Currently a resurgence of interest in peptide and protein drugs exists. The unusual characteristics of peptides and proteins present considerable challenges to pharmaceutical scientists in selecting a suitable route for their administration and formulation. Peptides and proteins cannot readily be given orally because of enzymatic breakdown in the gastrointestinal tract, their sensitivity to the acidic pH in the stomach and poor intrinsic permeability across intestinal epithelium (Maggio, 2005). In the past, the most commonly used route for protein and peptide delivery has been by parenteral administration. Most proteins and peptides have a relatively short half-life and therefore repeated administrations (injections) are often required (Veuilleux *et al.*, 2001). Development of suitable nonparenteral routes (such as the vagina) for introducing these agents into humans could significantly enhance patient compliance and increase the benefit derived from certain peptide or protein therapy.

Topical microbicide peptides are being developed to combat the transfer of HIV, but little is known about the permeation of these compounds through vaginal epithelium. Cationic peptides have activity against malaria parasites, and viruses (including HIV, HSV, influenza A virus and vesicular stomatitis virus). These properties make them promising candidates as new therapeutic agents. It was decided to investigate the permeation kinetics of three novel, synthetic FITC (fluorescein isothiocyanate)-labelled peptides MEA-5 (Mw = 2911.4 Da), MDY-19 (Mw = 2409.5 Da) and PCI (Mw = 2325 Da) across human vaginal mucosa by means of a continuous flow-through diffusion system. The FITC-labelled peptides were provided by EMPRO (European Microbicides Project). MEA-5 is an antibacterial peptide that binds to cell surfaces, but cannot be internalised. MDY-19 and PCI are transport peptides that have potential applications for transporting therapeutically active compounds into cells.

Permeability studies were conducted at concentrations of 1 mM, 0.75 mM and 0.5 mM in phosphate buffered saline (PBS) at 20 °C and 37 °C, respectively, and over a time period of 24 h, using fluorospectrophotometry as detection method. Effects of a surfactant on MDY-19 permeation and de-epithelialisation of the vaginal mucosa were also studied.

All three peptides readily penetrate vaginal mucosa. Microbicides may be coupled to MDY-19 and PCI to be transported transmucosally. Although increased size of the peptide/microbicides complex may decrease mucosal permeability this could possibly be overcome by the addition of a permeation enhancer, e.g. a surfactant. Removal of the vaginal epithelium increased the flux rates of the peptides across the mucosa and may have implications for a more rapid uptake of these and other microbicides *in vivo*. Concentration- and temperature dependency of peptide flux rates must be taken into consideration when performing *in vitro* permeability studies. The results of the vaginal mucosa study improved the understanding of the permeation characteristics of peptides through vaginal mucosal barriers. It demonstrated the usefulness of the *in vitro* flow-through diffusion model to study the absorption characteristics of vaginal epithelium, to investigate the effect of an absorption enhancer and to study the effect of different permeant concentrations and temperature on the diffusion kinetics of the peptide permeants.

2.2 Introduction

2.2.1 Peptides

Proteins are the most abundant organic molecules in animals, playing important roles in all aspects of cell structure and function. They are biopolymers of α -amino acids and the physical and chemical properties of a protein are determined by its constituent amino acids. Proteins are synthesised as a linear sequence of individual amino acids subunits linked together by peptide bonds, but they assume complex three-dimensional shapes in performing their function. There are approximately 300 amino acids present in various animal, plant, and microbial systems, but DNA which may potentially appear in proteins codes for only 20 amino acids, called standard amino acids. Many proteins also contain modified amino acids and accessory components termed prosthetic groups (Wade, 2003; Bhagavan, 1992).

The standard amino acids differ from each other in the structure of the side chains bonded to their α -carbon atoms. Each amino acid has a central carbon, called the α -carbon, to which four different groups are attached:

- A basic amino group ($-\text{NH}_2$)
- An acidic carboxyl group ($-\text{COOH}$)
- A hydrogen atom ($-\text{H}$)
- A distinctive side chain ($-\text{R}$)

All amino acids, except for glycine, contain at least one asymmetric carbon atom (the α -carbon atom), resulting in two isomers that are optically active. These isomers/enantiomers are chiral. The two amino acid configurations are called D- and L-configurations. All amino acids in proteins are of L-configurations, because proteins are biosynthesised by enzymes that insert only L-amino acids to chains.

The properties of each amino acid are dependent on its side chain ($-\text{R}$). The side chains are the functional groups that are the major determinants of the conformation and function of proteins. Amino acids with charged, polar or hydrophilic side chains are usually exposed on the surfaces of proteins, while the nonpolar hydrophobic residues are usually buried in the hydrophobic interior of a protein and are out of contact with water. Due to their varying amino acid composition, proteins have a wide range of

structural and catalytic properties. Because of this versatility, proteins serve an extensive variety of functions in living organisms (Wade, 2003; Bhagavan, 1992).

Currently a resurgence of interest in peptide and protein drugs exists (Veuillez *et al.*, 2001). Many of the latter are related to endogenous compounds regulating endocrine and other physiological processes in the body and are generally found at low concentrations in the tissues of mammals, but several different peptides can be found in a single type of tissue (Hancock and Rozek, 2002). These amino acid polymers are increasingly used in major research and development programs, especially due to advances in genetic engineering and biotechnology (Yu and Chien, 1997). They may act synergistically with each other and with other agents in the host, e.g. magainin 2 shows synergistic antimicrobial effects with the peptide PGLa.

The unusual characteristics of peptides and proteins present considerable challenges to pharmaceutical scientists in selecting a suitable route for their administration and formulation. In the past, the most commonly used route for protein and peptide delivery has been by parenteral administration. Most proteins and peptides have a relatively short half-life and therefore repeated administrations (injections) are often required (Veuillez *et al.*, 2001). Development of suitable nonparenteral routes for introducing these agents into humans could significantly enhance patient compliance and increase the benefit derived from peptide or protein therapy.

Peptides and proteins cannot readily be given orally because of enzymatic breakdown in the gastrointestinal tract, their sensitivity to the acidic pH in the stomach and poor intrinsic permeability across intestinal epithelium (Maggio, 2005). The fraction of intact peptide reaching the systemic circulation will depend on its ability to cross the mucosal barrier and also on its resistance to degradation by peptidases present at both the site of administration and in the mucosal barrier. Proteolysis can rapidly metabolise peptides at most mucosal routes of administration. Protein-like compounds are generally not well absorbed through mucosae, due to their molecular size, hydrophilicity and metabolism occurring at the site of absorption. Permeation enhancers and/or delivery vehicles can also be used to enhance membrane transport of proteins, peptides and other chemical compounds across biological membranes (Veuillez *et al.*, 2001).

Most small peptides, however, do not diffuse readily through mucosal membranes and diffusion enhancers must be added to increase their absorption. Currently much research involves studying the diffusion of small peptide molecules through biological membranes in the presence of chemical permeation enhancers.

2.2.1.1 Structure of peptides and physico-chemical properties

2.2.1.1.1 Molecular weight and size

The diffusion of a drug through the epithelial layer is influenced by its molecular weight and size. In general smaller molecules (<75-100 Da) appear to cross the mucosa barrier more readily than very large molecules (Veuillez *et al.*, 2001).

The apparent permeability coefficient of fluorescein isothiocyanate dextrans (FITCD), a neutral polysaccharide, decreases as molecular weight increase (Donovan *et al.*, 1990; Maiani *et al.*, 1989; Tavakoli-Saberi and Audus, 1989). Proteins and peptides have a very large dispersion in molecular weight (Mw) compared to most conventional drugs, ranging from less than 600 to greater than 100 000 Da (Sandow *et al.*, 1990). An *in vivo* study on humans showed that peptides such as protirelin (Mw: 362) and oxytocin (Mw: 1007) crossed the human buccal mucosa barrier, whereas buserelin (Mw: 1239) and calcitonin (Mw: 3500) did not (Veuillez *et al.*, 2001). In another study Merkle *et al.* (1992) proposed that the transfer of peptides with molecular weights above 500-1000 Da through buccal mucosa would require the use of an absorption enhancer.

2.2.1.1.2 Conformation, stereospecificity and immunogenicity

Peptides have unique structures and their diffusion characteristics are therefore different from other conventional drugs. Peptides have primary, secondary and tertiary structures and in solution may adopt different conformations depending on their size, which may present difficulties in preserving the pharmacologically active conformation (Bunrham, 1999; Delie *et al.*, 1995; Green *et al.*, 1991). During the processes of formulation and sterilisation of a peptide drug, the stereospecificity of the peptide must be reserved since this may influence membrane permeability and permeation systems are thought to be stereoselective (Palm *et al.*, 1996; Ho *et al.*, 1990).

Peptides may be immunogenic and the use of inert polymers such as polyethylene glycol (PEG), dextran, polyvinylpyrrolidone (PVP) and albumin for peptide delivery may increase resistance to proteolysis and simultaneously decrease peptide immunogenicity (Veuiliez *et al.*, 2001).

2.2.1.1.3 Electrostatic charges

Charge distribution on the peptide or protein chain may also play an important role in predicting their permeability through mucosae, since terminal charges on zwitterionic peptides decrease their membrane permeability (Ho *et al.*, 1990). The effect of charge density can be modified by altering the pH of the medium and therefore the degree of ionisation of the permeant to promote peptide absorption (Liaw *et al.*, 1992). It seems as if mucosae are considered to be permeability-selective towards positively charged peptides (Rojanasakul *et al.*, 1992).

2.2.1.1.4 Solubility, hydrophilicity and partition coefficient

Peptides are amphoteric and therefore usually have complex solubility versus pH profiles. The aqueous solubility is dependent on pH, metallic ions, ionic strength and temperature. The drug is usually neutral at the isoelectric point and therefore the aqueous solubility of the peptide is minimal at this point. Peptides are normally very hydrophilic, unless the N- and C-termini are blocked through cyclization, amide formation or esterification. These compounds have a low octanol-water partition coefficient and peptide absorption by passive diffusion can be enhanced by increasing their lipophilicity (Hansen *et al.*, 1992; Corbo *et al.*, 1989; Siegel *et al.*, 1981). Permeation properties may also be modified to a significant extent by the formation of hydrogen-bonds between peptides and the mucosal tissue (Saitoh and Aungst, 1997; Burton *et al.*, 1996). The pK_a and the local pH at the mucosal surface strongly influence the degree of ionisation of a permeant. Absorption of peptides is maximal at a pH at which they are mostly non-ionised, tailing off as the degree of ionisation increases. However, it has also been shown in an *in vitro* study that the permeability coefficient of protirelin through rabbit buccal mucosa was independent of the pH of the peptide solution (Dowty *et al.*, 1992).

2.2.1.1.5 Aggregation, self-association and hydrogen bonding

Self-aggregation may modify the intrinsic properties of peptides. Insulin usually exhibits aggregation. Ionic ingredients and phenolic preservatives may accelerate this

aggregation (Touitou, 1992). Therefore, zinc insulin complexes are more stable than zinc-free insulin. In another extensive study, it has been reported that non-ionic surfactants such as Pluronic F68 appear to be promising stabilisers (Massey and Shelga, 1989).

Another predictor of peptide absorption is the capacity of some peptides to form intermolecular hydrogen bonds with water. Selfassociation may involve formation of intermolecular H-bonds and hydrophobic interactions. The addition of hydroxyl groups generally promote hydrogen bonding with solvating water, leading to a decrease in the partition coefficient and a decrease in the permeation of a lipid membrane. However, the presence of hydroxyls can sometimes lead to an increased permeability. This is usually due to the formation of cyclic intermolecular hydrogen bonds, which appear to reduce hydrogen bonding and therefore increasing lipophilicity. Cyclisation of peptides appears to reduce hydrogen bonding and therefore increase lipophilicity (Veuilleux *et al.*, 2001).

2.2.1.2 Peptides as microbicides

About 20 years ago it was discovered that the lymph of insects, the granules of human's neutrophils and the skin of frogs contain peptides that could kill bacteria in culture. Since then more than 600 different cationic peptides have been observed in almost all species (Hancock, 2001). This class of small, positively charged peptides, namely cationic antimicrobial peptides, is known for its broad-spectrum antimicrobial activity and is found throughout nature. Cationic peptides are characterised by an overall positive charge and contain multiple lysine and arginine residues and 50 % or more hydrophobic residues. They are produced by most living organisms, from plants and insects to human beings, and form a major part of their immediate defences against infections. These peptides also have anti-viral and anti-cancer activity and the ability to modulate the innate immune responses. The main requirements for their antimicrobial activities are a cationic charge and an induced amphipathic conformation (Powers and Hancock, 2003). Interactions with membranes often induce the peptides to fold into an amphipathic or amphiphilic conformation (Powers and Hancock, 2003). The disulphide bridges cause them to fold into three-dimensional amphiphilic structures in which the positively charged and hydrophilic domains are well separated from the hydrophobic domains. This structural conformation equips the molecule to interact with membranes, especially with

bacterial membranes with their negatively charged and hydrophilic head groups and hydrophobic cores. The four structural classes of peptides include β -sheet molecules established by two or three disulphide bonds, amphipathic α -helices, extended molecules and loops due to a single bond (Hancock, 2001).

Due to the development of resistant pathogens, it is important to consider new classes of antibiotics, such as cationic peptides (Hancock, 2001). After the development of quinolones there were for more than 30 years no new antibiotic chemical structures, until the release of synergid and linezolid. An advantage of the cationic peptides over the traditional antibiotics is that cationic peptides have a wide variety of antimicrobial activities that include action against most Gram-positive and Gram-negative bacteria, fungi, enveloped viruses and protozoa. Most traditional antibiotics do not have activity against fungi and antifungal drugs do not act against bacteria. Cationic peptides are bactericidal and also able to combat the antibiotic-resistance mechanisms that limit the use of other antibiotics such as methicillin-resistant staphylococcus aureus. They kill bacteria very rapidly compared with conventional bactericidal antibiotics and there are only a few antibiotic-resistant mechanisms that affect antimicrobial peptides, but most only have a moderate effect on the MIC (Hancock, 2001; Zhang *et al.*, 2000).

These peptides may have minimal inhibitory concentrations (MIC) as low as 0.25-4 $\mu\text{g/ml}$ against microorganisms (Powers and Hancock, 2003). Some peptides have activity against malaria parasites and viruses (including HIV, HSV, influenza A virus and vesicular stomatitis virus). Examples of such peptides are defensins, indolicidin, polyphemusin and melittin (Zhang and Hancock, 2000). Mechanisms of action have been reported to include blockage of virus-cell fusion and inhibition of the activity of HIV long terminal repeats (Hancock, 2001). Cationic peptides may also possess anticancer activity and promote wound healing. There is still uncertainty as to whether the peptides have selectivity for malignant over normal cells. Recent studies have also shown their function as effectors of innate immune responses. These properties make cationic peptides exciting candidates as new therapeutic agents (Powers and Hancock, 2003). Where antibiotics only have activity against bacteria, cationic peptides have an extensive range of activities against bacteria, fungi, enveloped viruses and eukaryotic parasites. The pharmacological applications of cationic peptides to treat infections have therefore received much interest and are being developed for clinical trials (Hancock, 2001).

2.2.1.2.1 Mechanisms of action

The mechanism of action of cationic antimicrobial peptides is continuously investigated and information about their structures keeps expanding. Despite the enormous amount of investigations, there is still a disagreement in the literature on the role of membrane disruption/permeabilisation in determining the mechanism of action of cationic peptides. It seems that these peptides mainly exert their action by disrupting cytoplasmic membranes and may be cytotoxic by virtue of disturbing the bacterial inner or outer membranes (Hancock and Rozek, 2002). However, there is still uncertainty about precisely how these peptides perturb membranes and whether the latter process is related to the antimicrobial activities of these compounds (Epand and Vogel, 1999). It seems that two types of molecules exist: membrane disruptive and non-membrane disruptive antimicrobial peptides. Another proposal is that cationic antimicrobial peptides have multiple actions on cells including membrane permeabilisation, cell wall and division effects and macromolecular synthesis inhibition. It also seems as if the mechanism of action varies between different peptides and between different bacteria for a given peptide.

2.2.2 Vaginal mucosa

The safety and efficacy of vaginal administration have been well established (Alexander *et al.*, 2004). Although the vagina has been used since ancient times for drug delivery, it still remains a relatively unexplored route for drug administration. Some studies have suggested that the vagina's molecular weight cut-off point for absorption may be higher than that of other mucosal surfaces. Experience with a variety of compounds demonstrates that the vagina is a highly effective site for drug administration and particularly in women's health (Justin-Temu *et al.*, 2004). Advantages of vaginal administration of drugs include: the administration of lower doses, maintenance of steady drug levels, less frequent administration than with e.g. the oral route, avoidance of the first-pass effect and no effect of gastrointestinal (GI) disturbances on the absorption of the drug (Alexander *et al.*, 2004; Sanders and Matthews, 1990). This route also allows a woman to self-administer medication continuously for prolonged periods of time.

Modern technology has yielded vaginal drug delivery systems that provide optimised pharmacokinetic profiles, which make the vagina an excellent route for drug delivery.

The rate and extent of drug absorption after intravaginal administration may vary depending on formulation factors, vaginal physiology, age of the patient and menstrual cycle. Suppositories, creams, gels, tablets and vaginal rings are commonly used vaginal drug delivery systems for both systemic and local effects. In recent years, the vaginal route has been rediscovered as a potential route for systemic delivery of peptides and other therapeutically important macromolecules (Hussain and Ahsan, 2004).

2.2.2.1 Anatomy of vagina

The vagina is a muscular, tubular organ connecting the uterus to the exterior of the body. In adults the vagina varies from 8-12 cm, with the posterior wall approximately 1.50-2 cm longer than the anterior wall (Alexander *et al.*, 2004; Ahuja *et al.*, 1997). It is a collapsed organ with the anterior and posterior walls in contact with each other (Alexander *et al.*, 2004). Radiographic colpography has shown that the vagina has two distinct portions: a lower convex portion and a wider upper portion that lies in an almost horizontal plane when the woman is standing. The angle between the upper and lower axes is 130 degrees (Funt *et al.*, 1978). The vagina contains numerous folds called rugae, which provide distensibility and support and also increase the surface area of the vaginal wall (Namnoum and Murphy, 1997).

The vagina is provided by a nerve supply from two sources namely the peripheral, which primarily supplies the highly sensitive lower quarter of the vagina and the autonomic, which primarily supplies the upper three quarters. Autonomic fibres are not very sensitive to pain or temperature and responds mainly to stretch. Woman rarely feel localised sensations or discomfort when using vaginal products such as suppositories or vaginal rings and are often unaware of the presence of these products when used (Alexander *et al.*, 2004).

The vagina has an extensive blood vascular supply. Blood is supplied from different sources such as the uterine artery, the pudendal artery and the middle and inferior haemorrhoidal arteries. It also has an extensive venous system with the primary drainage through the pudendal veins. The vaginal, uterine, vesical and rectosigmoid veins from the middle and upper vagina provide drainage to the inferior vena cava and has the advantage that it bypass the hepatic portal system (Namnoum and Murphy,

1997). A so called “first uterine pass effect” has been hypothesised when hormones are administered vaginally, due to the extensive vascular connections between the vagina and uterus (De Ziegler *et al.*, 1997). Several groups have demonstrated that the endometrial concentrations of some compounds such as progesterone and estradiol (E2), were higher than the vaginal concentrations, with both vaginal and oral administration (Cicineli *et al.*, 2000; Tourgeman *et al.*, 1999; Fanchin *et al.*, 1997).

2.2.2.2 Histology of vagina

During the fertile period of a woman's life the vaginal mucosa consists of 4 main epithelial layers (Fig. 5). The most superficial layer consists of nonsecretory stratified squamous epithelium, which is non-keratinised and contains pycnotic nuclei. The next layer is the lamina propria or tunica, made of collagen and elastin, which also contains a rich supply of vascular and lymphatic channels. The muscle layer is the third layer, with smooth muscle fibres running in both circular and longitudinal directions. The final layer consists of areolar connective tissue and a large plexus of blood vessels. Vaginal tissue does not contain fat cells, glands or hair follicles (Herbst *et al.*, 1992). The thickness of the vaginal epithelial layer changes by approximately 200-300 μm during the menstrual cycle (Hussain and Ahsan, 2004). Although the vagina does not possess any glands, it does secrete a large amount of fluid (Paavonen, 1983). Secretions from the vaginal wall are transudate in nature and are produced by the engorgement of the vascular plexus that encompasses the vagina (Herbst *et al.*, 1992). The amount and composition of the vaginal fluid changes throughout the menstrual cycle (Hussain and Ahsan, 2004). Adult woman produce fluid at a rate of 3-4g/4h, while the discharge produced by postmenopausal women is 50 % less (Bergh, 1988). The contents of the fluid may include enzymes, enzyme inhibitors, proteins, carbohydrates, amino acids, alcohols, hydroxyl-ketones and aromatic compounds (Wagner and Levin, 1978). The volume and composition of the vaginal fluid may also be influenced by sexual arousal and this may lead to an alteration in the drug release pattern from a vaginal delivery system. Lactobacilli acidophilus are present in the vagina and produce lactic acid from glycogen. The lactic acid acts as a buffer to maintain the vaginal pH between 3.8 and 4.2. During menstruation, the pH of vaginal fluid increases and frequent acts of coitus may also cause an increase in the vaginal pH, because both ejaculate and vaginal transudate are

alkaline. The vaginal pH is also influenced by the presence of cervical mucus and the amount of vaginal transudate.

The epithelium contains densely packed cells present in the superficial layer with narrow, cycle-independent, intercellular spaces. The intercellular spaces widen and become deeper in the epithelium.

Paracellular diffusion barriers in non-keratinised epithelia can be represented by different types of junctional complexes. Lipids or glycoconjugates present in the upper levels of the vaginal epithelium may play a role in the inhibition of intercellular channels. Keratinised epithelia were found to have a different barrier function to the non-keratinised superficial epithelial cell layers of the vaginal mucosa. The intraepithelial transport system allows larger molecules, e.g. proteins, to pass (Forsberg, 1996).

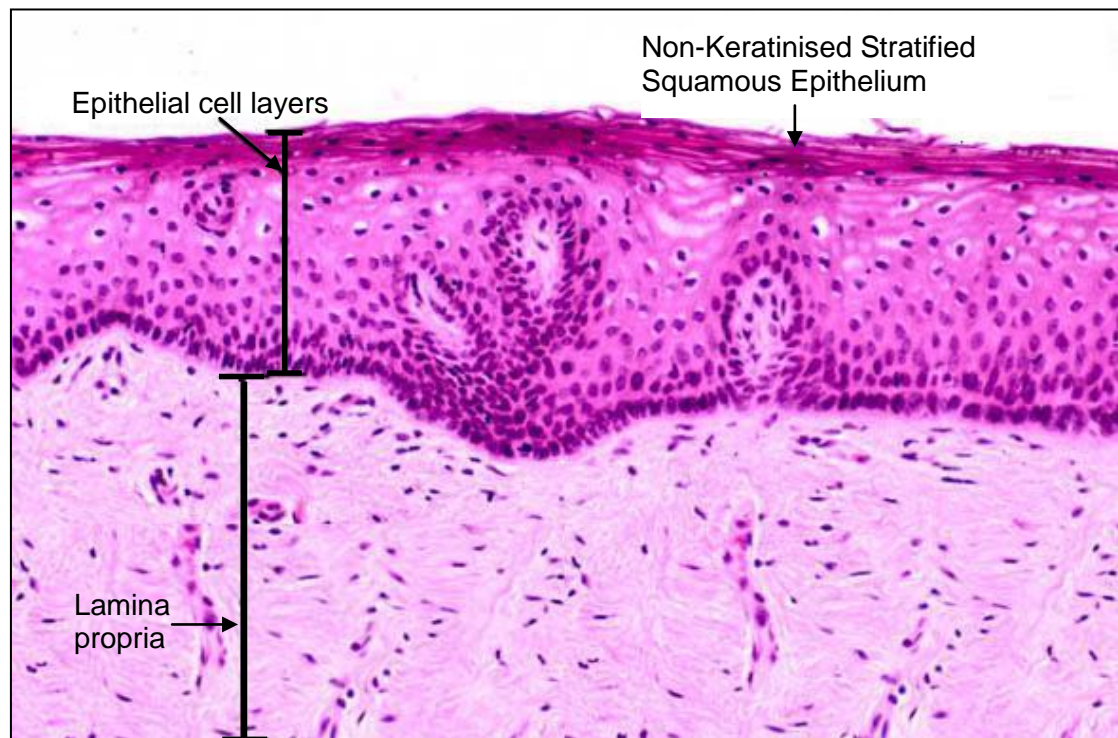


Fig. 5. Non-keratinised human vaginal epithelium (By courtesy of Prof. IOC Thompson)

2.2.2.3 Physiology of vagina and drug administration

Drugs administered *via* the vaginal route are absorbed similar to other mucosal routes. Compounds are absorbed through mucosa transcellularly *via* concentration dependent diffusion through cells; paracellularly by mediation of tight junctions and vesicularly or through receptor mediated transport. Absorption of the drug from the vaginal delivery system occurs through two main steps: drug dissolution in the vaginal lumen and membrane penetration. The absorption profile of vaginal drug delivery systems is therefore affected by any biological formulation factor that affects drug dissolution and membrane transport (Woolfson *et al.*, 2000).

The mucosal linings in the body have important protective functions and the vaginal defences include the epithelium, flora, immune cells and pH.

2.2.2.4 Vaginal enzymes

Most enzyme activity exists in the external layers and the basal cell layers of the vagina (Woolfson *et al.*, 2000; Wendel-Smith and Wilson, 1991; Schmidt and Beller, 1978). Different enzymes are present of which the proteases seem to be the prominent barrier for the absorption of intact peptide and protein drugs into the systemic circulation. Acatürk *et al.* (2001) did a comparison of enzymatic activities of four different aminopeptidases (aminopeptidase N, leucine aminopeptidase, aminopeptidase A and aminopeptidase B) in vaginal homogenates of various species. These authors reported that the enzyme activity in rat, rabbit, and human was significantly lower than that of sheep and guinea-pig. The aminopeptidase activity in the species studied showed the following order of activity: sheep>guinea-pig>rabbit≥human≥rat and they concluded that rat and rabbit vaginal tissue could be used as potential models for vaginal enzymatic activity studies and for the determination of degradation of protein and peptide drugs in the vagina.

2.2.2.5 Epithelium

When estrogen is present, the vaginal epithelium consists of 25 layers, which forms a physical barrier together with cervical mucus, vaginal secretions and local flora (Miller *et al.*, 2000). It is difficult for organisms to invade or access the basement membrane or

capillary bed, because the stratified squamous epithelium is continually renewing and shedding (Alexander *et al.*, 2004).

An example of the effect of vaginal epithelium thickness on drug transport has been shown in a study with vidarabine. Vidarabine has a 5-100 times higher permeability coefficient during the early dioestrous stage, than during the oestrus stage in guinea pigs (Pschera *et al.*, 1989). The vaginal absorption of estrogen is also higher in postmenopausal women compared to premenopausal women (Pschera *et al.*, 1989). Vaginal absorption is influenced by the thickness of vaginal epithelium (Carlsrom *et al.*, 1988). There are conflicting reports in the literature on the effect of an increase in vaginal epithelium on drug absorption through the mucosa. In women who received vaginal estrogen therapy, the vaginal progesterone absorption was found to be increased, even though prior estradiol therapy should have caused an increase in the vaginal epithelium (Villanueva *et al.*, 1981). This anomalous finding was explained by the fact that the absorption of progesterone was increased with increased vascularity of the vagina (Katz and Dunmire, 1993). The presence of cervical mucus could serve as a possible permeability barrier. The short- and long-term stability of intravaginal delivery systems and devices may be affected by the high activity of enzymes in the vaginal epithelium (Hussain and Ahsan, 2004).

2.2.2.6 Florae

Lactobacilli proliferate near the epithelium and use desquamated cells as a source of intracellular glycogen that can be converted to lactic acid. These organisms play an important role in vaginal health, because they compete with exogenous microbes for nutrients and produce lactic acid and hydrogen peroxide, which regulate the growth of other flora. An absence of hydrogen-producing lactobacilli in the normal vaginal flora may result in the overgrowth of catalase-negative organisms, resulting in vaginosis (Alexander *et al.*, 2004).

Estradiol stimulates glycogen production in the epithelial cells and therefore promotes the presence of lactobacillus. During pregnancy the high levels of estrogen may result in thick epithelium, proliferation of lactobacilli and a low pH. Other factors that may disrupt the vaginal milieu are antibiotics and some diseases e.g. diabetes. Healthy women of

reproductive age have vaginal secretions that contain a mixture of aerobic and anaerobic bacterial flora (Roy, 1994). The numbers and prevalence of different bacteria vary according to a woman's menstrual cycle (Priestley *et al.*, 1997; Schwebke *et al.*, 1997). Numbers decrease 10-fold to 100-fold in the week before menstruation, followed by dramatic increase in the number of bacteria as menstruation commences (Larsen and Galask, 1980).

2.2.2.7 Immune cells

The cellular and humoral systems provide protective immunity. The Langerhans' cells play an important role in the local immune system. It has dendritic extensions exposed to the lumen of the vaginal epithelium and these cells can pass antigens to dendritic cells that migrate to the lymph nodes, where they activate B and CD4⁺ T cells. The activated B lymphocytes return to the subepithelium, where they become IgA-secreting cells. The IgA is taken up by the epithelial cells and made into a dimer prior to release into the lumen.

The cervical mucus contains IgG and IgM, as well as IgA antibodies (McGhee *et al.*, 1993). Intraepithelial T lymphocytes, dendritic cells and a subepithelial population of B lymphocytes that synthesize IgA locally, all constitute to the protective immunity of the vagina.

Martin *et al.* (1998) have shown that long-term use of depot-medroxyprogesterone acetate results in thinning of the vaginal epithelium and increased susceptibility to HIV infection. Animal studies indicated that in progestogen-dominant environments infections such as chlamydia trachomatis and herpes simplex might also be worsened (Kaushic *et al.*, 2003; Kaushic *et al.*, 2000).

A recent human study demonstrated that changes in leukocyte subtype concentrations varied depending on whether depot-medroxyprogesterone acetate or levonorgestrel was administered. Estrogen treatment in monkeys made them completely resistant to simian immunodeficiency virus (SIV), whereas progestogen treatment made them susceptible. The presence of estrogen or exogenous products keeps the vagina's pH low and enables this organ to resist infection. It is still unclear whether the beneficial effects of

estrogen are due to its effect on the integrity and thickness of the cervicovaginal epithelium, or whether they are due to the inaccessibility of certain immune cells (Alexander *et al.*, 2004).

2.2.2.8 PH

The production of lactic acid by vaginal microflora maintains the vaginal pH at 3.8 to 4.2 (Herbst *et al.*, 1992). The pH may be rapidly influenced by the presence of semen, which is slightly alkaline (pH 7.0 to 8.0) and the effect may last for several hours (Voeller and Anderson, 1992). Female hygiene products may wash away a variety of the vaginal defences and can promote colonisation of bacteria or alter vaginal pH. This may lead to the proliferation of pathogenic bacteria and yeast. The absorbent material may also become media for bacterial colonisation and growth (Ness *et al.*, 2002). Therefore, products that will be used in the vagina for days, weeks, or months, must be made of a material that does not damage the surrounding tissue, should not interfere with the normal immune functions and must be non-absorbent (Alexander *et al.*, 2004).

2.2.2.9 Drug delivery systems and physiochemical properties of drugs

Gels, foams, solutions, suppositories and tablets have been traditionally used as vaginal formulations. The vaginal ring has also been introduced recently for hormone replacement and contraceptive therapy. Drug absorption, distribution and residence time may vary according to the delivery system used. Solutions, suspensions and foam show better distribution and coverage of vaginal tissue over a tablet form (Johnson and Masters, 1962). A vaginal drug delivery system that is intended for a local effect, should distribute uniformly throughout the vaginal cavity to be effective. A semi-solid formulation and a fast dissolving system are therefore required. Generally a bioadhesive dosage form or intravaginal ring system would be more preferable to attain a topical effect. It has been difficult for researchers to quantify the distribution of a drug after intravaginal administration, since it is uncertain if the administered formulation coated the whole organ (Hussain and Ahsan, 2004). Vaginal enzymes, cyclic changes in the thickness of vaginal epithelium, fluid volume and composition, pH and sexual arousal potentially affect drug release from intra vaginal delivery systems. Vaginal drug

absorption is also influenced by physiochemical properties of drugs such as molecular weight, lipophilicity, ionisation, surface charge and chemical nature.

The vaginal permeability of straight chain aliphatic alcohols increases in a chain dependant manner and similarly vaginal permeability is much greater to lipophilic steroids e.g. progesterone and estrone than to hydrophilic steroids such as hydrocortisone and testosterone (Brannon-Peppas, 1992; Hwang *et al.*, 1977). It is generally accepted that the absorption of low molecular weight lipophilic drugs is higher than large molecular weight lipophilic or hydrophilic drugs. A study on vaginal absorption of polyvinyl alcohol suggested that the vagina may absorb higher molecular weight compounds than other mucosal surfaces (Sanders and Matthews, 1990). Vaginal fluid contains a large amount of water and therefore any drug intended for vaginal delivery requires a certain degree of water solubility. Data on human vaginal permeability to drugs with different physiochemical properties is very limited and therefore much research is necessary to establish the effects of physicochemical parameters of drugs on vaginal absorption (Hussain and Ahsan, 2004).

A hydrophobic drug's absorption may increase when the vaginal fluid volume is higher, but the presence of overly viscous cervical mucus may present a barrier to drug absorption. Increased fluid volume may remove the drug from the vaginal cavity and subsequently reduce the absorption of the drug. Absorption of a drug by the vaginal epithelium is also influenced by the pH of the vaginal fluid since it determines the degree of ionisation of weak electrolyte drugs. An *in vitro* study has shown that the pH of the vaginal preparations affects the release of PGE₂ from the media (Johnson *et al.*, 1992). A change in the vaginal pH may therefore affect the release profiles of pH sensitive drugs from vaginal drug delivery systems (Hwang *et al.*, 1977).

2.2.2.9.1 Creams and gels

For creams and gels to be feasible as a drug delivery system, it has to be easy to use, non-toxic and non-irritating to the mucous membrane.

Creams and gels are often used for topical delivery of contraceptives and anti-bacterial drugs. Unfortunately, these products may have disadvantages of being uncomfortable, messy to apply and sometimes embarrassing when they leak into the undergarment.

These delivery methods may also not provide an exact dose, because of nonuniform distribution and leakage.

Applying a cream and gel may compare well with oral delivery of drug, such in the treatment of bacterial vaginosis, where metronidazole and clindamycin vaginal cream were found to be nearly as effective as orally administered drugs (DuBouchet *et al.*, 1998).

A gel formulation has also been used for intravaginal vaccine delivery, for cervical ripening and induction of labour. The cholera vaccine showed a greater mucosal response in female genital tract compared with the oral administration of the drug. Oxytocin, dinoprostone and misoprostone are examples of drugs commonly used for cervical ripening and induction of labour. There have been several studies done to compare the efficacy of intravaginal administration versus oral delivery. The dose required for the oral delivery of misoprostol is usually 4 times that of an intravaginal dose. There have been conflicting reports in the literature with respect to the efficacy of the route of misoprostol administration. Shetty *et al.* (2003) reported that vaginal administration of misoprostol was more efficacious than the oral route, but Hall *et al.* (2002) found that oral delivery of the drug had the potential to induce labour as safely and effectively as oral administration. This difference in findings occurred even though both researchers used the same oral and vaginal dose and the same intervals of drug administration. The disparity in their observation may be explained to their different principal outcome criteria assessed in each of the studies. From an analysis of different studies performed employing the oral and vaginal routes of misoprostol administration, it appears that the currently recommended vaginal routes of misoprostol dose (25 µg) is efficacious and safer than the 100 µg oral dose (Hussain and Ahsan, 2004).

Due to an absence of an effective prophylactic anti-HIV vaccine or therapy, there has been a current surge in interest at the development of a topical intravaginal formulation to combat the mucosal and perinatal HIV or microbicide transmission (D'Cruz and Uckun, 2004).

2.2.2.9.2 Vaginal rings

Vaginal rings are generally approximately 5.5 cm in diameter with a circular cross section diameter of 4-9 mm and are designed to release a drug in a controlled fashion after insertion in the vagina. They are usually made of a polymeric material such as the most commonly used polymer, poly (dimethylsiloxane) or silicone devices. Other elastomeric polymers such as ethylene vinyl acetate and styrene butadiene block copolymer have also been tested recently (Van Laarhoven *et al.*, 2002; Roumen and Dieben, 1999). The ethylene vinyl acetate polymers are classified by the content of vinyl acetate. Addition of vinyl acetate units in the polyethylene provides the following advantages: increased flexibility, improved optical properties, greater adhesion, increased impact and puncture resistance. The clinical acceptability of rings made of ethylene vinyl acetate is very high (Novák *et al.*, 2003; Roumen and Dieben, 1999). Roumen and Dieben (1999) evaluated the tolerability of ethylene vinyl acetate of a non-medicated vaginal ring. The ring had a diameter 54 mm and was inserted for 21 consecutive days, permitting temporary removal during coition. Most of the women judged the ring easy to insert and remove. No adverse effects were experienced among the test group during the study period and the ring received a 91 % acceptability.

Vaginal rings are most frequently used for contraceptive and hormone replacement therapy (Dezarnaulds and Fraser, 2002; Ballagh, 2001; Harwood and Mishell, 2001). When used for contraceptive applications, the rings are placed in vagina for 21 days followed by ring free period of a week. NuvaRing® was the first combined contraceptive vaginal ring available in the US market. It contains two active compounds, etonogestrel and ethinyl estradiol. The ring releases 120 µg/day of etonogestrel and 15 µg/day of ethinyl estradiol over a 3-week period of use. Clinical trials showed that NuvaRing® is an effective contraceptive ring with good cycle control and user acceptability (Novák *et al.*, 2003).

Femring® and Estring® are estrogen releasing rings used for hormone replacement therapy. Femring® is placed in the vagina once every trimester and releases acetate derivate of estradiol. The estradiol acetate is then hydrolysed to estradiol after being released from the delivery device. The vaginal ring Estring® contains estradiol (Hadgraft and Lane, 2005).

Advantages of vaginal rings are that it is user controlled, does not interfere with coition, does not require daily intake of pills and allows continuous delivery of low dose hormones (Hussain and Ahsan, 2004). In simple vaginal rings, the active compound is homogeneously dispersed within a polymeric ring. The drug at the surface of the ring is released more rapidly than the drug in the inner layer of the ring. This may lead to an initial burst release of the drug in the outermost layer and therefore sandwich or reservoir type rings have been developed to obtain a constant release of drug from the ring. Sandwich type devices consist of a narrow drug-containing layer located below the surface of the ring and positioned between a non-medicated central core and a nonmedicated outer band. In reservoir type rings, drugs are dispersed in a centralised core, which is then encapsulated by a drug free layer of polymer. It is possible to have several cores of different drugs in a single ring; allowing administration of several drugs from the same device. Changing the core diameter or thickness of the non-medicated coating can modify the rate of drug release (Hussain and Ahsan, 2004).

2.2.2.9.3 Suppositories and vaginal tablets

Some authors use the terms suppositories and pessaries interchangeably and consider vaginal tablets as a separate dosage form. Large numbers of vaginal medications are available in the form of tablets or suppositories. These formulations are designed to melt in the vaginal cavity and release the drug for several hours. Suppository systems are commonly used to administer drugs for cervical ripening prior to childbirth and for local delivery of drugs e.g. dehydroepiandrosterone sulphate for ripening effect on the uterine cervix, miconazole for vaginal candidacies and progesterone for hormonal replacement therapy (Yamashita *et al.*, 1991; Abrams and Weintraub, 1983; Vukovich *et al.*, 1977). Binders, disintegrants and other excipients that are used to prepare conventional oral tablets may also be used in vaginal tablets. It has the advantage of ease of manufacture and insertion. Mucoadhesive polymers are sometimes used in vaginal tablet formulation to increase vaginal residence time. Drugs that are administered as vaginal tablets include itraconazole, clotrimazole and prostaglandins. The presence of hydrophobic and release retarding materials may decrease the absorption of a drug from a vaginal formulation. Hydrophobic drugs may not be suitable for vaginal tablets, but the presence of penetration enhancers such as surfactants and bile salts may significantly enhance absorption.

2.2.2.9.4 Bioadhesive delivery systems

Bioadhesive drug delivery systems have been developed to circumvent the problems of leakage, low retention of the vaginal epithelium and unpleasantness of the conventional vaginal formulations. Polymers that have been used for vaginal formulation include polycarbohil, hydroxypropylcellulose and polyacrylic acid. The developed formulations showed good adhesive properties and the ability to hold the dosage form at the application site (Hussain and Ahsan, 2004). Various peptide and protein drugs have been hosted using this delivery system. An example of such a peptide is calcitonin, a polypeptide, which is used in the treatment of postmenopausal osteoporosis (Richardson and Armstrong, 1999). Other examples include nonoxynol-9, a spermicidal agent and the antimycotic agent, clotrimazole (Ceschel *et al.*, 2001; Lee and Chien, 1996).

2.2.2.9.5 User acceptability

Misperceptions and poor education about vaginal anatomy and physiology among patients may lead to reluctance to use vaginal medications. Counselling and educating of patients and health care workers may overcome this problem and help to establish the vaginal route of drug administration as safe, effective and convenient so that more women can experience the potential benefits.

2.2.2.9.6 Vaginal immunisation

Immunisations are conventionally administered *via* the oral or parenteral route, resulting in systemic rather than mucosal immunity. The mucosal-associated lymphoid tissues (MALT) of the gastrointestinal, respiratory, female and male genital tracts are the primary sites of entry of pathogens into the human body. They are structured to provide protection against pathogen transmission across the body surfaces and reduce dissemination to submucosal tissues, draining lymph nodes and blood. Mucosal immunisations therefore have the advantage of providing mucosal as well as systemic immunity by a non-invasive route (Hussain and Ahsan, 2004; Hobson *et al.*, 2003). In recent years, there have been several reports of successful immunisation with DNA vaccines delivered to a variety of mucosal surfaces (Kaneko *et al.*, 2000; Kanellos *et al.*, 2000; Klavinskis *et al.*, 1999; Roy *et al.*, 1999; Kuklin *et al.*, 1997).

Vaginal vaccine formulations have been researched to combat HIV. Bodgers *et al.* (2004) have reported a novel HIV-CCR5 receptor vaccine strategy in the control of

mucosal SIV/HIV infection and may serve as a novel strategy in the prevention of HIV transmission. A vaccine which targets both the virus and its CCR5 receptor was administered to female rhesus monkeys either by the vaginal route or by a para-mucosal route, targeting the proximity of the draining lymph nodes.

2.2.3 Permeation enhancers

Protein-like compounds are generally not well absorbed through mucosae, due to their molecular size, hydrophilicity and metabolism occurring at the site of absorption. Permeation enhancers and/or delivery vehicles can also be used to enhance membrane transport of proteins, peptides and other chemical compounds across biological membranes (Veuillez *et al.*, 2001).

Permeation enhancers promote the transport of drugs through a mucosal membrane. Several classes of enhancers have been evaluated for potential use in enhancing transmucosal delivery. It is clear from the literature that the choice of a permeation enhancer must be based on the following characteristics: effectiveness, safety, chemical inertness, lack of biological activity and rapidly reversibility of toxic and/or pharmacological effects, if any.

Examples of strategies to improve peptide absorption include the following: chemical enhancers, enzyme inhibitors, lipophilicity modification and formulation design (Veuillez *et al.*, 2001).

2.2.3.1 Chemical enhancers

Examples of chemical enhancers often studied or used are surfactants, bile salts, chelators, alcohols and fatty acids. Chemical enhancers may exert their effect by:

1. altering the rheological properties of the mucus layer
2. increasing the thermodynamic properties of the peptide
3. enhancing transcellular transport by interacting with phospholipids and/or proteins to increase membrane fluidity
4. enhancing paracellular transport
5. inhibiting enzyme activity

(Veuillez *et al.*, 2001)

2.2.3.1.1 Surfactant

Currently much research involves studying the diffusion of small peptide molecules through biological membranes in the presence of chemical permeation enhancers, such as surfactant.

Surfactants are used as emulsifier and as physical stabilising, wetting and suspending agents in many topical pharmaceutical formulations, cosmetic and food products. Surfactants may enhance partitioning by reducing the surface tension between the vehicle and the membrane surface and by influencing the barrier potential of the membrane and the tight junctions. It may also disrupt the barrier layers of the membrane (Fig. 6) (Barry and El Eini, 1976).

Furthermore, the use of surfactants or complexation agents is associated with a reduction in the thermodynamic activity of the drug due to micellar association or complexation. In turn, this leads to changes in the free concentration of drug available for transport or diffusion across the membrane (Poelma *et al.*, 1991).

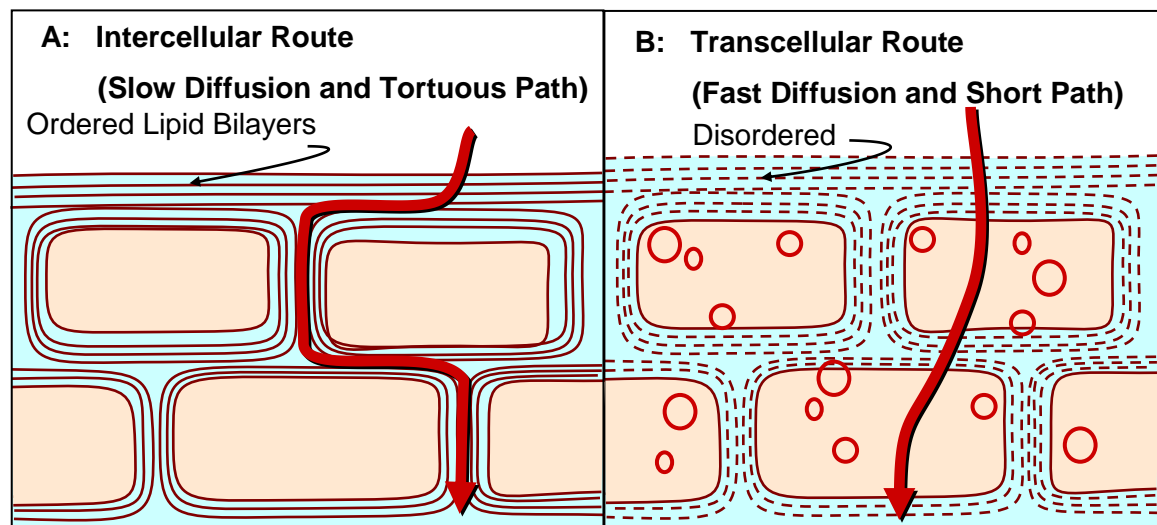


Fig. 6.

A: Permeation of drugs through the epithelial layer *via* the intercellular route.

B: Permeation of drugs through the epithelial layer *via* the transcellular route following disordering of the lipid layers by surfactant.

2.2.3.1.2 Vehicles and adjuvants (co-solvent)

A drug can be dissolved or dispersed in a solvent to improve transport by changing the thermodynamic activity and/or increasing the drug's solubility in the epithelial barrier of the mucosa (Veuillez *et al.*, 2001).

2.2.3.2 Enzyme inhibitors

Peptidase inhibitors may be employed to overcome both enzymatic barriers to permeation. It may be used alone or in combination with other permeation enhancers (Aungst, 1994).

2.2.3.3 Lipophilicity modification

The lipophilicity plays an important role in the diffusion of a peptide through a biological membrane. Acylation or alkylation may be used to conjugate the N-terminal with lipophilic molecules or prodrugs may be used to enhance lipophilicity and the diffusion of peptides (Veuillez *et al.*, 2001).

2.2.3.4 Formulation design

Formulation may have a dramatic impact on transmucosal absorption rates. The clinical efficacy of the formulation depends on the ability of the vehicle to release the drug in a controlled manner and to penetrate the mucosa in sufficient concentrations.

Liposomal formulations have shown to increase the local and decrease the systemic concentrations of peptides.

2.2.4 Transmembrane diffusion processes

Free diffusion or passive transport of substances through liquids, solids and membranes is an important process in pharmaceutical science. Transvaginal diffusion is a passive process, requiring a concentration differential as the driving force and each molecule requires kinetic energy to achieve a net movement down this gradient. The diffusion of a molecule through a membrane, biological or synthetic, *in vivo* or *in vitro*, would normally occur as follows (Smith, 1987). The molecule diffuses through the vehicle in which it is contained to the membrane interfaces and partition from the vehicle into the upper

lamina of the membrane. The molecule then diffuses within the membrane, equilibrating laterally and emerges under steady state conditions, from the distal surface of the medium. Absorptive interaction may be extensive in this layer, forming a reservoir of the molecule. It then partitions into the adjacent membrane strata or into the acceptor fluid under the influence of the concentration gradient and adsorption may occur once again. Diffusion through any of the layers or any of the partitioning events may control the overall rate of permeation.

There are two general mechanisms by which soluble macromolecules can be absorbed from the vaginal mucosa into the body. The molecules can pass either through the cells (transcellular transport/absorptive transcytosis) or between the cells (paracellular or intercellular transport). Transcellular transport may occur independently of a plasma membrane receptor (fluid phase or adsorptive transcytosis) or it may involve receptor mediated binding, followed by vesicular transport (receptor mediated transcytosis). Paracellular transport occurs through the junctional complex between two or three cells.

The physicochemical properties of a drug such as molecular weight, lipophilicity, ionisation, surface charge and chemical nature influence drug absorption. The governing factor for which pathway a molecule will follow when it diffuses through a membrane, is the partition coefficient. Hydrophilic molecules are expected to predominantly permeate through the paracellular route, while lipophilic molecules are expected to permeate mainly through the transcellular route (Williams, 2003). A mixed permeation model has been proposed for drugs that predominantly diffuse through tissue *via* the continuous intercellular lipid domains. Both the lipid and polar regions of the lipid bilayers could thus provide a micro-route through which the permeant diffuses, depending on the drug's partition coefficient (Williams, 2003).

The rate of absorption of a drug molecule is also a function of its molecular size and shape. An inverse relationship exists between the permeant flux and its molecular weight. Smaller molecules diffuse through the vaginal tissue at a faster rate than larger molecules. Organic molecules, which have a high melting point, have lower aqueous solubility at normal temperatures and pressures. This leads to the assumptions that the more lipophilic the molecule, the faster it would permeate through any tissue. Topical medication is usually applied from an aqueous formulation and the permeant should

contain some aqueous solubility to minimise the depletion of the permeant from the formulation over the time course of the application. Because many drug substances are weak acids or weak bases, the ionisation state of the drug will be important in its diffusion through membranes. Weak acids and weak bases will dissociate to various degrees depending on the pH of the formulation used and the pH of the membrane through which it diffuses. In the non-ionised state, the drugs will be more lipid soluble and will diffuse through membranes at a higher rate than in the ionised state (Williams, 2003).

2.2.5 Aim

Due to an absence of an effective prophylactic anti-HIV vaccine or therapy, there has been a current surge in interest at the development of a topical intravaginal formulation to combat the mucosal and perinatal HIV or microbicide transmission (D'Cruz and Uckun, 2004). Mucosal surfaces of the vagina are the portals for heterosexual transmission of HIV and therefore play a fundamental role in the pathogenesis of the primary infection. Topical microbicides, including peptides, are being developed for blocking the transfer of this virus, but little is known about the diffusion of these compounds into and through vaginal mucosal epithelium (Hussain and Ahsan, 2004).

The use of small peptide molecules as drugs has therefore gained a lot of interest in the last few years. These molecules, however, cannot readily be given orally, because they are metabolised by enzymes in the gastrointestinal tract and they are sensitive to the acidic pH in the stomach. They can be applied topically on mucosal membranes e.g. vaginal mucosa, thus bypassing first-pass metabolism and making them available to the systemic circulation directly. Most of these small peptides do not diffuse readily through the mucosal membranes and diffusion enhancers must be added to increase absorption. Currently, the research direction involves the diffusion of small peptide molecules through biological membranes, as well as the enhancement of their diffusion by using enhancers.

This study involves the investigation of diffusion in kinetics of three synthetic and novel peptides (MEA-5, MDY-19 and PCI) through human vaginal mucosa. Furthermore, the study entails an evaluation of the flow-through diffusion system's value to examine test conditions such as different temperatures, removal of epithelium and the use of a

surfactant. The three different peptides were chosen since they were manufactured to serve as potential microbicides or as transporters of microbicides against viruses such as HIV, which portal of entry is often the vaginal mucosa. MEA-5 is an antibacterial peptide that binds to cell surfaces, but cannot be internalised. It bears structural similarity to Maigainin 2 and can act synergistically with other existing microbicides. MDY-19 is a transport peptide that strongly binds to cell surfaces and can be internalised. This peptide has potential applications for transporting therapeutically active compounds into cells, where the active microbicides can be released and exert its activity against e.g. viruses such as HIV. The transport peptide PCI is comparable in size and has an amino acid sequence similar to MDY-19. However, it contains less of the basic acid, arginine (4 vs. 7 residues), thereby reducing the overall positive charge of the peptide.

The outcomes will hopefully improve the understanding of the permeation characteristics of peptides through vaginal mucosal barriers and the usefulness of the *in vitro* flow-through diffusion model to study the absorption characteristics of vaginal epithelium. It will also help to investigate the effect of an absorption enhancer, different permeant concentrations and temperature on the diffusion kinetics of the peptide permeants.

2.3 Materials and Methods

2.3.1 Human vaginal mucosa

Specimens were obtained from excess tissue removed from 43 postmenopausal patients, ages 40-81 years (mean age 58 ± 11 yr SD), following vaginal hysterectomies at the Louis Leipoldt Hospital, Bellville, South Africa. All surgical specimens obtained were immediately placed in a transport fluid and transferred to our laboratory within 1 h. The transport fluid consisted of a stock solution of Eagle's Minimum Essential Medium (MEM) without L-glutamine and sodium bicarbonate (Gibco, Paisley, Scotland), to which the latter as well as an antibiotic (penicillin/streptomycin, 100 IU/ml) and an antimycotic (amphotericin-B, 2.5 μg /ml) were added prior to using it for the transport of tissue specimens. Excess connective and adipose tissue were trimmed away and all specimens were snap-frozen in liquid nitrogen and stored at -85°C for periods up to 6 months, as previously prescribed (Van der Bijl and Van Eyk, 2004; Van der Bijl and Van Eyk, 1999; Van der Bijl *et al.*, 1998b; Van der Bijl *et al.*, 1997). No specimens were obtained where there was clinical evidence of any disease that might have influenced the permeability characteristics of the different specimens. The Ethics Committee of Stellenbosch University and the Tygerberg Academic Hospital approved the study. Project number: 95/019 (resubmitted and approved on 28/7/03).

2.3.2 Peptides

The three synthetic FITC (fluorescein isothiocyanate)-labelled peptides MEA-5 ($M_w = 2911.4$ Da), MDY-19 ($M_w = 2409.5$ Da) and PCI ($M_w = 2325$ Da) were obtained from the EMPRO (European Microbicides Project) consortium partner PEPSCAN, Lelystad, The Netherlands (Fig. 7). EMPRO is a European based research network investigating and developing new microbicides for the prevention of HIV infection. It is funded by the European Union. The EMPRO program is divided into different activities, e.g. covering development of new microbicides in the laboratory and also testing these candidates' permeability through different membranes. MEA-5 is an antibacterial peptide that binds to cell surfaces, but cannot be internalised. It bears structural similarity to Maigainin 2 and can act synergistically with other existing microbicides. MDY-19 is a transport peptide that strongly binds to cell surfaces and can be internalised. This peptide has potential applications for transporting therapeutically active compounds into cells, where the active microbicides can be released and exert its activity against e.g. viruses such as

HIV. The transport peptide PCI is comparable in size and has an amino acid sequence similar to MDY-19. However, it contains less of the basic acid, arginine (4 vs. 7 residues), thereby reducing the overall positive charge of the peptide.







MEA-5 (2922.4 Da) FITC-Ahx-GIGKFLHSAGKFGKAFVGEIMKS-NH ₂ MDY-19 (2409.5 Da) FITC-Ahx-GRQLRIAGRRLRGRSR-NH ₂ PCI (2325 Da) FITC-Ahx-GQQLRIAGRRLRGQSQ-NH ₂	Symbol	Amino Acid
	A	Alanine
	R	Arginine
	E	Glutamic Acid
	Q	Glutamine
	G	Glycine
	H	Histidine
	I	Isoleucine 
	L	Leucine 
	K	Lysine
	M	Methionine 
	F	Phenylalanine 
	S	Serine
	V	Valine 
	 Hydrophobic amino acids	

Fig. 7. Chemical structures of MEA-5, MDY-19, PCI (By courtesy of PEPSCAN)

Ahx (6-Aminohexanoic acid) was used to attach FITC-moiety into the peptides. A spacer between the peptide and the ligand (Figs. 8 and 9) minimises the inference in the folding of the peptide and/or it's binding to the receptor.

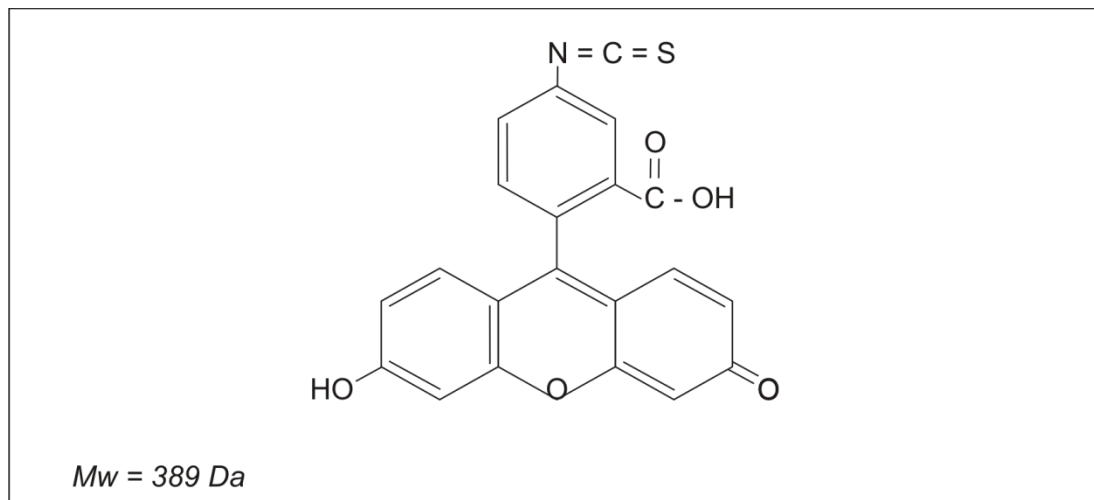


Fig. 8. FITC = Fluorescein isothiocyanate (Fluka/Riedel-de Haën Catalogue, 2007/2008)

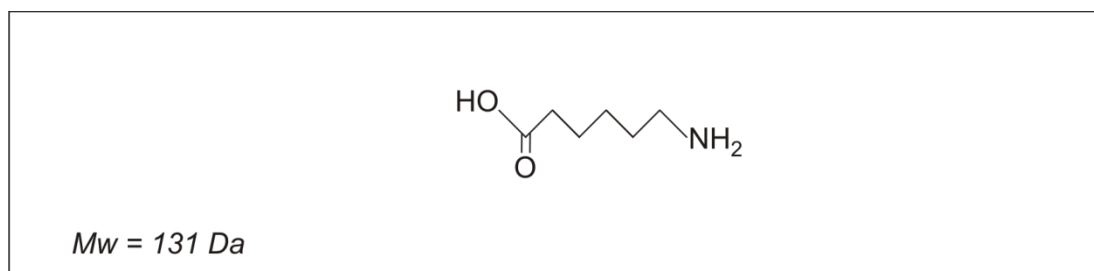


Fig. 9. Ahx = Aminohexanoic acid (Fluka/Riedel-de Haën Catalogue, 2007/2008)

2.3.3 Surfactant

A novel surfactant Biopolsurf (prepared in our laboratory by Prof. JM van Zyl) was used as an enhancer. It was decided to use this surfactant, since it was readily available and we wanted to investigate its effect on transvaginal peptide permeability. Initial studies on the effect of Biopolsurf on the bronchial, vaginal, buccal and rectal permeability of different chemicals showed that it significantly enhanced the permeability of most of the selected drugs across the different epithelia (Viljoen *et al.*, 2005).

Biopolsurf preparations were prepared by mixing DPPC (dipalmitoyl-L- α -phosphatidylcholine), cetyl alcohol and PG (1,2-dipalmitoyl-L- α -phosphatidylglycerol) in a 10:1.1:1 ratio (w/w) in chloroform. The organic solvent was then removed by rotary evaporation and the mixture was dried under a continuous stream of nitrogen at room

temperature. The dried phospholipids film was then hydrated with 0.1 M NaCl. A Branson B-15P ultrasonicator fitted with a microtip was then used to sonicate the mixture on ice under a stream of nitrogen (power of 20 watts for 7 x 13 s; 60 s intervals). Hereafter, tyloxapol was added to the preparation to a final concentration of 1 mg/ml and the tube was sealed under nitrogen before use. The Biopolsurf suspension contained 13.5 mg/ml DPPC and 1.35 mg/ml PG.

2.3.4 Permeability Experiments

Prior to each permeability experiment, vaginal tissue specimens were thawed at room temperature in phosphate buffered saline (PBS, pH 7.4). The diffusion kinetics of FITC-labelled peptides through thawed, frozen vaginal mucosa were then determined (Basson *et al.*, 2007). After equilibration of the specimens in PBS, they were carefully cut, so as not to damage the epithelial surfaces, into sections (4 mm in diameter) and then mounted in flow-through diffusion cells (exposed areas 0.039 cm²) with the epithelial surfaces facing upwards. Previous studies showed that the vaginal mucosa could be harvested without damaging the underlying connective tissue layer. Histological examination of formalin-fixed specimens of fresh, thawed frozen and post 24 h experiment vaginal mucosa showed that the structural integrity of it was preserved after harvesting and freezing. Individual epithelial cells, however, demonstrated early signs of autolysis and some minor degenerative changes. In spite of these degradative changes, there was no evidence of a break-down in properties as barriers to the various permeants tested (Van der Bijl and Van Eyk, 2004; Van der Bijl *et al.*, 1998).

Six permeation studies were performed for each investigation, on 7 tissue replicates for each of the six patients' tissue. Prior to commencing each permeability experiment, tissue disks were equilibrated for 10 min with PBS (pH 7.4) at 20 °C in both the donor and acceptor compartments of the diffusion cells. Following equilibration, the PBS was removed from the donor compartment and replaced with 0.5 ml of PBS containing the FITC-labelled peptide. PBS at 20 °C was pumped through the acceptor chambers at a rate of 1.5 ml/h and collected, by means of a fraction collector, at 2 h intervals for 24 h. FITC-labelled peptides in the acceptor chambers were quantified by means of fluorospectrophotometry. Intra- and inter-experiment variations were calculated for the peptide experiments (CV= SD/mean x 100).

2.3.5 Methodology

Various test conditions were applied such as different temperatures, different concentrations of the test peptides, the use of a surfactant and the de-epithelisation of the mucosa.

The *in vitro* permeation of a FITC-labelled synthetic transport peptide (MDY-19) and a FITC-labelled microbicidal peptide (MEA-5) through human vaginal mucosa was studied. Permeability studies of MDY-19 and MEA-5 were conducted at a concentration of 1 mM (in PBS) at a temperature of 20 °C and over a time period of 24 hours. The permeation enhancing effects of a phospholipid surfactant on MDY-19 permeation and the effect of vaginal de-epithelisation on MEA-5 flux rates were also studied. De-epithelisation mimics the situation when integrity of vaginal epithelium is breached due to disease or local trauma.

Initially de-epithelisation was conducted by two methods to establish the most effective and convenient method. The one method involves the vaginal tissue being submerged in 80 °C water for 30 s and the quick stripping of the epithelial layer with tweezers (heat stripping). The second method entailed the removal of the epithelial cells by scraping the tissue with a scalpel (mechanical stripping). Concentrations of 1 mM (in PBS) were used for the forgoing mentioned permeability studies.

Initial experiments were conducted at 20 °C (room temperature), since it is less expensive to have a flow-through system without a waterbath to control temperature and previous vaginal permeability studies in our laboratory with selected chemicals showed that permeability data of experiments at 20 °C could be extrapolated and correlated with experimental data at temperatures of 37 °C (Van der Bijl and Van Eyk, 2004; Van der Bijl and Van Eyk, 1999; Van der Bijl *et al.*, 1997). Precipitation of peptides on the vaginal mucosa during the permeability experiments at room temperature (20 °C) and at a concentration of 1 mM was noticed. For this reason, further permeability studies of MDY-19 and PCI were conducted at a temperature of 37 °C. PEPSCAN stopped the production of MEA-5 and therefore studies with MEA-5 could not be repeated at 37 °C.

Permeability studies of MDY-19 and PCI were conducted at concentrations of 1, 0.75 and 0.5 mM (in PBS) at a temperature of 37 °C and the respective extraction ratios were calculated.

Only limited amounts of MDY-19 and PCI were manufactured by PEPSCAN and it was then decided to conduct further experiments with FITC-labelled peptides at 37 °C and at a concentration of 0.75 mM to prevent precipitation and to economise the use of the peptides. The effect of vaginal de-epithelisation on MDY-19 and PCI flux rates were also studied.

2.3.6 Detection of FITC-labelled peptides

Fluorospectrophotometry (emission: 520 nm and excitation: 497 nm) using a Perkin-Elmer spectrophotometer (Perkin-Elmer, MA, USA), was used for the detection of FITC peptides (Figs. 10-15). The standards of the three selected peptides were dissolved in the same PBS as the drug containing samples collected from the flow-through diffusion system. Fresh or new standards were made for each experiment and all the samples were analysed on one day. The coefficient of determination was better than 0.99 for all the peptides (R^2) and the reproducibility of 3 injections from standards from 3 different vials were better than a % RSD of 2 % for all analytes. The stability of the peptides was not tested in the carrier medium and no information is available on the stability of the peptides at the temperatures used in the experiment.

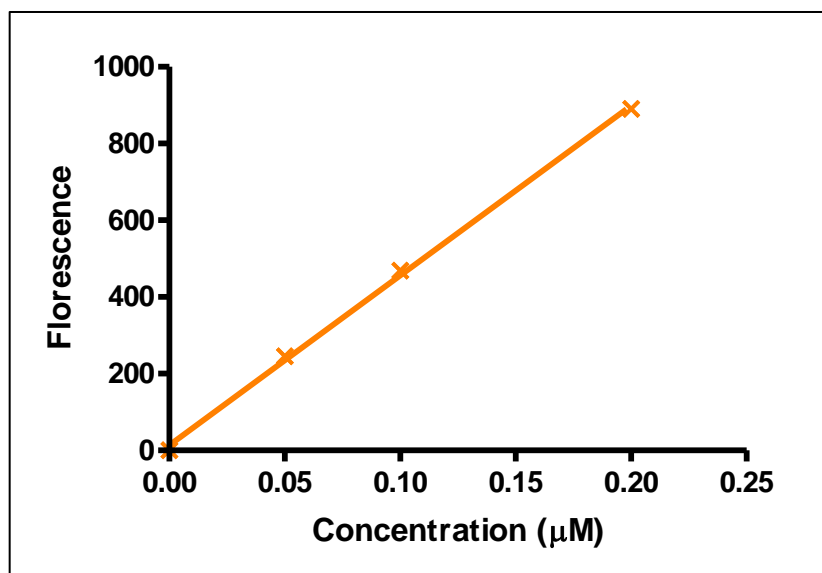


Fig. 10. Calibration curve of MEA-5

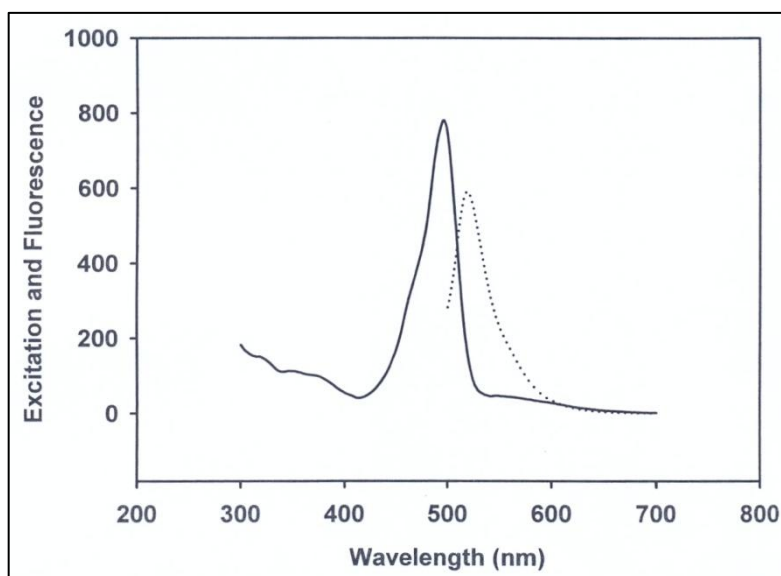


Fig. 11. Excitation and fluorescence spectra of MEA-5

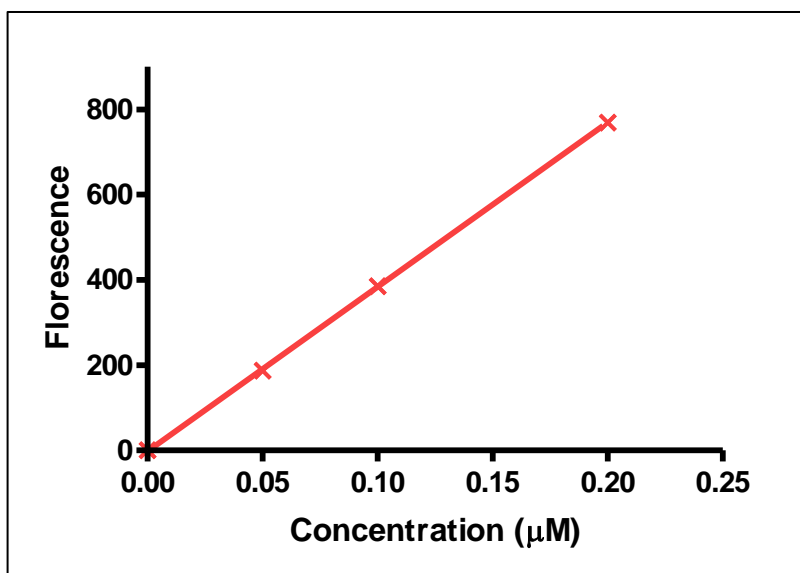


Fig. 12. Calibration curve MDY-19

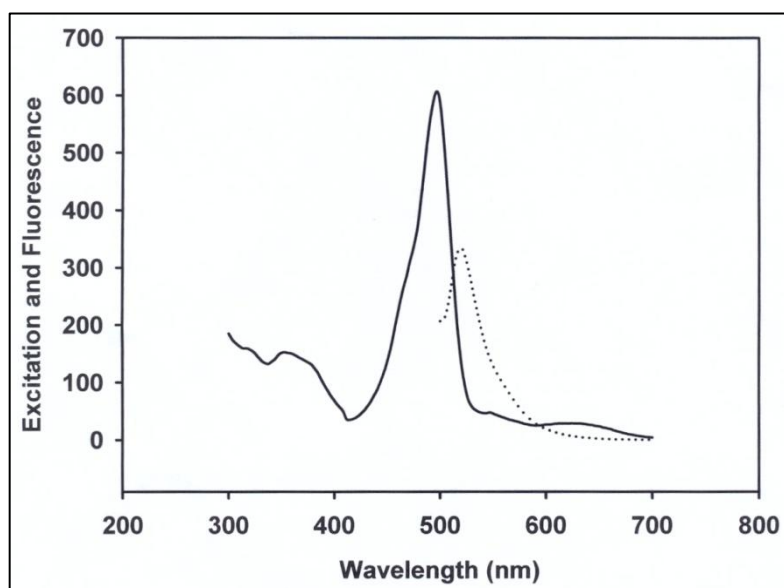


Fig. 13. Excitation and fluorecence spectra of MDY-19

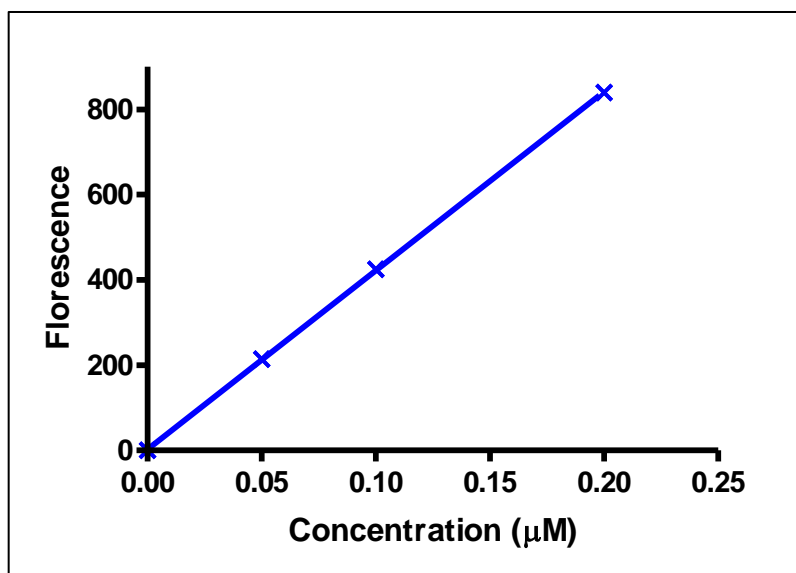


Fig. 14. Calibration curve of PCI

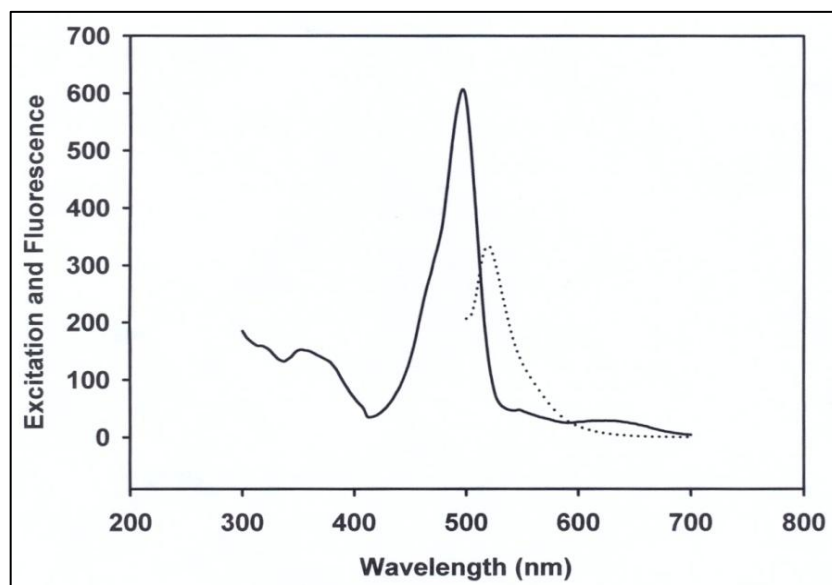


Fig. 15. Excitation and fluorescence spectra of PCI

2.3.7 Calculation of flux values

Flux (J) values of the various chemicals compounds across the different membranes were calculated by means of the relationship: $J = Q/(A \times t)$, where Q = quantity of compound crossing membrane (pmoles), A = membrane area exposed (cm²) and t = time of exposure (min) (Basson *et al.*, 2005). The trapezoid method of obtaining area under curve (AUC) was used.

2.3.8 Steady state kinetics and statistical analysis of data

Repeated measure ANOVA over all time periods and a Bonferroni multiple comparison procedure was used to determine steady state. If at consecutive time periods p-values exceeded 0.05, these flux values do not differ significantly, therefore indicating steady state. Non-linear regression analyses (third-order polynomial) were performed using GraphPad Prism, version 4, 2003 computer programme. A F-test was used to compare entire curves (Motulsky, 1995). A significant level of 5 % was used for all tests and comparisons.

2.4 Results

The overall mean flux values of MEA-5 and MDY-19 versus time across vaginal tissue (20 °C) are shown in Fig. 16. Both MEA-5 and MDY-19 permeated vaginal mucosa well. MDY-19 had a significantly higher ($p<0.05$) flux rate than MEA-5, commensurate with its smaller molecular size (weight). Both MEA-5 and MDY-19 reached steady state at 18 h (Fig. 16a).

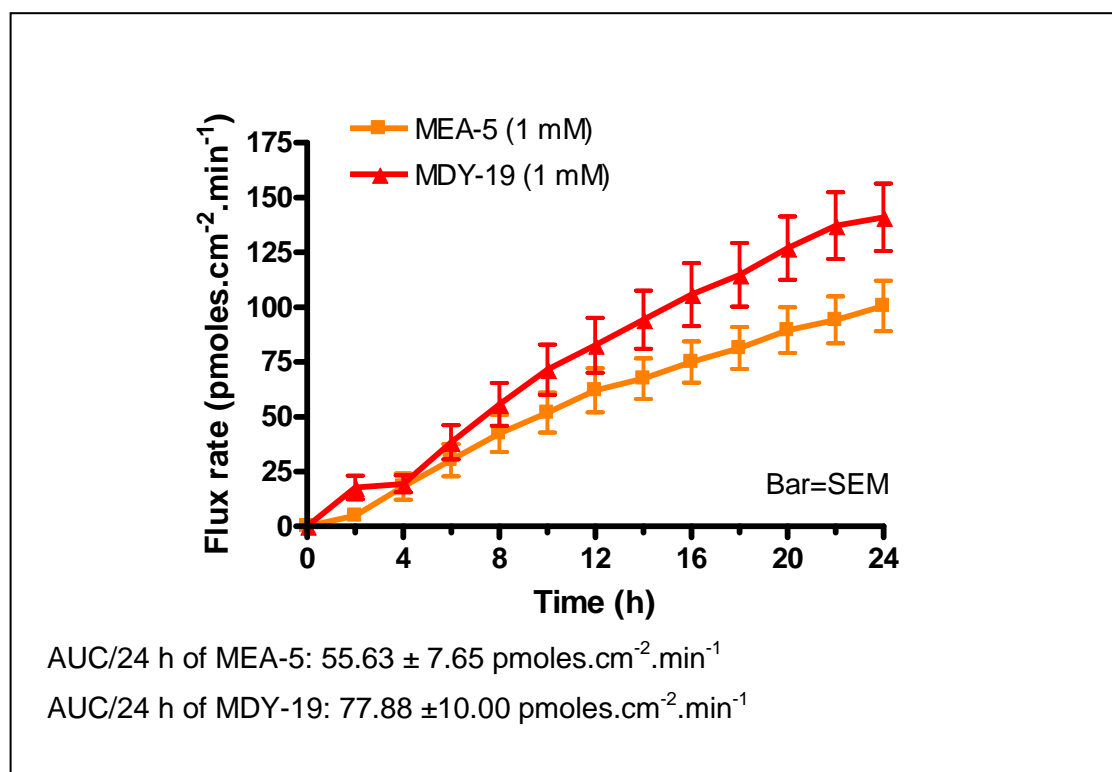


Fig. 16. The overall mean flux values at 20 °C of MEA-5 and MDY-19 versus time across vaginal tissue

Bonferroni test; variable DV_1 (MEA-5)													
Error: Within MS = 726.11, df = 451.00													
Cell No.	Time (h)	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}
		4.926	18.106	30.205	42.351	51.909	62.052	67.398	74.995	81.355	89.542	94.204	100.54
1	2		1	0.00139	0	0	0	0	0	0	0	0	0
2	4	1		1	0.00294	1E-06	0	0	0	0	0	0	0
3	6	0.00139	1		1	0.01655	7E-06	0	0	0	0	0	0
4	8	0	0.00294	1		1	0.05771	0.00165	3E-06	0	0	0	0
5	10	0	1E-06	0.01655	1		1	0.57585	0.00659	5.2E-05	0	0	0
6	12	0	0	7E-06	0.05771	1		1	1	0.07314	0.00026	5E-06	0
7	14	0	0	0	0.00165	0.57585	1		1	1	0.01241	0.00044	2E-06
8	16	0	0	0	3E-06	0.00659	1	1		1	0.90625	0.07725	0.00114
9	18	0	0	0	0	5.2E-05	0.07314	1	1		1	1	0.07825
10	20	0	0	0	0	0	0.00026	0.01241	0.90625	1		1	1
11	22	0	0	0	0	0	5E-06	0.00044	0.07725	1	1		1
12	24	0	0	0	0	0	0	2E-06	0.00114	0.07825	1	1	

Bonferroni test; variable DV_1 (MDY-19)													
Error: Within MS = 1603.7, df = 462.00													
Cell No.	Time (h)	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}
		17.665	19.382	38.347	55.635	71.481	82.669	94.307	105.77	114.74	126.98	137.21	140.96
1	2		1	1	0.0009	0	0	0	0	0	0	0	0
2	4	1		1	0.00214	0	0	0	0	0	0	0	0
3	6	1	1		1	0.00938	2.8E-05	0	0	0	0	0	0
4	8	0.0009	0.00214	1		1	0.12261	0.00063	1E-06	0	0	0	0
5	10	0	0	0.00938	1		1	0.56073	0.00549	5.1E-05	0	0	0
6	12	0	0	2.8E-05	0.12261	1		1	0.51068	0.01514	2.8E-05	0	0
7	14	0	0	0	0.00063	0.56073	1		1	1	0.01159	6.3E-05	7E-06
8	16	0	0	0	1E-06	0.00549	0.51068	1		1	0.9541	0.02003	0.00358
9	18	0	0	0	0	5.1E-05	0.01514	1	1		1	0.63243	0.167
10	20	0	0	0	0	0	2.8E-05	0.01159	0.9541	1		1	1
11	22	0	0	0	0	0	0	6.3E-05	0.02003	0.63243	1		1
12	24	0	0	0	0	0	0	7E-06	0.00358	0.167	1	1	

Fig. 16a. Both MEA-5 and MDY-19 reached steady state at 18 h

The overall mean flux values of MDY-19 across vaginal tissue (20 °C) with and without surfactant as a penetration enhancer are shown in Fig. 17. The surfactant significantly ($p < 0.05$) enhanced the flux rate of MDY-19. The surfactant increased the AUC of the mean flux values (average flux over 24 h experiment) of MDY-19 1.3 times and also decreased the lag time of the peptide. The control group reached steady state after 18 h and the group with the surfactant enhancer had a lag time of 16 h (Fig. 17a).

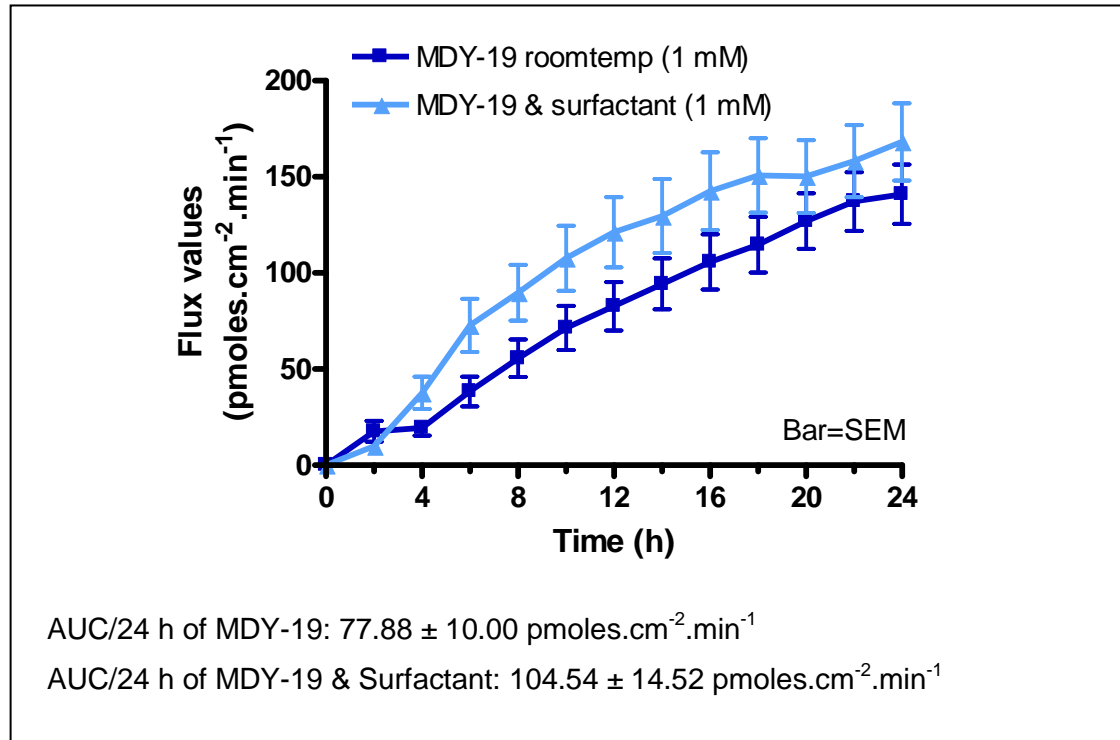


Fig. 17. The overall mean flux values at 20 °C of MDY-19 across vaginal tissue with and without surfactant as a penetration enhancer

Bonferroni test; variable DV_1 (MDY-19)													
Error: Within MS = 1603.7, df = 462.00													
Cell No.	Time (h)	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}
		17.665	19.382	38.347	55.635	71.481	82.669	94.307	105.77	114.74	126.98	137.21	140.96
1	2		1	1	0.0009	0	0	0	0	0	0	0	0
2	4	1		1	0.00214	0	0	0	0	0	0	0	0
3	6	1	1		1	0.00938	2.8E-05	0	0	0	0	0	0
4	8	0.0009	0.00214	1		1	0.12261	0.00063	1E-06	0	0	0	0
5	10	0	0	0.00938	1		1	0.56073	0.00549	5.1E-05	0	0	0
6	12	0	0	2.8E-05	0.12261	1		1	0.51068	0.01514	2.8E-05	0	0
7	14	0	0	0	0.00063	0.56073	1		1	1	0.01159	6.3E-05	7E-06
8	16	0	0	0	1E-06	0.00549	0.51068	1		1	0.9541	0.02003	0.00358
9	18	0	0	0	0	5.1E-05	0.01514	1	1		1	0.63243	0.167
10	20	0	0	0	0	0	2.8E-05	0.01159	0.9541	1		1	1
11	22	0	0	0	0	0	0	6.3E-05	0.02003	0.63243	1		1
12	24	0	0	0	0	0	0	7E-06	0.00358	0.167	1	1	

Bonferroni test; variable DV_1 (MDY-19 & Surfactant)													
Error: Within MS = 991.95, df = 209.00													
Cell No.	Time (h)	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}
		9.895	37.66	72.805	89.755	107.7	121.17	129.63	142.65	150.74	150.2	158.19	168.21
1	2		0.38261	0	0	0	0	0	0	0	0	0	0
2	4	0.38261		0.03388	2.7E-05	0	0	0	0	0	0	0	0
3	6	0	0.03388		1	0.03705	0.00016	3E-06	0	0	0	0	0
4	8	0	2.7E-05	1		1	0.12185	0.00572	1.8E-05	0	0	0	0
5	10	0	0	0.03705	1		1	1	0.03633	0.00158	0.00198	5.8E-05	0
6	12	0	0	0.00016	0.12185	1		1	1	0.22024	0.26053	0.01712	0.00028
7	14	0	0	3E-06	0.00572	1	1		1	1	1	0.30143	0.00947
8	16	0	0	0	1.8E-05	0.03633	1	1		1	1	1	0.72447
9	18	0	0	0	0	0.00158	0.22024	1	1		1	1	1
10	20	0	0	0	0	0.00198	0.26053	1	1	1		1	1
11	22	0	0	0	0	5.8E-05	0.01712	0.30143	1	1	1		1
12	24	0	0	0	0	0	0.00028	0.00947	0.72447	1	1	1	

Fig. 17a. MDY-19 and MDY-19 with surfactant (MDY-19 & Surfactant) as a penetration enhancer, reached steady state respectively at 18 and 16 h

Vaginal mucosal surfaces were de-epithelialised by means of mechanical and heat stripping. The overall mean flux values of MEA-5 across human vaginal mucosa across intact vaginal tissue and after heat and mechanical de-epithelialisation are shown in Fig. 18. Both heat and mechanical de-epithelialisation of mucosa significantly ($p < 0.05$) enhanced flux values of MEA-5 (heat de-epithelialisation: 1.9 x; mechanical de-epithelialisation: 1.8 x) compared with controls (20 °C), although no marked significant differences ($p > 0.05$) or advantages could be discerned between the two methods. MEA-5 across intact vaginal tissue reached steady state after 18 h, but de-epithelialisation (heat and mechanical) of the vaginal tissue caused MEA-5 not to reach steady state during the 24 h experiment (Figs. 18a and b). Precipitation of MEA-5 and MDY-19 was noted with the initial experiments, therefore further permeability studies were conducted at 37 °C. The overall mean flux values of MDY-19 and PCI across intact vaginal tissue (control group) versus de-epithelialised tissue are respectively shown in Figs. 19 and 20. De-epithelialisation of the vaginal mucosa significantly ($p < 0.05$) enhanced flux values of MDY-19 (1.5 x) and PCI (1.3 x) compared with the controls (37°C) and it also decreased the lag time of the two peptides (Figs. 19a and 20a).

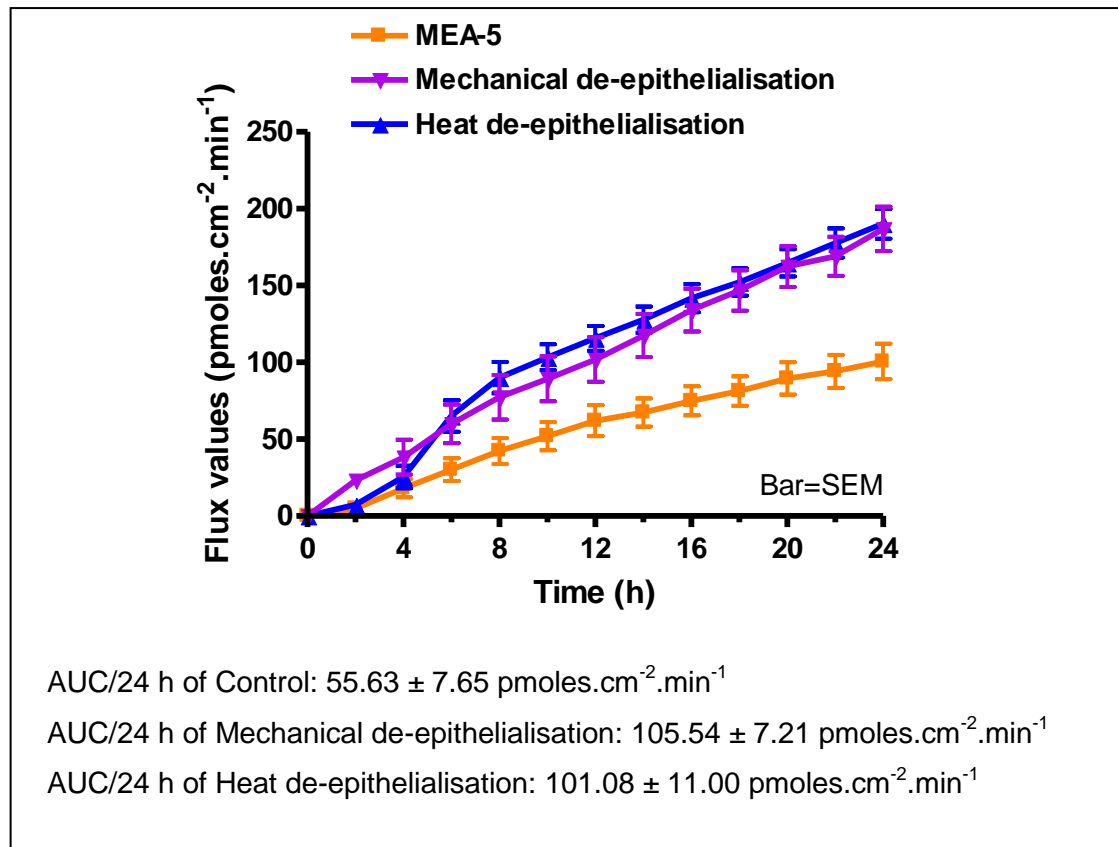


Fig. 18. Overall mean flux values at 20 °C of MEA-5 versus time across intact vaginal mucosa (control) and de-epithelialised mucosa by means of heat and mechanical stripping

Bonferroni test; variable DV_1 (MEA-5)													
Error: Within MS = 726.11, df = 451.00													
Cell No.	Time (h)	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}
		4.926	18.106	30.205	42.351	51.909	62.052	67.398	74.995	81.355	89.542	94.204	100.54
1	2		1	0.00139	0	0	0	0	0	0	0	0	0
2	4	1		1	0.00294	1E-06	0	0	0	0	0	0	0
3	6	0.00139	1		1	0.01655	7E-06	0	0	0	0	0	0
4	8	0	0.00294	1		1	0.05771	0.00165	3E-06	0	0	0	0
5	10	0	1E-06	0.01655	1		1	0.57585	0.00659	5.2E-05	0	0	0
6	12	0	0	7E-06	0.05771	1		1	1	0.07314	0.00026	5E-06	0
7	14	0	0	0	0.00165	0.57585	1		1	1	0.01241	0.00044	2E-06
8	16	0	0	0	3E-06	0.00659	1	1		1	0.90625	0.07725	0.00114
9	18	0	0	0	0	5.2E-05	0.07314	1	1		1	1	0.07825
10	20	0	0	0	0	0	0.00026	0.01241	0.90625	1		1	1
11	22	0	0	0	0	0	5E-06	0.00044	0.07725	1	1		1
12	24	0	0	0	0	0	0	2E-06	0.00114	0.07825	1	1	

Fig. 18a. MEA-5 across intact vaginal mucosa (control) reached steady state at 18 h

Bonferroni test; variable DV_1 (Heat de-epithelialisation)													
Error: Within MS = 316.40, df = 165.00													
Cell No.	Time (h)	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}
		7.495	25.446	65.129	90.156	103.32	115.65	127.82	141.76	152.28	164.78	177.65	190.24
1	2		0.32118	0	0	0	0	0	0	0	0	0	0
2	4	0.32118		0	0	0	0	0	0	0	0	0	0
3	6	0	0		0.00681	1E-06	0	0	0	0	0	0	0
4	8	0	0	0.00681		1	0.00511	1E-06	0	0	0	0	0
5	10	0	0	1E-06	1		1	0.00939	0	0	0	0	0
6	12	0	0	0	0.00511	1		1	0.00348	2E-06	0	0	0
7	14	0	0	0	1E-06	0.00939	1		1	0.0096	1E-06	0	0
8	16	0	0	0	0	0	0.00348	1		1	0.02238	3E-06	0
9	18	0	0	0	0	0	2E-06	0.0096	1		1	0.00552	1E-06
10	20	0	0	0	0	0	0	1E-06	0.02238	1		1	0.00522
11	22	0	0	0	0	0	0	0	3E-06	0.00552	1		1
12	24	0	0	0	0	0	0	0	0	1E-06	0.00522	1	

Bonferroni test; variable DV_1 (Mechanical de-epithelialisation)													
Error: Within MS = 648.85, df = 165.00													
Cell No.	Time (h)	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}
		23.17	38.31	59.958	77.335	89.234	101.87	117.48	134.02	146.8	162.33	168.99	186.83
1	2		1	0.00453	1E-06	0	0	0	0	0	0	0	0
2	4	1		1	0.00168	4E-06	0	0	0	0	0	0	0
3	6	0.00453	1		1	0.09208	0.00044	0	0	0	0	0	0
4	8	1E-06	0.00168	1		1	0.47108	0.00101	0	0	0	0	0
5	10	0	4E-06	0.09208	1		1	0.13359	0.00011	0	0	0	0
6	12	0	0	0.00044	0.47108	1		1	0.03089	0.0001	0	0	0
7	14	0	0	0	0.00101	0.13359	1		1	0.09082	0.00011	3E-06	0
8	16	0	0	0	0	0.00011	0.03089	1		1	0.13085	0.00983	2E-06
9	18	0	0	0	0	0	0.0001	0.09082	1		1	0.97337	0.00106
10	20	0	0	0	0	0	0	0.00011	0.13085	1		1	0.47651
11	22	0	0	0	0	0	0	3E-06	0.00983	0.97337	1		1
12	24	0	0	0	0	0	0	0	2E-06	0.00106	0.47651	1	

Fig. 18b. MEA-5 across de-epithelialised mucosa by means of both heat (heat de-epithelialisation) and mechanical stripping (mechanical de-epithelialisation) did not reach steady state during the 24 h period

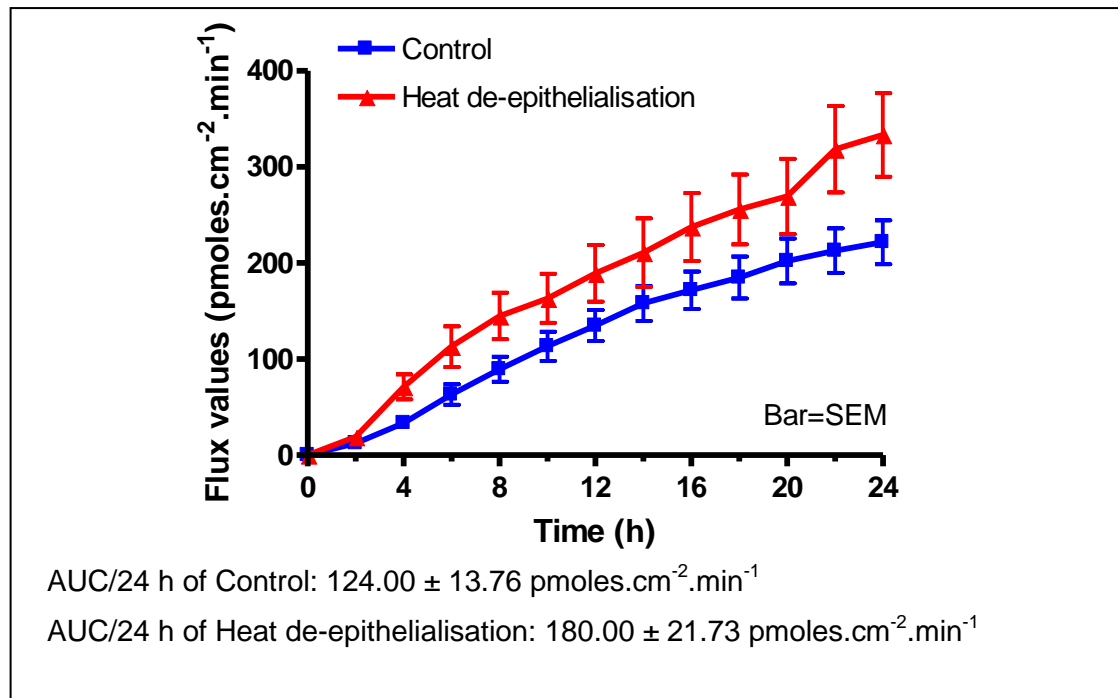


Fig. 19. Overall mean flux values at 37 °C of MDY-19 versus time across intact vaginal mucosa and de-epithelialised mucosa by means of heat

Bonferroni test; variable DV_1 (MDY-19)													
Error: Within MS = 4049.0, df = 451.00													
Cell No.	Time (h)	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}
		12.64	33.445	63.248	89.405	113.37	134.98	158.13	171.69	184.97	202.16	212.97	221.68
1	2		1	0.01973	4E-06	0	0	0	0	0	0	0	0
2	4	1		1	0.00432	1E-06	0	0	0	0	0	0	0
3	6	0.01973	1		1	0.02252	2.4E-05	0	0	0	0	0	0
4	8	4E-06	0.00432	1		1	0.07331	6.9E-05	0	0	0	0	0
5	10	0	1E-06	0.02252	1		1	0.08957	0.00212	2.5E-05	0	0	0
6	12	0	0	2.4E-05	0.07331	1		1	0.55911	0.02329	0.00012	2E-06	0
7	14	0	0	0	6.9E-05	0.08957	1		1	1	0.10725	0.00599	0.0004
8	16	0	0	0	0	0.00212	0.55911	1		1	1	0.20518	0.02336
9	18	0	0	0	0	2.5E-05	0.02329	1	1		1	1	0.56037
10	20	0	0	0	0	0	0.00012	0.10725	1	1		1	1
11	22	0	0	0	0	0	2E-06	0.00599	0.20518	1	1		1
12	24	0	0	0	0	0	0	0.0004	0.02336	0.56037	1	1	

Bonferroni test; variable DV_1 (Heat de-epithelialisation)													
Error: Within MS = 9739.3, df = 198.00													
Cell No.	Time (h)	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}
		19.183	71.227	113.08	144.96	163.21	189.29	210.83	237.56	255.89	269.39	318.53	333.37
1	2		1	0.24809	0.0078	0.00077	1.9E-05	1E-06	0	0	0	0	0
2	4	1		1	1	0.29783	0.01932	0.00138	3.3E-05	2E-06	0	0	0
3	6	0.24809	1		1	1	1	0.17003	0.00911	0.0009	0.00014	0	0
4	8	0.0078	1	1		1	1	1	0.28084	0.04297	0.00916	1.1E-05	1E-06
5	10	0.00077	0.29783	1	1		1	1	1	0.27864	0.07162	0.00016	1.9E-05
6	12	1.9E-05	0.01932	1	1	1		1	1	1	0.86887	0.00512	0.00076
7	14	1E-06	0.00138	0.17003	1	1	1		1	1	1	0.06098	0.01147
8	16	0	3.3E-05	0.00911	0.28084	1	1	1		1	1	0.80701	0.20591
9	18	0	2E-06	0.0009	0.04297	0.27864	1	1	1		1	1	1
10	20	0	0	0.00014	0.00916	0.07162	0.86887	1	1	1		1	1
11	22	0	0	0	1.1E-05	0.00016	0.00512	0.06098	0.80701	1	1		1
12	24	0	0	0	1E-06	1.9E-05	0.00076	0.01147	0.20591	1	1	1	

Fig. 19a. MDY-19 across intact vaginal mucosa and de-epithelialised mucosa by means of heat (heat de-epithelialisation) reached steady state respectively after 18 and 16 h

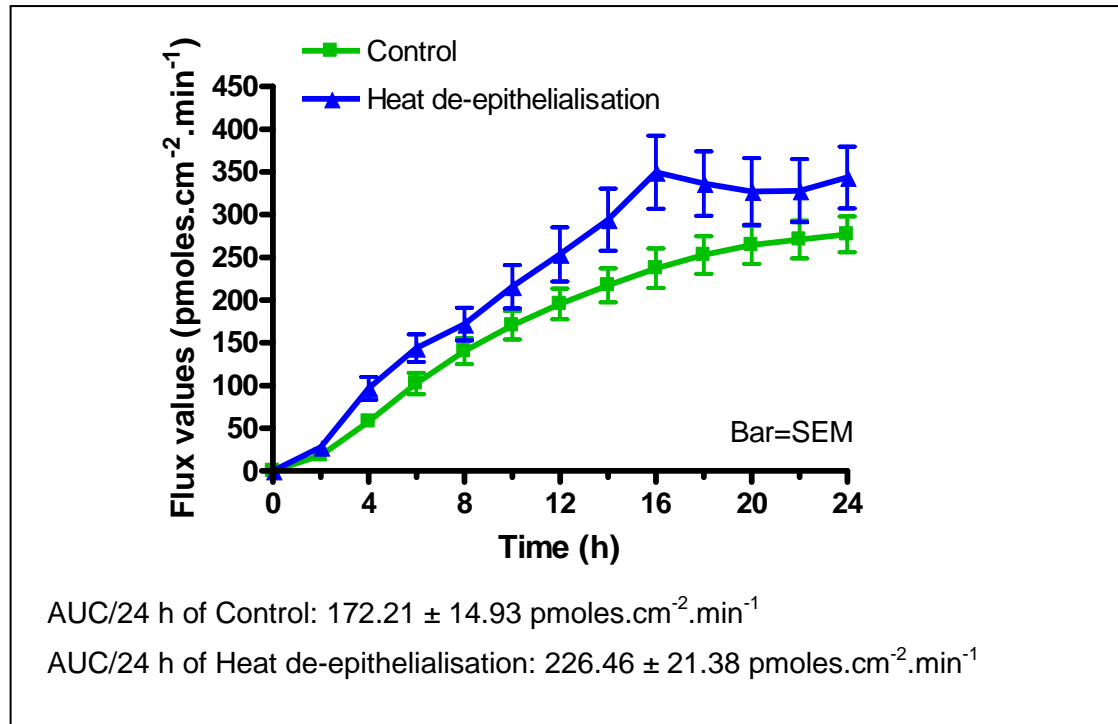


Fig. 20. Overall mean flux values at 37 °C of PCI across intact vaginal mucosa and de-epithelialised mucosa by means of heat.

Bonferroni test; variable DV_1 (PCI)													
Error: Within MS = 3856.5, df = 484.00													
Cell No.	Time (h)	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}
		18.079	57.992	102.5	140.33	170.62	195.62	217.31	237.37	252.78	264.53	270.95	277.05
1	2		0.16002	0	0	0	0	0	0	0	0	0	0
2	4	0.16002		0.04821	0	0	0	0	0	0	0	0	0
3	6	0	0.04821		0.26571	1.9E-05	0	0	0	0	0	0	0
4	8	0	0	0.26571		1	0.00191	1E-06	0	0	0	0	0
5	10	0	0	1.9E-05	1		1	0.02629	3.2E-05	0	0	0	0
6	12	0	0	0	0.00191	1		1	0.10019	0.00102	1.4E-05	1E-06	0
7	14	0	0	0	1E-06	0.02629	1		1	0.4608	0.02257	0.00323	0.00042
8	16	0	0	0	0	3.2E-05	0.10019	1		1	1	0.70052	0.16952
9	18	0	0	0	0	0	0.00102	0.4608	1		1	1	1
10	20	0	0	0	0	0	1.4E-05	0.02257	1	1		1	1
11	22	0	0	0	0	0	1E-06	0.00323	0.70052	1	1		1
12	24	0	0	0	0	0	0	0.00042	0.16952	1	1	1	

Bonferroni test; variable DV_1 (Heat de-epithelialisation)													
Error: Within MS = 12476., df = 286.00													
Cell No.	Time (h)	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}
		28.097	96.786	143.81	171.99	215.8	253.61	294.12	349.7	336.54	327.26	327.98	343.52
1	2		1	0.01138	0.00023	0	0	0	0	0	0	0	0
2	4	1		1	0.92003	0.00746	3.1E-05	0	0	0	0	0	0
3	6	0.01138	1		1	1	0.02369	8.6E-05	0	0	0	0	0
4	8	0.00023	0.92003	1		1	0.50696	0.00497	1E-06	9E-06	3.9E-05	3.5E-05	3E-06
5	10	0	0.00746	1	1		1	0.69174	0.00099	0.00596	0.01935	0.01771	0.00234
6	12	0	3.1E-05	0.02369	0.50696	1		1	0.11495	0.44651	1	0.99232	0.22167
7	14	0	0	8.6E-05	0.00497	0.69174	1		1	1	1	1	1
8	16	0	0	0	1E-06	0.00099	0.11495	1		1	1	1	1
9	18	0	0	0	9E-06	0.00596	0.44651	1	1		1	1	1
10	20	0	0	0	3.9E-05	0.01935	1	1	1	1		1	1
11	22	0	0	0	3.5E-05	0.01771	0.99232	1	1	1	1		1
12	24	0	0	0	3E-06	0.00234	0.22167	1	1	1	1	1	

Fig. 20a. PCI across intact vaginal mucosa and de-epithelialised mucosa by means of heat (heat de-epithelialisation) reached steady state respectively after 16 and 12 h

The mean flux values of MDY-19 and PCI compared at different concentrations and temperatures (20 °C and 37 °C) are shown in Figs. 21-24 and the AUC/24 h (average flux over 24 h) of these figures are shown as column bars in Fig. 25. The AUCs of the mean flux values of MDY-19 and PCI increased with an increase in concentration and temperature (20 °C to 37 °C). The times to steady state are shown in Figs. (21a,22a,23a,24a). The AUCs of the mean flux values of 1 mM MDY-19 and PCI at 37 °C were the highest.

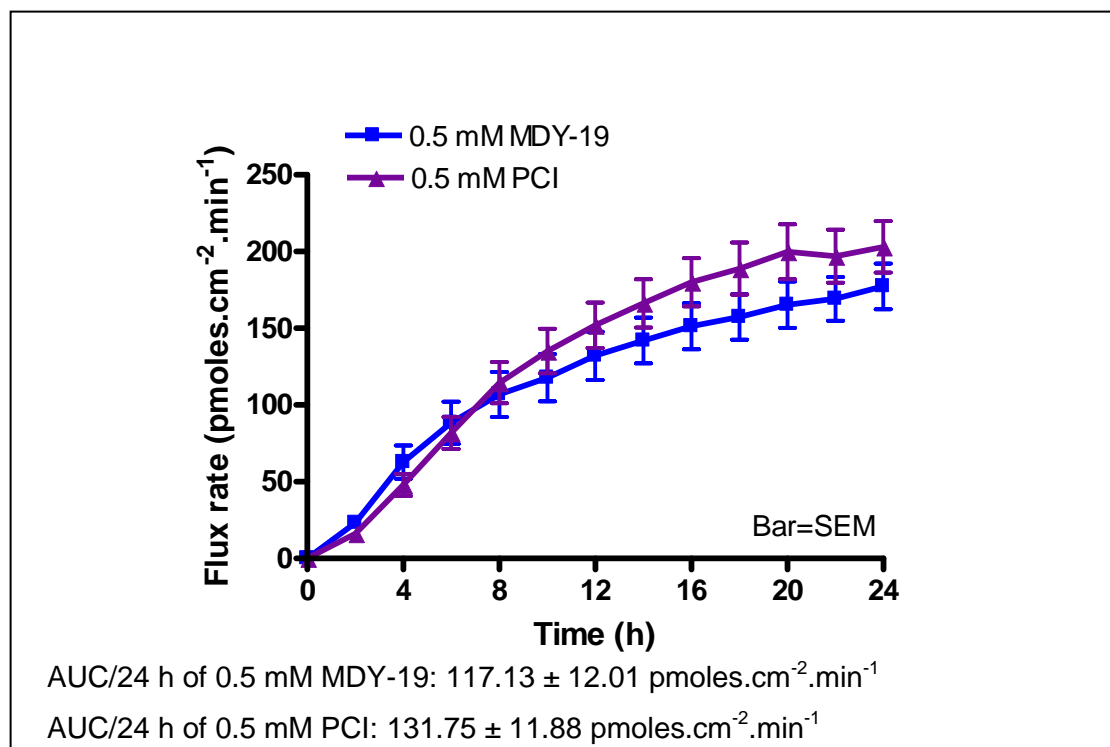


Fig. 21. Overall mean flux values at 37 °C of 0.5 mM MDY-19 and PCI across intact vaginal mucosa.

Bonferroni test; variable DV_1 (MDY-19)													
Error: Within MS = 2612.4, df = 495.00													
Cell No.	Time (h)	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}
		23.178	62.81	88.4724	106.898	117.802	132.116	142.065	151.324	157.435	165.407	169.182	177.348
1	2		0.00098	0	0	0	0	0	0	0	0	0	0
2	4	0.00098		0.31694	0.0001	0	0	0	0	0	0	0	0
3	6	0	0.31694		1	0.0849	0.00013	0	0	0	0	0	0
4	8	0	0.0001	1		1	0.36822	0.00776	8.4E-05	3E-06	0	0	0
5	10	0	0	0.0849	1		1	0.50442	0.01579	0.00098	1.5E-05	2E-06	0
6	12	0	0	0.00013	0.36822	1		1	1	0.35587	0.01742	0.0033	5.4E-05
7	14	0	0	0	0.00776	0.50442	1		1	1	0.67756	0.19119	0.00738
8	16	0	0	0	8.4E-05	0.01579	1	1		1	1	1	0.28012
9	18	0	0	0	3E-06	0.00098	0.35587	1	1		1	1	1
10	20	0	0	0	0	1.5E-05	0.01742	0.67756	1	1		1	1
11	22	0	0	0	0	2E-06	0.0033	0.19119	1	1	1		1
12	24	0	0	0	0	0	5.4E-05	0.00738	0.28012	1	1	1	

Bonferroni test; variable DV_1 (PCI)													
Error: Within MS = 3280.2, df = 484.00													
Cell No.	Time (h)	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}
		16.0809	47.9187	81.7305	114.521	135.149	151.956	166.277	180.002	188.937	199.825	196.906	203.12
1	2		0.13419	0	0	0	0	0	0	0	0	0	0
2	4	0.13419		0.06984	0	0	0	0	0	0	0	0	0
3	6	0	0.06984		0.09831	1.9E-05	0	0	0	0	0	0	0
4	8	0	0	0.09831		1	0.01937	4.3E-05	0	0	0	0	0
5	10	0	0	1.9E-05	1		1	0.16841	0.001	1.6E-05	0	0	0
6	12	0	0	0	0.01937	1		1	0.42969	0.02288	0.00027	0.00096	5.7E-05
7	14	0	0	0	4.3E-05	0.16841	1		1	1	0.07636	0.197	0.02406
8	16	0	0	0	0	0.001	0.42969	1		1	1	1	1
9	18	0	0	0	0	1.6E-05	0.02288	1	1		1	1	1
10	20	0	0	0	0	0	0.00027	0.07636	1	1		1	1
11	22	0	0	0	0	0	0.00096	0.197	1	1	1		1
12	24	0	0	0	0	0	5.7E-05	0.02406	1	1	1	1	

Fig. 21a. 0.5 mM MDY-19 and PCI reached steady state at 16 h

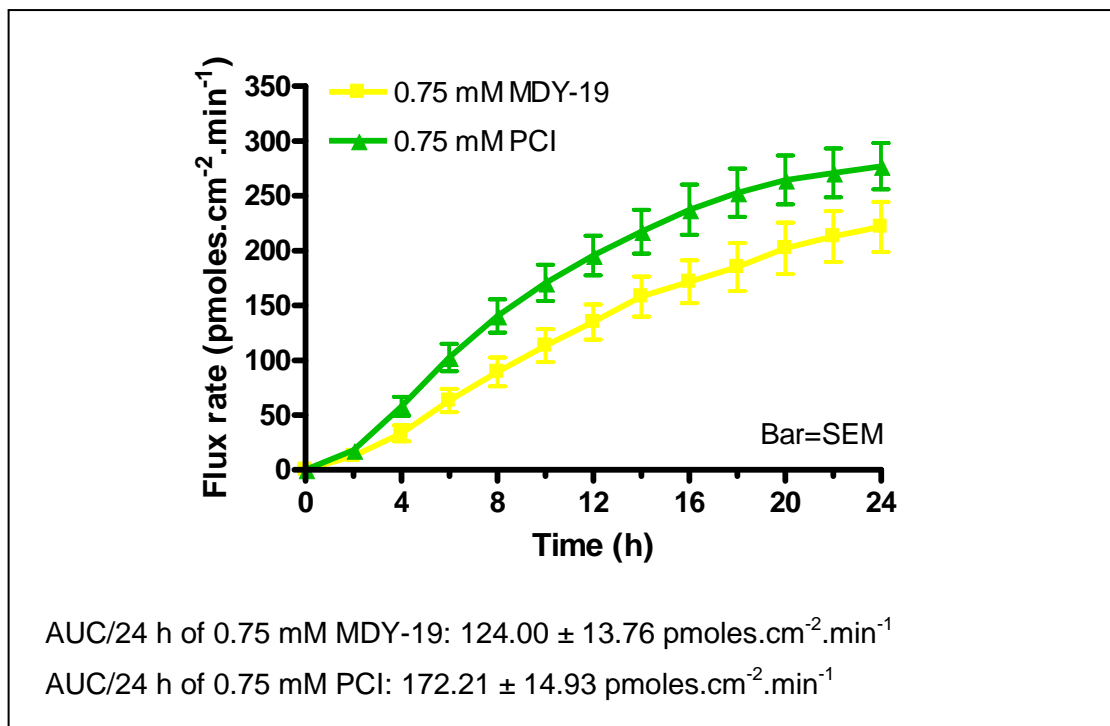


Fig. 22. Overall mean flux values at 37 °C of 0.75 mM MDY-19 and PCI across intact vaginal mucosa

Bonferroni test; variable DV_1 (MDY-19)													
Error: Within MS = 4049.0, df = 451.00													
Cell No.	Time (h)	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}
		12.64	33.445	63.248	89.405	113.37	134.98	158.13	171.69	184.97	202.16	212.97	221.68
1	2		1	0.01973	4E-06	0	0	0	0	0	0	0	0
2	4	1		1	0.00432	1E-06	0	0	0	0	0	0	0
3	6	0.01973	1		1	0.02252	2.4E-05	0	0	0	0	0	0
4	8	4E-06	0.00432	1		1	0.07331	6.9E-05	0	0	0	0	0
5	10	0	1E-06	0.02252	1		1	0.08957	0.00212	2.5E-05	0	0	0
6	12	0	0	2.4E-05	0.07331	1		1	0.55911	0.02329	0.00012	2E-06	0
7	14	0	0	0	6.9E-05	0.08957	1		1	1	0.10725	0.00599	0.0004
8	16	0	0	0	0	0.00212	0.55911	1		1	1	0.20518	0.02336
9	18	0	0	0	0	2.5E-05	0.02329	1	1		1	1	0.56037
10	20	0	0	0	0	0	0.00012	0.10725	1	1		1	1
11	22	0	0	0	0	0	2E-06	0.00599	0.20518	1	1		1
12	24	0	0	0	0	0	0	0.0004	0.02336	0.56037	1	1	

Bonferroni test; variable DV_1 (PCI)													
Error: Within MS = 3856.5, df = 484.00													
Cell No.	Time (h)	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}
		18.079	57.992	102.5	140.33	170.62	195.62	217.31	237.37	252.78	264.53	270.95	277.05
1	2		0.16002	0	0	0	0	0	0	0	0	0	0
2	4	0.16002		0.04821	0	0	0	0	0	0	0	0	0
3	6	0	0.04821		0.26571	1.9E-05	0	0	0	0	0	0	0
4	8	0	0	0.26571		1	0.00191	1E-06	0	0	0	0	0
5	10	0	0	1.9E-05	1		1	0.02629	3.2E-05	0	0	0	0
6	12	0	0	0	0.00191	1		1	0.10019	0.00102	1.4E-05	1E-06	0
7	14	0	0	0	1E-06	0.02629	1		1	0.4608	0.02257	0.00323	0.00042
8	16	0	0	0	0	3.2E-05	0.10019	1		1	1	0.70052	0.16952
9	18	0	0	0	0	0	0.00102	0.4608	1		1	1	1
10	20	0	0	0	0	0	1.4E-05	0.02257	1	1		1	1
11	22	0	0	0	0	0	1E-06	0.00323	0.70052	1	1		1
12	24	0	0	0	0	0	0	0.00042	0.16952	1	1	1	

Fig. 22a. 0.75 mM MDY-19 and PCI respectively reached steady state at 18 and 16 h

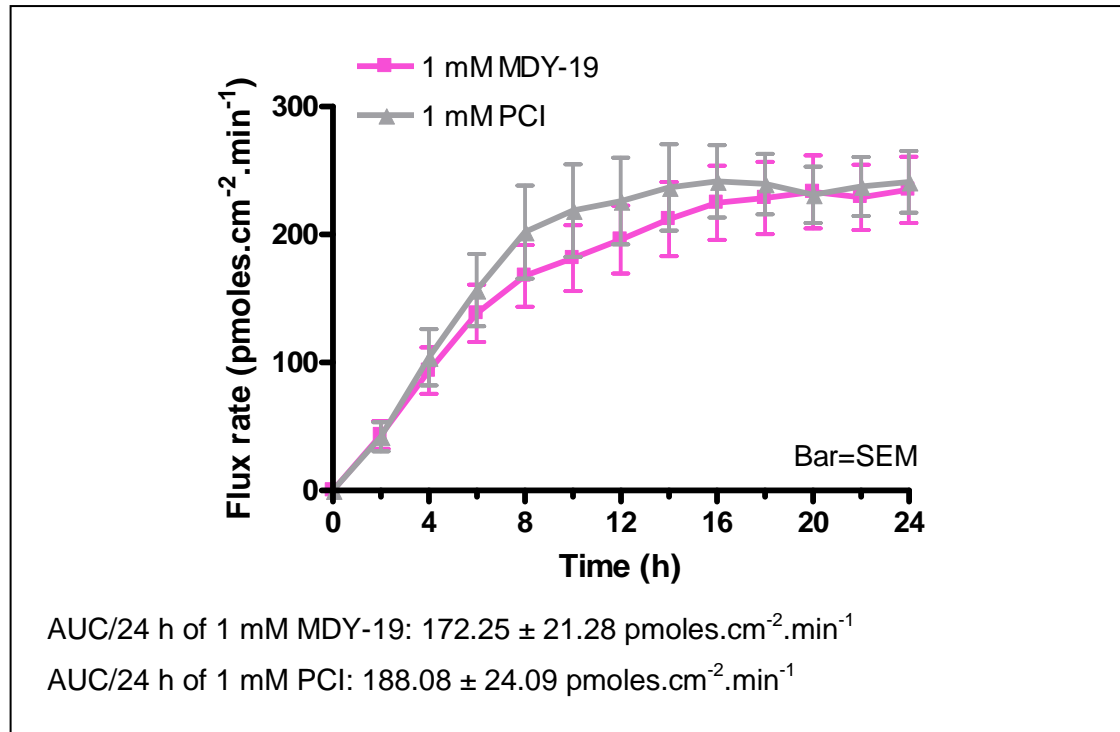


Fig. 23. Overall mean flux values at 37 °C of 1 mM MDY-19 and PCI across intact vaginal mucosa

Bonferroni test; variable DV_1 (MDY-19)													
Error: Within MS = 6777.5, df = 506.00													
Cell No.	Time (h)	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}
		43.471	93.692	138.49	167.87	181.55	196.11	212.16	224.85	228.59	233.34	229.13	235.1
1	2		0.21444	2E-06	0	0	0	0	0	0	0	0	0
2	4	0.21444		0.56804	0.001	2.2E-05	0	0	0	0	0	0	0
3	6	2E-06	0.56804		1	0.76054	0.04918	0.00114	3.4E-05	1.1E-05	3E-06	9E-06	1E-06
4	8	0	0.001	1		1	1	0.61908	0.05634	0.02528	0.00862	0.02247	0.00569
5	10	0	2.2E-05	0.76054	1		1	1	0.73109	0.38321	0.15906	0.34824	0.11287
6	12	0	0	0.04918	1	1		1	1	1	1	1	1
7	14	0	0	0.00114	0.61908	1	1		1	1	1	1	1
8	16	0	0	3.4E-05	0.05634	0.73109	1	1		1	1	1	1
9	18	0	0	1.1E-05	0.02528	0.38321	1	1	1		1	1	1
10	20	0	0	3E-06	0.00862	0.15906	1	1	1	1		1	1
11	22	0	0	9E-06	0.02247	0.34824	1	1	1	1	1		1
12	24	0	0	1E-06	0.00569	0.11287	1	1	1	1	1	1	

Bonferroni test; variable DV_1 (PCI)													
Error: Within MS = 6970.1, df = 462.00													
Cell No.	Time (h)	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}
		42.057	104.06	156.68	202.04	218.8	226.13	236.84	241.62	239.54	231.12	237.54	241.15
1	2		0.04132	0	0	0	0	0	0	0	0	0	0
2	4	0.04132		0.24056	6E-06	0	0	0	0	0	0	0	0
3	6	0	0.24056		0.7978	0.04035	0.00864	0.0007	0.00021	0.00036	0.00279	0.00059	0.00024
4	8	0	6E-06	0.7978		1	1	1	1	1	1	1	1
5	10	0	0	0.04035	1		1	1	1	1	1	1	1
6	12	0	0	0.00864	1	1		1	1	1	1	1	1
7	14	0	0	0.0007	1	1	1		1	1	1	1	1
8	16	0	0	0.00021	1	1	1	1		1	1	1	1
9	18	0	0	0.00036	1	1	1	1	1		1	1	1
10	20	0	0	0.00279	1	1	1	1	1	1		1	1
11	22	0	0	0.00059	1	1	1	1	1	1	1		1
12	24	0	0	0.00024	1	1	1	1	1	1	1	1	

Fig. 23a. 1 mM MDY-19 and PCI respectively reached steady state at 10 and 8 h

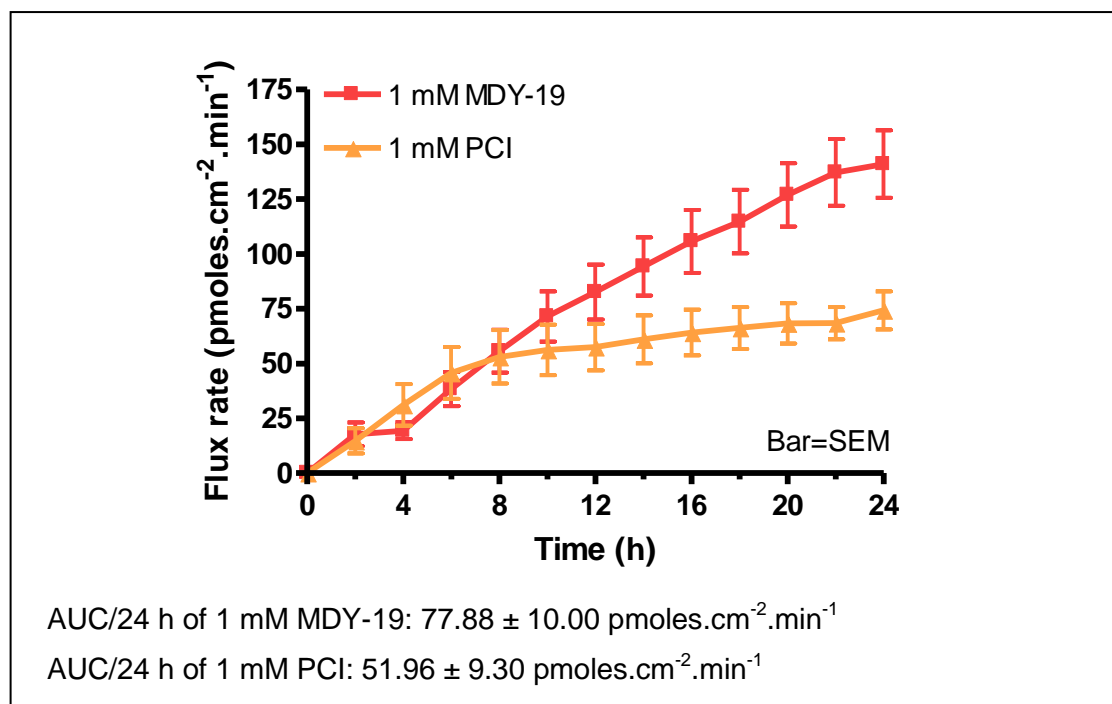


Fig. 24. Overall mean flux values at 20 °C of 1 mM MDY-19 and PCI across intact vaginal mucosa

Bonferroni test; variable DV_1 (MDY-19)													
Error: Within MS = 1603.7, df = 462.00													
Cell No.	Time (h)	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}
		17.665	19.382	38.347	55.635	71.481	82.669	94.307	105.77	114.74	126.98	137.21	140.96
1	2		1	1	0.0009	0	0	0	0	0	0	0	0
2	4	1		1	0.00214	0	0	0	0	0	0	0	0
3	6	1	1		1	0.00938	2.8E-05	0	0	0	0	0	0
4	8	0.0009	0.00214	1		1	0.12261	0.00063	1E-06	0	0	0	0
5	10	0	0	0.00938	1		1	0.56073	0.00549	5.1E-05	0	0	0
6	12	0	0	2.8E-05	0.12261	1		1	0.51068	0.01514	2.8E-05	0	0
7	14	0	0	0	0.00063	0.56073	1		1	1	0.01159	6.3E-05	7E-06
8	16	0	0	0	1E-06	0.00549	0.51068	1		1	0.9541	0.02003	0.00358
9	18	0	0	0	0	5.1E-05	0.01514	1	1		1	0.63243	0.167
10	20	0	0	0	0	0	2.8E-05	0.01159	0.9541	1		1	1
11	22	0	0	0	0	0	0	6.3E-05	0.02003	0.63243	1		1
12	24	0	0	0	0	0	0	7E-06	0.00358	0.167	1	1	

Bonferroni test; variable DV_1 (PCI)													
Error: Within MS = 282.54, df = 462.00													
Cell No.	Time (h)	{1}	{2}	{3}	{4}	{5}	{6}	{7}	{8}	{9}	{10}	{11}	{12}
		14.711	31.079	45.767	53.074	56.219	57.504	61.076	64.152	66.266	68.304	68.437	74.249
1	2		0.00053	0	0	0	0	0	0	0	0	0	0
2	4	0.00053		0.00394	0	0	0	0	0	0	0	0	0
3	6	0	0.00394		1	0.27198	0.0853	0.00192	3.8E-05	2E-06	0	0	0
4	8	0	0	1		1	1	1	0.15663	0.0201	0.0021	0.0018	1E-06
5	10	0	0	0.27198	1		1	1	1	0.38326	0.06113	0.05372	6.1E-05
6	12	0	0	0.0853	1	1		1	1	1	0.20076	0.17844	0.00033
7	14	0	0	0.00192	1	1	1		1	1	1	1	0.02048
8	16	0	0	3.8E-05	0.15663	1	1	1	1	1	1	1	0.36738
9	18	0	0	2E-06	0.0201	0.38326	1	1	1		1	1	1
10	20	0	0	0	0.0021	0.06113	0.20076	1	1	1		1	1
11	22	0	0	0	0.0018	0.05372	0.17844	1	1	1	1		1
12	24	0	0	0	1E-06	6.1E-05	0.00033	0.02048	0.36738	1	1	1	

Fig. 24a. 1 mM MDY-19 and PCI respectively reached steady state at 18 and 16 h

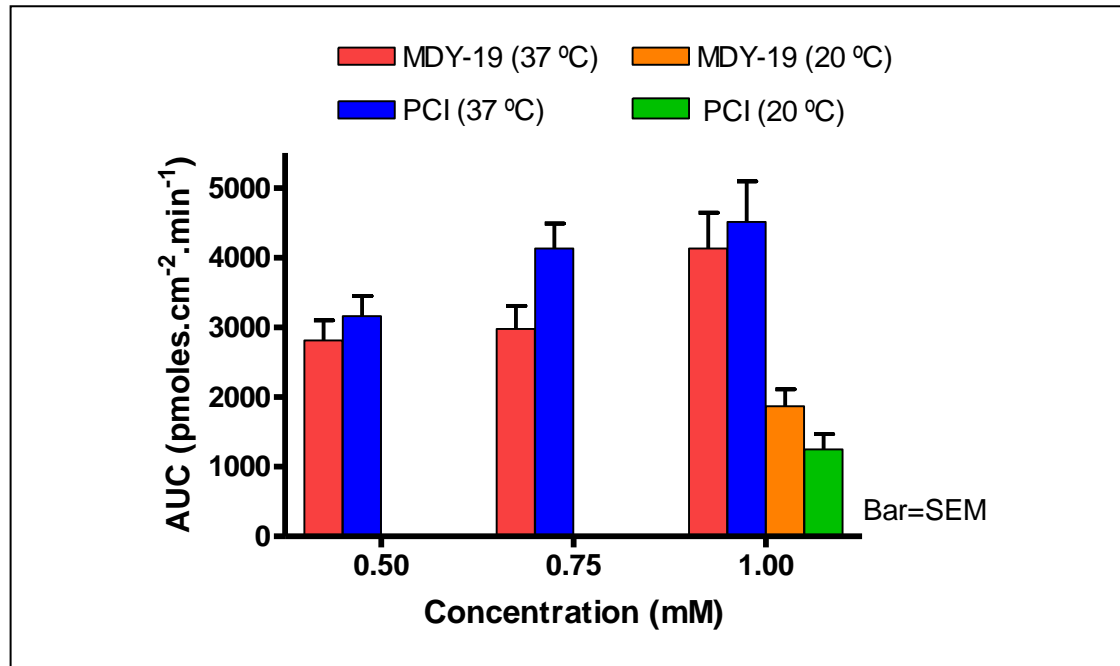


Fig. 25. The mean AUC/24h of MDY-19 and PCI at different concentrations, over a 24 h period

The normalised, mean flux values of 0.5, 0.75 and 1 mM MDY-19 and PCI over the 24 h period, at 37 °C are shown in Fig. 25 and 26.

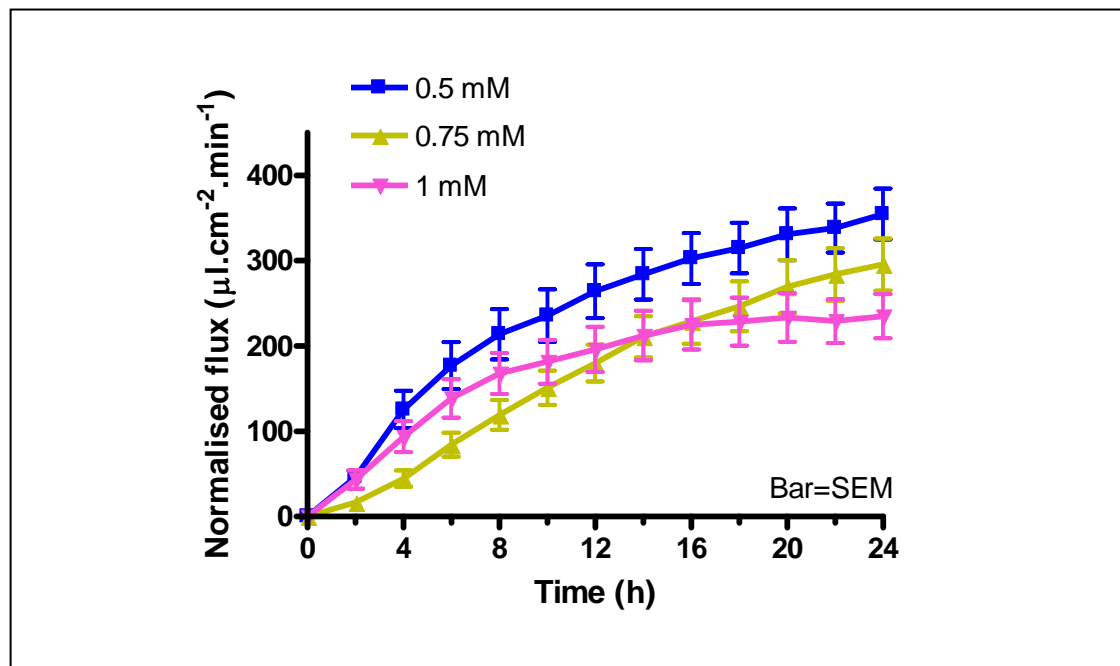


Fig. 26. The normalised flux values of 0.5, 0.75 and 1 mM MDY-19 (37 °C)

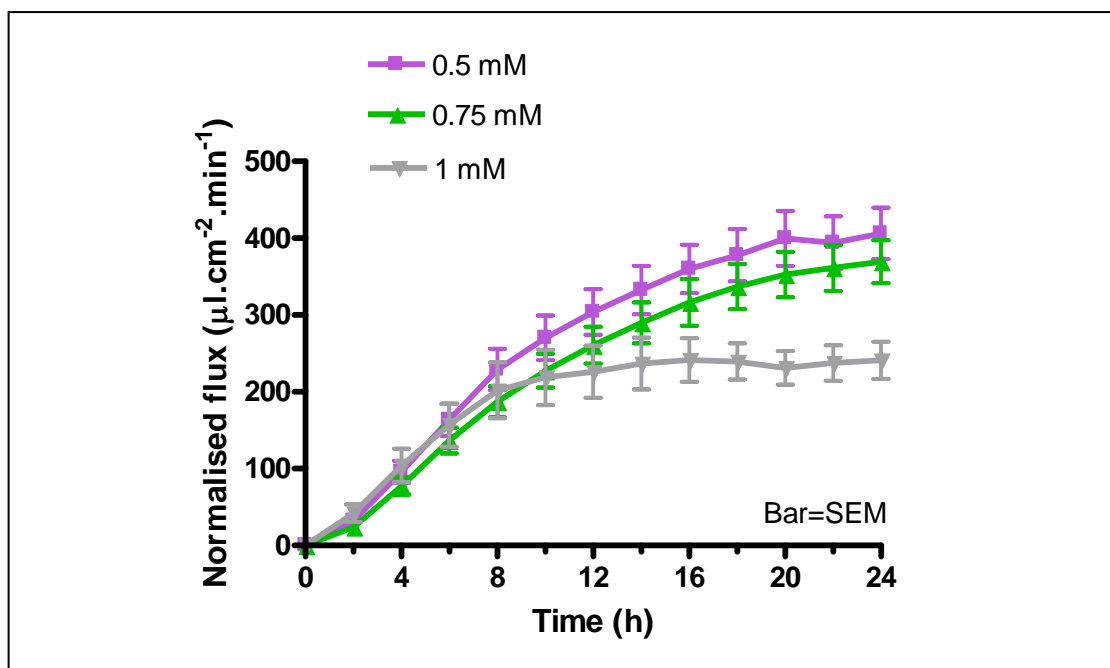


Fig. 27. The normalised flux values of 0.5, 0.75 and 1 mM PCI (37 °C)

The intra-experiment variation for the peptides was 43.4 % the inter-experiment variation was 43.1 %.

2.5 Discussion

HIV is spreading rapidly especially in sub-Saharan Africa and Southeast-Asia. The usage of condoms and the teaching of altered sexual behaviour, however, seem to be ineffective and therefore new prophylactic strategies are being investigated. The use of microbicidal vaginal formulations, which have the advantage that women can take control of their own safety, are being investigated (Garg *et al.*, 2001). Cationic peptides have activity against malaria parasites, and viruses (including HIV, HSV, influenza A virus and vesicular stomatitis virus). These properties make them promising candidates as new therapeutic agents (Powers and Hancock, 2003). Where antibiotics only have activity against bacteria, cationic peptides have a wide range of activities against bacteria, fungi, enveloped viruses and eukaryotic parasites. The pharmacological applications of peptides to treat infections are therefore receiving much interest, because they have an enormous attraction as potential therapeutic agents (Hancock, 2001).

Even though aging and estrogen deficiencies may influence vaginal-cervical epithelial paracellular permeability, previous studies have shown that postmenopausal vaginal mucosa can successfully be utilised as an *in vitro* model to predict *in vivo* permeation (Viljoen *et al.*, 2005; Gorodeski, 2001).

MEA-5, MDY-19 and PCI readily penetrated vaginal mucosa. MDY-19 had a significantly ($p < 0.05$) higher flux rate than MEA-5 (Fig. 16), commensurate with its smaller molecular size (weight). This may also possibly be explained by precipitation of MEA-5 at 20 °C. Further test could not be conducted with MEA-5, since EMPRO stopped the production of this compound.

Novel microbicides may be coupled to the transport peptide MDY-19 to be transported transmucosally or into cells. Although the increased size of a MDY-19/microbicide complex may decrease mucosal permeability, this could possibly be offset by the addition of an appropriate surfactant-containing permeation enhancer. Even though the peptides readily penetrated the vaginal mucosa, the surfactant used in this study further enhanced the AUC of MDY-19 by approximately 1.3 times (Fig. 17).

De-epithelialisation mimics the situation when integrity of vaginal epithelium is breached due to disease or local trauma. De-epithelialisation of the vaginal mucosa significantly

($p < 0.05$) increased the permeability of all three peptides (MEA-5, MDY-19 and PCI) over their controls. This supports the premise that the main barrier is located in the epithelium and it may have possible implications for increased *in vivo* uptake of microbicides as well as HIV by traumatised vaginal mucosa. It is in keeping with the finding that other sexually transmitted diseases e.g. genital herpes, chlamydia infection, trichomoniasis (ulcerative and non-ulcerative) are known to cause disruption of the vaginal epithelium and can increase HIV transmission 3- to 10-fold (Fleming and Wasserheit, 1999; Stratton and Alexander, 1993). Initially two methods e.g. mechanical and heat de-epithelialisation were used to establish the most effective and convenient method to remove epithelium from the vaginal mucosa. The MEA-5 peptide was used to evaluate these two de-epithelialisation methods. Although it was not more effective, overall de-epithelialisation by heat appeared to be a more convenient method. It was therefore used for mucosal de-epithelialisation of the peptides MDY-19 and PCI. Interestingly, 1 mM MEA-5 (at 20 °C) did not reach a steady state (prolonged the lag time) when the vaginal tissue was de-epithelialised by means of mechanical and heat stripping, but heat de-epithelialisation decreased the lag time of 0.75 mM MDY-19 and PCI (37 °C).

Precipitation of the peptides MDY-19 and PCI on the vaginal mucosa during the permeability experiments at room temperature (20°C) and at a concentration of 1 mM was noticed. For this reason, permeability studies of MDY-19 and PCI were repeated at concentrations of 1 mM, 0.75 mM and 0.5 mM in PBS buffer, at a temperature of 37 °C.

The permeability of 1 mM MDY-19 and PCI at 37 °C were significantly ($p < 0.05$) higher than at 20 °C. At 37 °C the AUCs of the overall mean flux values of MDY-19 and PCI increased with concentration according to well-established diffusion theory. At room temperature, the AUCs of 1 mM MDY-19 is 1.5x higher than 1 mM PCI and at 37 °C the AUC of 1 mM PCI is 1.1x higher than the corresponding value found for 1 mM MDY-19. This may be explained as follows. PCI has a less overall positive charge than MDY-19 since it contains less of the basic acid, arginine. At high concentrations (1 mM) and lower temperatures precipitation of PCI may occur, since it is less water-soluble than MDY-19 and therefore the flux values of the PCI across vaginal tissue were decreased. The solubility of PCI in water however increased with an increase in temperature (20-37 °C), leading to less precipitation on the vaginal tissue, which in turn yields in higher flux.

The foregoing combined with the fact that PCI are more lipid soluble than MDY-19 may explain why at 37 °C, 1 mM PCI's permeability is higher than MDY-19. The normalised, mean flux values of MDY-19 and PCI (Figs. 26 and 27) varied with concentration and may possibly be explained by the precipitation of the two peptides at higher concentrations, especially at 1 mM. However, these findings may also indicate that the flow-through diffusion system's ability to predict permeability of test compounds is influenced by the concentration and the solubility of the test compound. Further studies are therefore necessary to determine the exact influence of different concentrations of the test compound on the flow-through diffusion system's ability to predict permeability.

Chapter 3: Skin tissue

3.1 Summary

The objectives of the skin tissue study were to determine the permeability of different terbinafine hydrochloride formulations through human skin and to measure the respective concentrations of each formulation within the skin tissue. A further objective of the study was to investigate the effects of increased temperature on *in vitro* permeability of tritiated water across human skin.

Relatively little information is available in the literature about the concentration of terbinafine in tissues. A possible reason could be due to a lack of availability of an analytical method (Yeganeh and McLachlan, 2000). Since the skin is the main target for topical antifungal drug treatment, terbinafine's clinical efficacy can be determined by evaluating the concentration of the drug at its target site. The permeation of three commercial available 1 % (10 mg/ml) terbinafine hydrochloride formulations and three terbinafine hydrochloride solutions of 10, 20 and 30 mg/ml through human skin was determined using our *in vitro* flow-through diffusion system. The terbinafine retained in the exposed skin was extracted and analysed. The terbinafine from the different formulations readily diffused into the skin tissue. However, no flux values for any of the terbinafine hydrochloride formulations through the skin into the acceptor fluid were found. The mean terbinafine concentrations in the skin after 24 h exposure to the three commercial available formulations were 3.589, 1.590 and 4.219 µg/ml respectively. The mean terbinafine concentration in the skin exposed to the 10 mg/ml PBS/Methanol solution was higher than those from the three commercial formulations. Terbinafine seems to accumulate in skin, rather than to diffuse through the skin into the acceptor compartment. These unique pharmacokinetic properties of terbinafine may enhance its efficacy as topical antifungal and reduce systemic side effects. The *in vitro* model used in the present study may possibly be expanded into a model with the potential to assess bioequivalence of different formulations.

There has been relatively little investigation of the effect of temperature on skin permeation compared to other methods of penetration enhancement. Increasing the temperature of the skin and its environment may well provide the potential for overcoming the barrier properties of the stratum corneum and this prompted us to

investigate the effects of increased temperature on *in vitro* permeability of the chemical marker tritiated water across human skin. The results of this temperature study demonstrated the temperature-dependency of water flux rates across skin tissue. An increase of 5 °C caused a significant increase in flux values of tritiated water across skin. The average flux for tritiated water across skin at 37 °C was on average double those at a temperature of 32 °C. This *in vitro* temperature model may therefore be useful for predicting flux rates at higher temperatures and to investigate temperature as a potential enhancer of transdermal delivery.

3.2 Introduction

3.2.1 Topical administration of drugs

Percutaneous administration of drugs offers many advantages compared to traditional drug delivery methods e.g. oral delivery and injections. Firstly, compared to oral delivery, it avoids gastrointestinal drug metabolism (since it circumvents the hepatic first-pass effect), reduces elimination by the liver, provides less chance of an over- or underdose, allows easy termination (e.g. removing the drug from the skin) and permits both local and systemic treatment of conditions. Secondly, the pain, fear and the possibility of infections associated with injections, often results in low patient compliance. Transdermal drug transport seems to be an answer to some of these problems. The research on methods to increase absorption of drugs transcutaneously constitutes a huge area of pharmaceutical research.

Although topical drugs are usually applied at a convenient site, the target for the drug action may be local (e.g. cortisone creams, analgesics) or systemic (e.g. scopolamine to prevent motion sickness). Regardless of the target, all transcutaneous drugs that penetrate the skin and enter the vascular system will have some systemic effects.

For a drug to be a successful topical agent, it has to have efficacy at the target site and also have the ability to reach that site. This may involve delivery *via* the systemic circulation and direct penetration. The topical agent consists of a vehicle or base, which often contains an active ingredient. Possible vehicles include: lotions, creams, gels, patches, ointments and pastes. The clinical effect is achieved through direct transport to the tissue or by systemic absorption and redistribution (Vaile and Davis, 1998). The clinical efficacy of the formulation therefore depends on the ability of the vehicle to release the drug which then must penetrate the stratum corneum. The diffusion and partitioning processes are a succession of steps and the slowest event will determine the rate of local drug availability (Higuchi, 1960).

One of the functions of the skin is to act as a diffusional barrier. The skin has metabolic activity and some of the topically administered drugs are metabolised during skin diffusion. The metabolic activity of the skin should therefore be kept in mind when developing transdermal drug formulations (Martin *et al.*, 1987).

Various sources are used for obtaining human skin, including cosmetic surgery and amputations. Skin from a wide variety of animal species is used for *in vitro* and *in vivo* studies e.g. pigs, rats, guinea pigs, snakes and rabbits. Although animal and synthetic skin models provide a good indication of the diffusion characteristics of chemicals, it does not necessarily reproduce the complex nature of the stratum corneum of human skin (Katritzky *et al.*, 2006).

Skin penetration studies play an important role in the selection of drugs for dermal or transdermal application. Therefore, the choice of predictive *in vitro* penetration models is very important and the use of especially human skin to evaluate penetration properties of candidate drugs can be a useful tool (Schmook *et al.*, 2001).

3.2.2 Skin

The skin is an extensive organ covering the exterior of the body and varies in thickness, colour and the presence of hairs, glands and nails. It regulates heat and water loss from the body and protects the body from the penetration of harmful chemicals or microorganisms, including agents applied to the skin. The human skin also plays a role in the absorption and blockage of radiation, temperature regulation, sensory perception and immunological surveillance. It therefore helps us to withstand a considerable range of environmental challenges. It has a surface area of approximately 2 m² and receives about a third of the body's blood circulation (MacKie, 1987). Since it is the biggest organ (that of an average adult male weighs 4.5 to 5 kg) and easily accessible, it offers great opportunities for the administration of therapeutic compounds (Williams, 2003).

Skin (*in vivo*) is metabolically active and in a state of continual regeneration. It has immunological and histological responses to different chemicals. Most transdermal drug delivery studies tend to use *in vitro* skin, due to ethical and experimental difficulties. It should be kept in mind that skin from *in vitro* studies serves only as a model and data obtained cannot always be translated directly to the *in vivo*-situation, since some of the abovementioned active processes are lost in these studies.

The skin is composed of three layers: an outer layer of epithelial cells called the epidermis, middle layer of connective tissue called the dermis, and a variable inner layer called the hypodermis (Figs. 28 and 29).

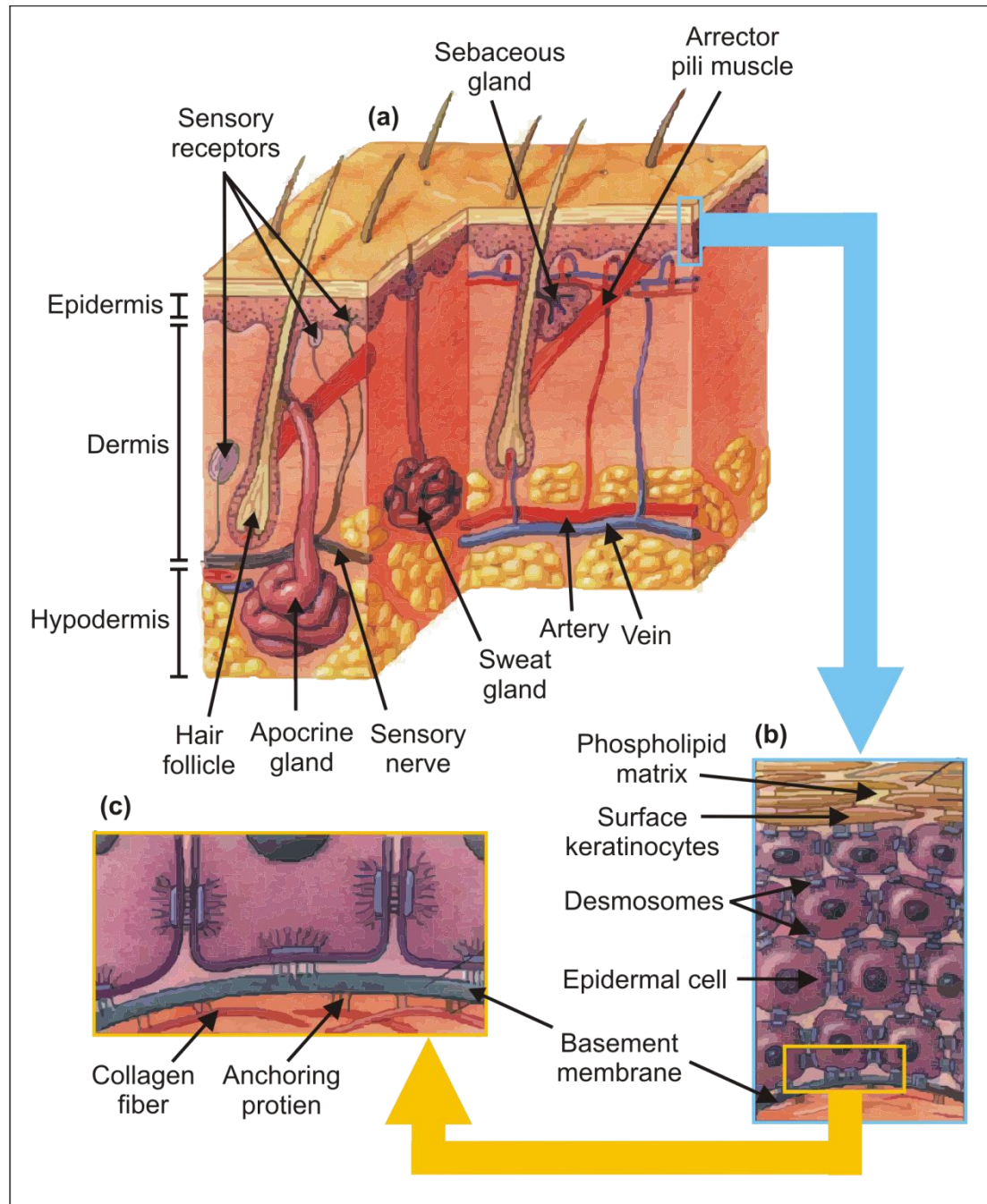


Fig. 28. (Silverthorn, 1998)

(a) The different layers of the skin

(b) Epidermis

(c) Basement membrane and the connection between epidermis and dermis

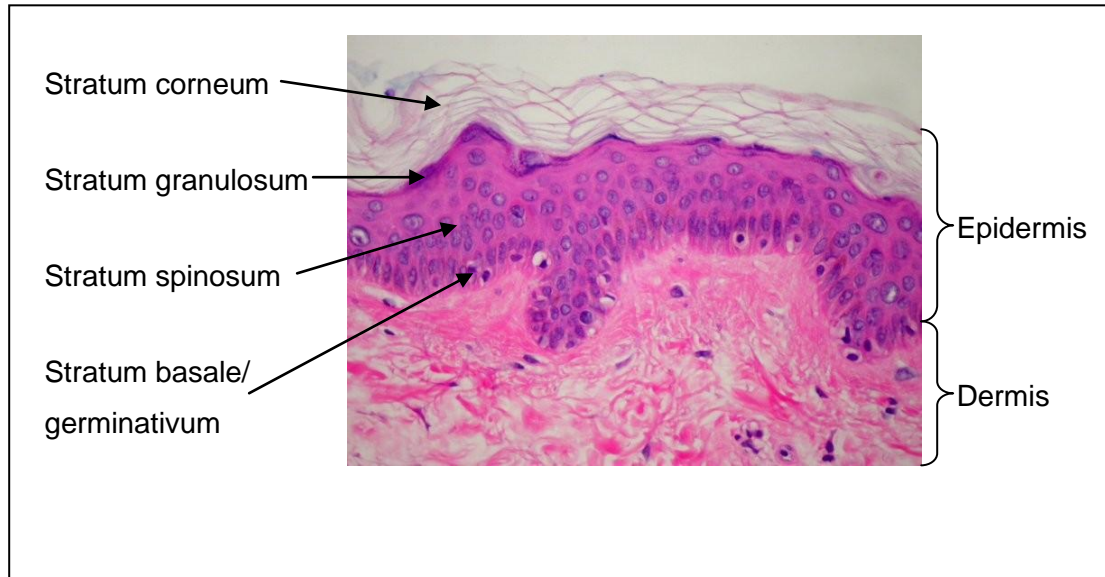


Fig. 29. Histology of human skin (By courtesy of Prof. IOC Thompson)

3.2.2.1 Epidermis

The epidermis, the outermost part of the skin, is a continually renewing, stratified, squamous epithelium. It varies in thickness from around 0.06 mm on the eyelids to around 0.8 mm on the load-bearing areas of the skin. Marcello Malpighi (1628-1694), who believed that it was a gelatinous membrane, first described the epidermis and he divided it into an inner layer of viable cells (now known as the stratum malphigii) and an outer layer of anucleated keratinised cells (stratum corneum).

The epidermis consists of four histologically distinct layers, which represent the stages of maturation of keratin by keratinocytes: the stratum basale or germinativum, stratum spinosum, stratum granulosum and the outermost layer the stratum corneum (Fig. 29). Some literatures describe a fifth layer, the stratum lucidum, but this layer is usually considered the lower layer of the stratum corneum (Williams, 2003).

It does not contain any blood vessels and therefore molecules have to permeate to the dermis to be cleared or taken up by the systemic circulation. The main cell of the epidermis is the keratinocyte, which produces the protein keratin. Keratins are high-molecular-weight polypeptide chains and are the major constituent of the stratum corneum. The stratum corneum comprises keratin (65 %), along with soluble proteins

(10 %), amino acids (10 %), lipids (10 %) and cell membranes (5 %). The keratinocytes are tied to each other by an extensive system of desmosomes (Fig. 28b). As these cells mature, they synthesize numerous keratin fibres and secrete a hydrophobic phospholipid matrix that acts as the skin's main waterproofing agent. By the time the older keratinocytes are pushed to the surface of the epidermis by newer cells, their cytoplasm is thick with keratin fibres. At this point the cells die and their nuclei and organelles disappear. The mats of keratin fibres that remain behind are still linked to each other by the protein fibres of the desmosomes. Dispersed between the keratinocytes are the melanocytes, the cells that produce melanin, and the cells of Langerhans, which have a defensive function (Williams, 2003).

The stratum corneum seems to be the main permeability barrier and provides an incredibly effective barrier to penetration, even though it is merely 15-20 μm thick (Hadgraft, 2001b). It comprises densely packed disc-like keratinocytes, which are anucleate keratinised cells, and separated by multi-cellular lipid bilayers which function as cement (Joshi and Jaideep, 2002). These lipid bilayers consist of regions of ceramides, fatty acids, cholesterol, cholesterol esters and triglycerides and form regions of semi crystalline gel and liquid crystal domains. The lipids form long lamellae parallel to the corneocyte surfaces. Inside the lamellae, at physiological temperatures, the lipids are arranged in bilayers consisting of ordered, crystalline phases on both sides of a narrow, central band of fluid lipids. The stratum corneum resembles a 'brick and mortar' structure, analogous to a wall, and this highly ordered construction makes the stratum corneum impermeable to many drugs. It forms the rate-controlling barrier for diffusion for almost all compounds (Barry, 2001).

Most of the molecules penetrate through skin *via* the intercellular micro route and many of the transdermal enhancement techniques are aimed at disrupting this layer's molecular architecture. This layer is also lipophilic and unionised drugs penetrate the stratum corneum more readily, while the rest of the epidermis is more hydrophilic (Singh and Roberts, 1994). A drug that exhibits both hydrophilic and lipophilic characteristics will therefore have optimal penetration.

The epidermis does not contain any blood vessels or lymph nodes, but does contain a large number of nerve endings. Between the epidermis and the underlying layer of

connective tissue is an acellular basement membrane. The cells of the epidermis and fibres of the dermis are anchored to each other by protein fibres that run throughout the basement membrane (Fig. 28c).

3.2.2.2 Dermis

The dermis is the layer below the epidermis. It is typically 3-5 mm thick and composed of a network of connective tissue, which consists primarily of collagen fibres embedded in a mucopolysaccharide gel (Wilkes *et al.*, 1973).

The dermis, in contrast with the epidermis, does contain blood vessels. The blood vessel supply plays an essential role in the regulation of body temperature whilst also delivering oxygen and nutrients to the tissue and removing toxins and waste products. The blood flow is responsible for removing molecules that have permeated the outer skin layers. This ensures a concentration gradient, and therefore a driving force, between the applied formulation on the skin surface and the vasculature. It also contains lymphatic vessels, nerve endings, pilosebaceous units (hair follicles and sebaceous glands) and sweat glands (eccrine and apocrine). The lymphatic flow seems to play a bigger role with the clearance of larger molecules (Williams, 2003). Hair follicles are present over the entire body surface, except over the load-bearing areas and on the lips. The sebaceous glands are found together with the hair follicles and secrete sebum that plays a role in maintaining the skin's pH at approximately 5 (Williams, 2003).

This layer is often viewed as consisting of essentially gelled water and therefore provides a minimal barrier to the delivery of most polar drugs. The dermal barrier properties are more pronounced when delivering lipophilic compounds (Williams, 2003).

3.2.2.3 Hypodermis

The hypodermis is the deepest layer of the skin. This subcutaneous fat layer forms the bridge between the overlying dermis and the underlying body organs. It serves as an insulator of the human body, protects it against physical shock and provides a reservoir of high-energy molecules. In most areas of the body, it is a few millimetres thick, but in some areas, e.g. the eyelids, it is absent. The hypodermis contains blood vessels and

nerves (Williams, 2003). Below the hypodermis is the subcutaneous tissue, which forms the internal boundary of the skin.

3.2.3 Processes of percutaneous absorption of drugs

Percutaneous absorption of a topically applied drug involves a sequence of individual transport processes. A drug molecule has three possible pathways through skin tissue: appendageal (through hair follicles with associated sebaceous glands or *via* sweat ducts), inter- and transcellularly (Byl, 1995).

The area available for diffusion through skin appendages is almost negligibly small (approximately 1 %) (Barry, 2001). This pathway may be important for ions and large polar molecules. If a drug follows the transcellular pathway, the drug crosses the skin by directly passing through the phospholipid membranes and the cytoplasm of the dead keratinocytes that constitute the stratum corneum. Since the drug must pass through several lipophilic membranes of the different cells (lipid bilayers) and the hydrophilic cytoplasm, only a few drugs have the chemical properties to penetrate the skin through this method. Drugs that follow the intercellular route must pass through the small spaces between the cells of the skin. The drugs have to follow a tortuous route between the cells and the diffusion path of most chemicals penetrating the skin is approximately 400 μm , although the thickness of the stratum corneum is only 15-20 μm . The intercellular spaces contain a mixture of ceramides, free acids (and their esters), cholesterol (and its sulfate). The lipids are arranged into structured bilayers and the reasons for the impermeability of the skin are due to the combined effect of the tortuous route that the compounds have to follow and the repeated partition and diffusion across these lipid bilayers (Hadgraft, 2001a; Flynn, 1996). Once diffusion through the stratum corneum has taken place, the molecules permeate the dermis and are absorbed by the capillary plexus and transported into the circulating bloodstream (Hadgraft and Lane, 2005). For many years it was thought that percutaneous drugs all entered the dermal capillary network and reached the central blood compartment only to return to the local area above which they were applied. However, it now appears that there is a system of local delivery separate from systemic delivery *via* the central blood compartment (Byl, 1995). As mentioned the molecule follows a tortuous route and has to cross, sequentially and repeatedly, a number of hydrophilic and lipophilic domains. The time necessary to reach

C_{\max} is approximately 10 times longer than that when the equivalent oral administration is given (Heyneman *et al.*, 2000).

The transmembrane diffusion process is passive in nature, requiring a concentration differential as the driving force and each molecule requires kinetic energy to effect a net movement down this gradient. The lipid-water partitioning characteristics of the permeant play an important role in its penetration. Drugs that are either extremely hydrophilic or extremely hydrophobic are poorly absorbed, since the skin is a heterogeneous membrane. Molecular size plays a role in penetration of the skin and so does the pH of the vehicle (Payne-James *et al.*, 1992). The pH of the skin is approximately 4-5 and seems to have a good buffer capacity. Formulations used on the skin should therefore preferably have a pH between 4 and 7 (Hadgraft, 2001b). Most drugs are weak acids or weak bases which dissociate to various degrees depending on the pH of the formulation used and the pH of the membrane through which it must diffuse. Other physiochemical properties of a drug include solubility/melting point and the state of ionisation, since the skin behaves as a lipophilic membrane. The governing factor for which pathway the permeant will follow when it diffuses through the tissue, is the partition coefficient. There is an inverse relationship between the permeant flux and its molecular weight. Smaller molecules diffuse through the skin tissue at a faster rate than larger molecules. Topical medication is usually applied in an aqueous formulation and the permeant from the formulation should thus be somewhat watersoluble (Williams, 2003; Heyneman *et al.*, 2000). Another significant factor that influence percutaneous diffusion is the number of functional groups on the drug that are able of hydrogen bonding. The diffusion coefficients of the permeant decreases as the number of hydrogen bonding groups increase until three groups are present. With a further increase of hydrogen bonding groups, there is little effect. It seems that this effect is related to the ability of the permeant to form a hydrogen bond with the polar head groups of the ceramides (Hadgraft, 2001b).

Permeability of the skin is also influenced by age, disease, the appearance of skin appendages (e.g. hair follicles, apocrine sweat glands, erector pili muscles, sebaceous glands), the nature of its physiochemical condition (e.g. thickness, lipid structure, membrane capacity), as well as the viscosity and the extent of cross-linking of the collagen (Wester and Maibach, 1991). Hydration of the stratum corneum plays a vital

role. The amount of water in the stratum corneum increases the permeability of this layer to hydrophilic drugs. It is possible for the stratum corneum to absorb three to five times its own weight in water, resulting in a two- to threefold enhancement in the permeability to water and other polar molecules (Byl, 1995).

Transdermal drug delivery is often chosen to bypass hepatic metabolism, but it must be kept in mind that after drug penetration through the stratum corneum, substances may be subjected to metabolism by the viable epidermis. Venkatesha *et al.* (1992) studied the cytochrome P450 (P450) content, the cytochrome reductase activity, the metabolism of a variety of P450 substrates and the presence and role of flavin-containing monooxygenase (FMO) in xenobiotic metabolism, in mouse skin, and compared it to those of liver. The cytochrome P450 content of skin was approximately 6.8 % of the liver P450 content. By comparison, cytochrome reductase activity in skin microsomes was high, being equivalent to approximately one-third of the liver microsomal enzyme activity. Skin microsomes metabolized several known P450 substrates and, depending upon the substrate used, the specific activity ranged from 2.5 to 13.4 % of the corresponding rates seen in liver microsomes. Metabolism of a chemical may result in significant modification of the molecule in terms of reduced or increased pharmacological and/or toxicological activity (Wester and Maibach, 1983). Examples of cutaneous metabolism of drugs include steroids (estradiol, progesterone, testosterone) and nitroglycerin. Usually drug metabolism by the skin is less compared to the oral administration (Rohatagi *et al.*, 1997; Zobrist *et al.*, 2001). Berner and John (1994) found regional variations in cutaneous metabolism which may cause differences in drug bioavailability. Scrotal application of a testosterone transdermal formulation resulted in an increased systemic dihydrotestosterone concentration compared to non-scrotal application (Berner and John, 1994). Some excipients (e.g. permeation enhancers) in formulations may affect skin metabolism. This can be explained through the inhibition or induction of enzymatic activity by these chemicals (Berner and John, 1994). Therefore, the formulation characteristics might be considered as a potential reason for inter- and intra-individual variations in metabolism and bioavailability of transdermally administered drugs. The foregoing must be kept in mind when conducting *in vitro* permeability studies.

3.2.4 Predicting skin permeation of compounds

Many efforts have been made to measure percutaneous absorption (Schnetz and Fartasch, 2002). The permeation rates of compounds can be measured by employing or using:

- *In vivo* drug release in live animals or human volunteers
 - Excised skin from human or animal sources in *in vitro* experiments
 - Synthetic model membranes as diffusion barriers in *in vitro* experiments
- (Cronin *et al.*, 1999)

Both human and animal skin *in vitro* models are used to predict percutaneous penetration in humans. Skin from a wide range of animals has been suggested as a suitable replacement for human skin. Pig and rat skin are most commonly employed (Katritzky *et al.*, 2006). Animal skin may provide a good indication of the diffusion characteristics of chemicals, but animal skin tends to be more permeable than human skin and it does not necessarily reproduce the complex nature of the human stratum corneum. Excised human skin is therefore regarded as the “gold standard” for *in vitro* penetration experiments (Barbero and Frasch, 2009). Previous workers have demonstrated that pig skin is a good *in vitro* model for human skin, since it had permeability properties closer to humans than rodents. Pig skin can only be stored for 2 months before the barrier properties begin to deteriorate. Frozen human skin retains its barrier properties for at least one year (Davies *et al.*, 2004). Human skin from various sources, including cosmetic surgery and amputations, has been used for the *in vitro* assessment of percutaneous penetration (Katritzky *et al.*, 2006).

Predicting skin permeation of compounds has advantages for the pharmaceutical, cosmetic and agrochemical fields, but the development of reliable mathematical models to predict the diffusion of drugs through skin remains a challenging objective. Numerous attempts have been made to predict skin permeability coefficients. Since experimental data for skin permeability are still relatively scarce, predictive models are usually based on the data set published by Flynn (1990) or subsets thereof. The prediction of partitioning and diffusivity of compounds remains a challenge due to the complexity of skin structure and variability in skin lipid composition and thickness. Changes in skin induced by solvents and permeants and the effects of metabolizing enzymes on permeants may also influence skin permeation of compounds.

Mechanistic and empirical modelings have been developed in an attempt to predict skin permeation of compounds (Yamashita and Hashida, 2003). Both approaches certainly have their merits and have reached a relatively high level of sophistication, but unfortunately the heterogeneity of the data in terms of skin origin and experimental conditions, contribute to the variance in existing models. Statistics demonstrate that some published models are based on nonsignificant parameters and this may diminish mechanistic insight and will lead to over-interpretation of the data (Geinoz *et al.*, 2004). Mechanistic and empirical approaches should not be exclusive, but compensative in nature. Ideally, empirical models should be used to compensate for uncertainties that mechanistic models overlook and yet-to-be-known relationships that mechanistic models cannot deal with (Yamashita and Hashida, 2003).

3.2.4.1 Mechanistic analysis of skin permeation of drugs

Mechanistic approaches provides information on understanding of skin permeation of compounds, such as structure-permeability relationship, contribution of each barrier step, mechanisms of penetration enhancers and *in vivo-in vitro* relationships. Mechanistic approaches are also used to predict the time-dependent change of concentration of a compound in the layers of the skin e.g. the dynamics of skin penetration. It also yields information about the additional factors influencing skin permeation e.g. the contribution of the various barriers or the effect of penetration enhancers (Yamashita and Hashida, 2003).

3.2.4.1.1 Analysis based on simple diffusion models

3.2.4.1.1.1 Fick's diffusion law

Compounds are thought to transfer through the skin by a predictable system of passive diffusion, which is defined by Fick's Law and the rate of permeation. Diffusion for most low molecular weight substances seems to occur uniformly through the stratum corneum. Fick's first law assume that the rate of transfer of the permeant through a unit area of a section tissue is proportional to the concentration gradient. It is used to describe steady state diffusion and can be represented as follows:

$$J = DK\Delta c/h \quad \text{or} \quad J = C_v/C_{s,v} \cdot D \cdot C_{s,m}/H$$

Where:

J is the flux per unit area

D is the diffusion coefficient of skin

K is the skin-vehicle partition coefficient

Δc is the concentration difference across the skin

H is the diffusional pathlength

C_v is the drug concentration dissolved in the vehicle

$C_{s,v}$ and $C_{s,m}$ are the solubility of the drug in the vehicle and in the barrier respectively

$C_v/C_{s,v}$ represents the degree of saturation of the drug in the formulation

Often his equation is simplified when *in vitro* studies, such as the present study, are conducted. The applied concentration (in the donor chamber) is much larger than the concentration under the skin (acceptor chamber). The equation is then simplified to:

$$J = kp \cdot C_{app}$$

Where:

kp is a permeability coefficient (= KD/h).

C_{app} is the applied concentration

The second law is a mass-balance equation that is derived from the first law and boundary conditions are given to solve the equation (Yamashita and Hashida, 2003).

3.2.4.1.1.2 Quantitative structure-permeability relationship (QSAR)

QSAR methods are used to predict the permeability of compounds through skin by statistically relating the experimentally measured percutaneous penetration of a range of compounds to known physiochemical parameters. The majority of QSAR studies were based on the analysis of homologous, series of compounds, until Flynn published a data set of human skin permeability for 94 compounds (Roy and Flynn, 1989; Anderson *et al.*, 1988b; Scheuplein, 1965). Flynn also compiled 97 human skin permeability coefficients from 15 different literature sources, but these data showed a high degree of experimental errors due to inter-laboratory variability (Degim *et al.*, 1998; Flynn, 1990). Flynn's studies showed that the skin permeability could be predicted by the permeants' octanol-water partition coefficient and molecular weight (Yamashita and Hashida, 2003; Flynn, 1990).

It is important that all biological data are reproducible and consistently measured, when developing any QSAR. It is therefore advisable to use the same protocol, type of skin, anatomical region, temperature, receptor medium and to measure the skin permeation in one laboratory, by the same researchers (Degim *et al.*, 1998).

3.2.4.1.2 Analysis based on heterogeneous diffusion models

3.2.4.1.2.1 Development of heterogeneous diffusion models

Due to the heterogeneity of the skin structure, the structure-permeability relationship model becomes more complex when a wide range of compounds, especially highly hydrophilic compounds are involved. Skin is often considered to exist out of two layers namely the stratum corneum and aqueous viable layer. Polar and nonpolar pathways exist in the former layer. Therefore, the processes of drug absorption through the skin play an important role. The permeability coefficient of polar compounds is independent of their oil-water partition coefficient. These drugs follow the polar or aqueous pathway through the skin. The highly lipophilic drugs have an almost constant permeability coefficient, since the more aqueous viable layer underneath the stratum corneum limits their permeation rate. Due to mathematical simplicity, Fick's diffusion law cannot be used to analytically solve the permeants' mass-balance equation and therefore only the steady state portion of the permeation profile is analysed when using the heterogeneity model of skin (Anderson *et al.*, 1988b).

3.2.4.2 Empirical analysis of skin permeation of drugs

Empirical modeling has the potential to overcome inaccuracies of mechanistic models caused by the existence of uncertainties and gives better predictions from the practical point of view. Empirical approaches usually predict the steady state properties of skin permeation from molecular parameters such as molecular size and lipophilicity. These two parameters (or slight variations, e.g. molecular volume) have been used repeatedly in predictive models and seem to affect the passive diffusion of drugs through any biological barrier to a large extent. Various other descriptors (e.g. polarity, hydrogen-bond donor acidity and hydrogen-bond acceptor basicity) have also been employed in models predicting skin permeability (Geinoz *et al.*, 2004).

3.2.4.2.1 Linear empirical modeling for prediction of skin permeability

This approach includes the selection of molecular or structural descriptors without mechanistic consideration (Yamashita and Hashida, 2003).

3.2.4.2.2 Neural network modeling for prediction of skin permeability

Artificial neural network (ANN) is a powerful model for non-linear modeling of complex causal-effect relationships. It is an information-processing paradigm that is inspired by the biological nervous systems, such as the brain. Application of ANNs includes structure-activity relationship analysis, structure-property relationship analysis, pharmacokinetic modeling and the design of formulation (Yamashita and Hashida, 2003).

3.2.5 Concentration gradient and supersaturation

The concentration gradient is often considered as the main driving force for percutaneous diffusion. Strictly, it should be the chemical potential gradient. Fick's law states that the flux (J) should increase linearly with concentration until C_{app} reaches solubility limit. When the solubility limit is reached, a further increase in concentration of the same drug in the same solution will result in the same flux (Hadgraft, 2001b).

However, a drug solution can be supersaturated and this may enhance permeation through and uptake into, *in vitro* skin. The supersaturated state may be produced by temperature changes, the uptake of water from the skin, the loss of a volatile solvent and the use of cosolvent mixtures. A drug formulation in this state is innately unstable, but if the supersaturation state can be maintained for the duration of the administration, fluxes greater than those from a saturated solution can be accomplished. Maintaining the supersaturated state of the drug can be accomplished by the addition of polymers such as hydroxypropylmethyl cellulose (HPMC), which can impart stability for many hours. The thermodynamic activity of the drug in the vehicle is increased above unity, thus enhancing the driving force for drug delivery and increasing skin permeation. It is dependent on the nature of the compound, but the precise mechanism is not fully understood (Hadgraft, 2001b).

3.2.6 Methods to breach the skin barrier

Scientists have looked at different means to enhance the transport of drugs through the skin and especially trying to decrease the barrier properties of the stratum corneum - the main barrier of the skin. The barrier properties of the skin are primarily attributed to the intercellular lipid bilayers of the stratum corneum. The ability of a drug to penetrate the skin is closely related to its molecular weight and its affinity for the stratum corneum (Akomeah *et al.*, 2004; Joshi and Jaideep, 2002).

Transdermal delivery is often only considered after a novel compound has been selected and shown to be active, but then was proven to be problematic for oral administration. The drugs that are available for transdermal formulation consequently seldom have ideal physicochemical properties for transdermal administration. Redesigning of the chemical is often too expensive and time consuming, after the compound's toxicological, pharmacological and pre-formulation studies have already been conducted (Williams, 2003).

Many of the topically applied agents do not penetrate skin optimally (Wenkers and Lippold, 2000; Cordero *et al.*, 1997; Singh and Roberts, 1994). In order to overcome the significant barrier posed by stratum corneum (SC), several enhancement strategies are being explored to expand the number of drugs delivered through skin. While product formulation may have a dramatic impact, not only on transcutaneous absorption rates, but also on depth of penetration into the underlying tissues, further optimisation may be desirable (Heyneman *et al.*, 2000). A number of approaches to enhance transport across skin and to expand the range of drugs delivered have been investigated (Guy and Hadgraft, 2003). Despite intensive research during the last three decades, there are still limited transdermal products available on the market for transdermal transport. Those available are mainly low-molecular weight lipophilic drugs ($M_w < 500$ Da). Expanding the range of topically available drugs, especially to include macromolecules, is primarily hindered by low skin permeability. Strategies for transdermal drug absorption are primarily targeted at enhancing the permeability of the stratum corneum, the main barrier of the skin. These strategies involve the selection of the correct drug or prodrug and chemical potential adjustment. Hydration of the stratum corneum, chemical methods and increased temperature can also possibly be used.

Penetration enhancers may improve the percutaneous penetration of drugs by modifying the thermodynamic activity of the drug (e.g. changes in partitioning tendencies) or by altering the skin barrier properties (e.g. changes in fluidity of extracellular lipids) (Jantharaprapap and Stagni, 2007). A good enhancer may also act by increasing the effective concentration of the drug in the vehicle. Chemical enhancers include surfactants, solvents, lecithin gels, liposomes and submicron emulsions. Surfactants may enhance partitioning by reducing the surface tension between the vehicle and the membrane surface and by influencing the barrier potential of the membrane (Barry and El Eini, 1976).

Physical methods, e.g. iontophoresis, electroporation, magnetophoresis, photomechanical waves, sonophoresis and increased temperature are other options (Barry, 2001). These techniques are based on two principles: enhancing skin permeability and/or providing driving force acting on the drug.

3.2.6.1 Temperature

It is an established fact that when skin is heated, its permeability increases and transport of a variety of chemical substances across this tissue is enhanced (Barry, 1983; Fritsch and Stoughton, 1963). Even though the use of heat to enhance percutaneous absorption has been well documented, it has never been fully exploited as a means of aiding drug delivery across the skin (Blank *et al.*, 1967). There has been relatively little investigation of the effect of temperature on skin permeation compared to other methods of penetration enhancement.

In order to understand the mechanism of permeation enhancement it is essential to investigate the effect of temperature on permeation, the cellular components (keratin, ceramides etc.) and the functions of the skin. The Food and Drug Administration (FDA) recently produced an article describing the possibility of toxicity, due to the percutaneous absorption of topically applied material, as a result of the increased temperature and exposure to UV radiation associated with the sun (Lewis, 2002). Consequently, increasing the temperature of the skin and its environment may well provide the potential for overcoming the barrier properties of the stratum corneum and warrants a systematic investigation. This increased permeability has been attributed to an increased fluidity of

the stratum corneum intercellular lipids, which are thought to constitute the major barrier towards penetration of chemical substances across human skin (Tanojo *et al.*, 1997). Furthermore, thermal energy increases the molecular diffusivity of the permeant molecules (Cagnie *et al.*, 2003; Byl, 1995).

3.2.7 Antifungals

Superficial fungal infections are commonly encountered in the dermatologic practice. It is second to acne the most common condition prompting dermatological care. A rise in the frequency of fungal infections over the past few years has occurred in response of a combination of factors. This include disease states such as AIDS, an aging population, the increase prevalence of diabetes mellitus, immunosuppression employed in transplantation or resulting from chemotherapy and advances in invasive surgical techniques which allow for opportunistic pathogen access. Topical antifungals are generally considered as the first-line therapy for uncomplicated, superficial dermatomycoses due to their high efficacy and low potential for systemic adverse effects, unless the infection is widespread, covers an extensive area, or is resistant to initial therapy (Wolverton, 2007; Huang *et al.*, 2004; Elewski and Smith, 2001; Jensen-Pergakes *et al.*, 1998).

The three main classes of topical antifungals currently employed in the topical treatment of superficial fungal infections are the polyenes, azoles and allylamines/benzylamines. Other topical antifungal agents include morpholine derivatives, hydroxypyridone, ciclopirox olamine and selenium sulphide (Katz, 2000; Jensen-Pergakes *et al.*, 1998).

The azoles are mainly fungistatic and can be subdivided into the imidazoles and the triazoles. All azoles work by means of the same mechanism of action. It inhibits the cytochrome P450 dependent enzyme, lanosterol 14- α -demethylase, an essential enzyme in ergosterol synthesis (Katz, 2000).

The topical polyenes, e.g. nystatin and amphotericin B, work by increasing cell membrane permeability by binding to ergosterol. They have been in use since the 1950's, but their use is limited by the fact that they have no significant activity against dermatophytes. They are mainly used for the candidal intertrigo, candidal vaginitis and oral thrush (Katz, 2000).

The introduction of allylamine/benzylamine compounds in the 1980s has improved antifungal therapy, since these two classes exhibit fungicidal mechanisms of action by acting at an earlier stage of the metabolic pathway than the azoles. They are a relatively new class of antifungal drugs and were inadvertently discovered during efforts to synthesize an active central nervous system compound. Examples of this group of antifungal drugs include terbinafine, naftifine and butenafine. They inhibit the enzyme squalene epoxidase, an enzyme required for ergosterol synthesis (Katz, 2000; Jensen-Pergakes *et al.*, 1998).

All the topical antifungal agents mentioned may be effective, but the allylamine/benzylamine-type drugs are more potent *in vitro* and have a higher efficacy *in vivo* than the azole type antifungal agents in the treatment of dermatophytoses. The allylamines/benzylamines are fungicidal while the azoles are primarily fungistatic against common dermatophytes. The allylamines/benzylamines also exhibit a “reservoir” effect due to their highly lipophilic nature and therefore has the advantage that patients continue to improve after cessation of therapy. This retention effect, together with a fungicidal activity, results in shorter duration of therapy and lower relapse rates in patients (Jensen-Pergakes *et al.*, 1998).

3.2.7.1 Terbinafine hydrochloride

Terbinafine hydrochloride is classified as a synthetic allylamine antifungal. It is the most representative of this new chemical class of antimycotic compounds. Chemically, terbinafine is (E)-N,6,6-trimethyl-N-(naphthalen-1-ylmethyl) hept-2-en-4-yn-1-amine and the empirical formula is $C_{21}H_{25}N$ (Figs. 30 and 31). It is marketed for oral and topical use and is highly lipophilic and keratophilic, leading to high concentrations and efficient binding to the stratum corneum, sebum and hair follicles. It therefore reduces the probability of reinfection (Schuster *et al.*, 1988) and tends to accumulate in skin, nails and fatty tissues (Hosseini-Yeganeh and McLachlan, 2002). Terbinafine has a molecular mass of 291.43 g/mol and is typically administered orally (250 or 500 mg per day) or topically (10 mg/ml cream, gel, solution/spray or powder; applied one/twice daily) (James *et al.*, 2007).

It has a broad spectrum of antifungal activity at relatively low concentrations. Terbinafine is fungicidal against dermatophytes (of the genera trichophyton, epidermophyton and microsporum), moulds and certain fungi. The activity against yeasts is fungicidal or fungistatic, depending on the species (Terbicil 1 % Cream® package insert by Aspen, 2005). Terbinafine inhibits ergosterol synthesis by inhibiting fungal squalene epoxidase, an enzyme that is part of the fungal cell wall synthesis pathway. Squalene epoxidase is involved in the biosynthesis of 2,3-oxidosqualene from squalene and thereby inhibits biosynthesis of ergosterol, an essential component of the fungal cell membrane. This leads to intracellular accumulation of squalene that is toxic to the fungal cell and results in rapid death. Terbinafine's effects are selective for fungi, because its affinity for squalene epoxidase in fungal cells is 400 to 4000 times greater than in animal/human cells and it has few adverse reactions (Tanuma *et al.*, 2000; Gupta and Shear, 1997). It is applied twice a daily when administered topically and is prescribed for fungal infections of the skin caused by trichophyton (e.g. T. rubrum, T. mentagrophytes, T. verrucosum, T. violaceum), mycrosporum canis and epidermophyton floccosum. It is also indicated for yeast infections of the skin, principally those caused by candida albicans and pityriasis (tinea) vesicolor due to pityrosporum orbiculare (also known as malassezia furfur) (Terbicil 1 % Cream® package insert by Aspen, 2005; Brunton *et al.*, 2006). Its site of action is within the stratum corneum and therefore terbinafine's local bioavailability in this most superficial layer of the skin is pertinent to its ultimate clinical efficacy *in vivo* (Balfour and Faulds, 1992; Faegermann *et al.*, 1991).

Terbinafine hydrochloride is a fine, white crystalline powder that is freely soluble in methanol and methylene chloride, soluble in ethanol and slightly soluble in water. It is highly lipophilic and keratophilic (Gupta and Shear, 1997). In a study by Tatsumi *et al.* (2002), the affinity to keratin powder was established. Terbinafine showed a 96 % binding to the keratin. The release of terbinafine from drug-preloaded keratin powder after 10-minute washings with saline at 37 °C was 21.6 %. Uchida and Yamaguguchi (1993) also reported that terbinafine showed a high rate of keratin binding when incubated in a buffer with 10 % keratin. These authors found a binding of 88.4 %.

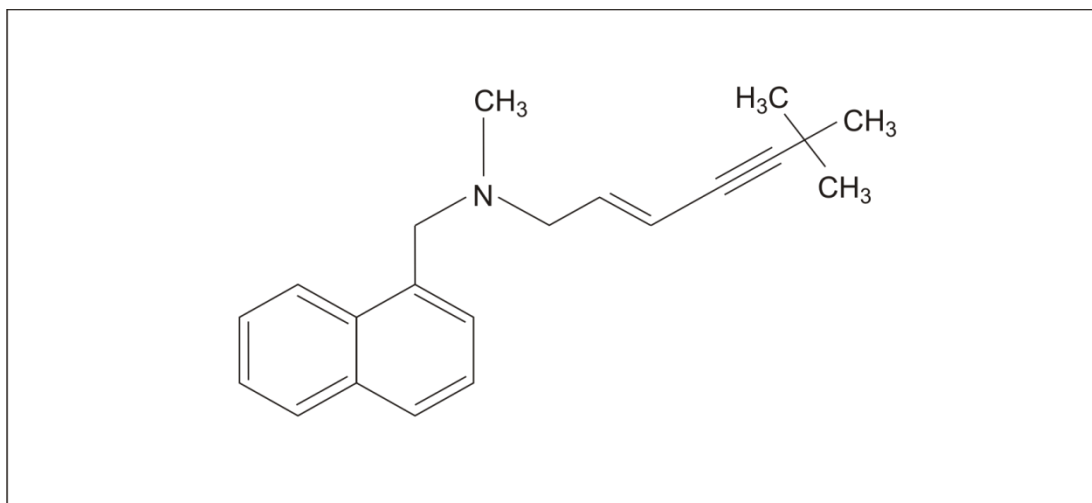


Fig. 30. Terbinafine (The Merck Index, 2001)

drug	terbinafine hydrochloride
therapeutic category	antifungal
octanol/water partition coefficient	3.3
pKa	7.1
solubility	water (1 in 160) alcohol (>1 in 14)
MW (g/mol)	327.9

Fig. 31. Physicochemical characteristics of terbinafine hydrochloride (Alberti *et al.*, 2001b; Dollery *et al.*, 1999)

Topical therapy is often preferred to oral drug administration in the treatment of superficial fungal infections of the skin, since generally a high oral dose are necessary to reach a therapeutic drug concentration in the skin which may lead to the increased risk of adverse reactions and drug interactions. Usually, topical administration results in much lower and often undetectable plasma levels and the required concentration for antimycotic activity at the skin target site may be more efficiently achieved by topical administration (Alberti *et al.*, 2001b).

Terbinafine undergoes extensive biotransformation in the liver, with the production of about 15 metabolites. It is unknown whether or not there is significant skin metabolism of topically applied terbinafine (Dollery *et al.*, 1999; FDA website).

3.2.8 Tritiated water

Tritiated water is a form of water in which the usual hydrogen atoms are replaced with tritium. In its pure form it may be called tritium oxide ($^3\text{H}_2\text{O}$). *In vitro* transdermal tritiated water flux measurements are frequently used to evaluate skin barrier integrity for quality control purposes, so that any damaged tissues can be eliminated from subsequent *in vitro* studies (Meidan and Roper, 2008). Hence, understanding how tritiated water flux values vary with respect to different variables such as anatomic site, temperature etc. is crucial within this quality control context (Meidan and Roper, 2008).

3.2.9 Aim

Dermal and transdermal drug delivery serve as an attractive alternative to conventional drug delivery methods. Topical administration of drugs can be used to achieve systemic or local effects. If the skin is the site of action, the effectiveness of a topical dermatological formulation depends on the bioavailability of the drug within this target organ. The drug has to reach the target skin at the required concentration and achieve the desired therapeutic action, with minimal systemic exposure. The barrier properties and the subsequent impermeability of the skin are a considerable problem in the delivery of active compounds both to and through skin. The pharmaceutical industry is therefore investing ever increasing amounts of resources on the development of new products that are able to overcome the barrier properties of the skin (Akomeah *et al.*, 2006). Skin is a complex organ, which have a wider variety of cell types than the brain. Even though the skin is very accessible for drug administration and research, some aspects of transdermal drug penetrations are still unknown. The precise penetration route of chemicals and how this can be affected by formulations are still not fully established.

Due to modern high-throughput technologies large numbers of potential drugs are produced by parallel synthesis and combinatorial chemistry. The pharmaceutical industry therefore requires rapid and accurate methods to screen drug leads for tissue permeability potential in the early stages of drug discovery. *In vivo* human dermal

studies are not always feasible due to the invasiveness of biopsies and the high costs. Even though mathematical models are available for the selection of appropriate transdermal drugs, these models are still ambiguous in predicting skin permeability (Moss *et al.*, 2002). Reliable *in vitro* models can be applied to determine permeation of the test compounds, which will help avoid the wasting of valuable resources on the development of drugs that are destined to fail in preclinical and clinical phases due to insufficient absorption properties. Well-designed and standardised *in vitro* models and studies may play an important role to enable the accurate prediction of the optimum choice of formulations and solvent type enhancers for *in vivo* percutaneous absorption (Moss *et al.*, 2002; Alberti *et al.*, 2001). One would prefer to use human skin to evaluate the penetration properties of new candidate drugs, since *in vitro* studies using human skin to measure drug diffusion and metabolism, provide more accurate absorption data than animal skin studies (Schmook *et al.*, 2000; Collier *et al.*, 1991).

Terbinafine hydrochloride is classified as a synthetic allylamine antifungal. It is the most representative of this new chemical class of antimycotic compounds and has a broad spectrum of antifungal activity at relatively low concentrations. It is marketed for oral and topical use and is highly lipophilic and keratophilic, resulting in high concentration in the stratum corneum, sebum and hair follicles and therefore reducing the probability of reinfection (Alberti *et al.*, 2001; Schuster *et al.*, 1988). Ideally, a topical antifungal drug should be absorbed by the skin and achieve the desired therapeutic action with minimal systemic exposure. Relatively little information is available in the literature about the concentration of terbinafine in tissues. A possible reason could be due to a lack of availability of an analytical method (Yeganeh and McLachlan, 2000).

Choosing the appropriate vehicle/formulation for a specific drug plays a critical role in the clinical efficacy of a dermatological topical drug. The formulation has to exhibit qualities such as optimised drug release and at the same time be able to facilitate drug transport across or into the skin. The foregoing prompted the use of a flow-through diffusion system to determine the permeability of different terbinafine hydrochloride formulations through human skin and to measure the drug's concentrations within the skin tissue. Since the skin (stratum corneum) is the main target for topical antifungal drug treatment, terbinafine's clinical efficacy can be determined by evaluating the concentration of the drug at its target site. The results will present a potential *in vitro* model for of evaluating

different formulations and may possibly be developed into a method for assessing the bioequivalence of alternative (generic) vehicles or formulations containing the same drug.

A further objective of the study was to investigate the effects of increased temperature on *in vitro* permeability of tritiated water across human skin. Tritiated water flux measurements are frequently used to evaluate barrier integrity of the skin. There has been relatively little investigation of the effect of temperature on skin permeation compared to other methods of penetration enhancement. Increasing the temperature of the skin and its environment may well provide the potential for overcoming the barrier properties of the stratum corneum. Establishing an *in vitro* model in which skin temperature can be controlled will therefore have the potential to investigate and predict the effect of increased temperature on *in vivo* skin.

3.3 Materials and Methods

3.3.1 Skin tissue

Skin specimens were obtained from excess tissue removed from 23 females, mean age 43 ± 16 SD (range: 18-75) yr, during breast reduction procedures and abdominal plastic surgery at the Louis Leipoldt Hospital, Bellville, South Africa. No specimens were obtained where there was clinical evidence of any disease that might have influenced the permeability characteristics of the skin.

All skin specimens were immediately placed in a transport fluid after removal and kept at 4 °C. The transport fluid consisted of a stock solution of Eagle's Minimum Essential Medium (MEM) without L-glutamine and sodium bicarbonate (Gibco, Paisley, Scotland), to which the latter as well as an antibiotic (penicillin/streptomycin, 100 IU/ml) and an antimycotic (amphotericin-B, 2.5 µg /ml) were added prior to using it for the transport of tissue specimens. The skin tissue was transferred to our laboratory within 2 hours. In the laboratory excess connective tissue was trimmed away using a forceps and scalpel. Specimens from each patient (10 x 10 mm) were snap-frozen in liquid nitrogen and stored at -85 °C. Full thickness skin was obtained, since the presence of both the epidermis and dermis makes for a more realistic model for *in vivo* drug transport. Human skin was used since it is regarded as the "gold standard" for *in vitro* penetration experiments (Barbero and Frasc, 2009). Prior to use, the frozen samples were thawed and hydrated in PBS for 24 hours at 4 °C.

The study was approved by the Ethics Committee of Stellenbosch University and the Tygerberg Academic Hospital. Project number: 95/019 (resubmitted and approved on 28/7/03).

3.3.2 Terbinafine hydrochloride

Terbinafine hydrochloride powder and three commercial terbinafine hydrochloride 10 mg/ml formulations (Topical1, Topical2, and Topical3) were obtained from different pharmaceutical companies. The manufacturer who provided some of the formulations do not want to be revealed and it was therefore one of the conditions I had to accept before receiving the formulations. Therefore no mention will be made to the manufacturer, trade names or batch numbers in this thesis. Topical1 and Topical2 were

generic cream formulations (Topical1 was obtained from a local generic pharmaceutical company) and contained 1.0 % benzyl alcohol as preservative, while Topical3 was a gel formulation and had 0.5 % (m/m) benzyl alcohol as well as 10 % ethanol (96 %, v/v) as preservatives.

Benzyl alcohol is a widely used low-risk preservative and fragrance additive in the pharmaceutical and cosmetic industry. It is not only able to solvate strongly hydrophobic compounds such as terbinafine by forming micelles, but also able to maintain contact with aqueous solutions due to its moderate hydrophilicity, thus making it ideal for penetration through the stratum corneum (Mikulak *et al.*, 1998). The inclusion of benzyl alcohol in topical formulations has been shown to increase drug retention and accumulation within the skin significantly, whilst lack of binding in the dermis and metabolic biotransformation reactions within the epidermis aid the removal of the drug from the skin and ensure sink conditions (Peng and Nimni, 1999; Mikulak *et al.*, 1998; Boehnlein *et al.*, 1994; Barry *et al.*, 1985).

Methanol is a solvent that interferes with the skin's barrier function by exerting an effect on the stratum corneum's lipids and proteins (Hadgraft, 2001a; Goates and Knutson, 1994). It has the ability to extract lipids in the stratum corneum. It removes lipids that are covalently bonded to the corneocyte envelope which leads to a decreased barrier function by the skin (Hadgraft, 2001b). It is therefore known to cause rapid and irreversible damage to stratum corneum. The extent of damage increase with contact time (Nangia *et al.*, 1998; Dugard *et al.*, 1984).

Ethanol is commonly used as a solvent and permeation enhancer (Femenia-Font *et al.*, 2005; López-Pinto *et al.*, 2005; Ho *et al.*, 2004). It extracts some of the lipids of the stratum corneum, but is a much milder solvent than methanol. Ethanol consists of a short two carbon chain, which enable it to interact with and dissolve relatively non-polar molecules. It also has a hydroxyl group, which gives ethanol the ability to interact with relatively polar molecules and to form hydrogen bonds. Ethanol therefore has the ability to act as a solvent for organic molecules with a wide range of octanol/water partition coefficients (Van der Merwe and Riviere, 2005). Its synergistic effect with other permeation enhancers has been widely studied (Gwak *et al.*, 2004; Kim and Chien, 1995). Ethanol can exert its permeation enhancing activity by extracting lipids from the

stratum corneum to increase drug flux, increasing the lipid fluidity of the skin, effecting the putative pore pathway, enhancing drug solubility in stratum corneum lipids, changing stratum corneum hydration and altering the keratinised protein (Trommer and Neubert, 2006). As a solvent it can increase the solubility of the drug in the vehicle. It is also feasible that the rapid permeation of ethanol or evaporative loss of this volatile solvent from the donor phase modifies the thermodynamic activity of the drug within the formulation. Heard and Screen investigated this effect of ethanol further and showed the transit of the ethanol permeating through skin gave rise to co-transportation of the drug (the so called pull or drag effect) (Heard and Screen, 2008).

3.3.3 Permeability experiments

The human skin specimens were mounted in flow-through diffusion cells (exposed circular areas 0.196 ± 0.002 SD cm²) and permeation studies were performed on 7 tissue replicates for each patient (Van der Bijl *et al.*, 2006; Basson *et al.*, 2005; Van der Bijl *et al.*, 2003). The skin was mounted between donor and acceptor compartments and the stratum corneum faced towards the donor compartment. 0.5 ml Topical1 was placed in the donor compartment of each flow-through diffusion cell and covered with adhesive tape to avoid evaporation.

PBS at 32 °C was pumped through the acceptor chambers at a rate of 1.5 ml/h and collected by means of a fraction collector, at 2-h intervals for 24 h. Alternatively 0.5 ml (~ 5 mg) Topical2 and Topical3 were used. The above experiments were repeated with terbinafine hydrochloride solutions (PBS/Methanol 1:1) of 10, 20 and 30 mg/ml respectively. 0.5 ml of the respective terbinafine hydrochloride solutions was added to the donor compartments of the flow-through diffusion cells and fractions were collected at 2-h intervals for a 24 h period.

The terbinafine in the acceptor chambers was quantified by means of LC/MS analysis and terbinafine extractions of the exposed skin were conducted.

For studying the effect of temperature on ³H₂O flux rates, skin tissue was mounted in the flow-through diffusion cells, as previously described. Prior to each permeability experiment, the thawed skin specimens were equilibrated for 10 min in PBS (pH 7.4) at 32 or 37 °C in the diffusion cells. The PBS was then removed from the donor

compartment and replaced with 1.0 ml of PBS containing 1 μCi ^3H -water (Amersham Laboratories, Little Chalfont, Amersham, UK). Aliquots (100 μl) were removed within minutes from each of the seven donor compartments for the determination of donor cell concentration at time zero. A flow rate of 1.5 ml/h was maintained and fractions collected, by means of a fraction collector, at 2-h intervals. Temperatures were controlled by using a circulator water bath. Scintillation cocktail (10 ml) (PCS Scintillation Cocktail; Amersham Biosciences, Uppsala, Sweden) was added to each sample collected and the radioactivity (dpm) determined using a Beckman LS 5000TD liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA). The counting of the samples was continued until a 2-s value of 1 % was reached. Quenching for each sample was automatically corrected in the counter.

Intra- and inter-experiment variations were calculated for the terbinafine and $^3\text{H}_2\text{O}$ experiments (SD/mean x 100).

3.3.4.1 Terbinafine extractions from skin

After each 24 h transdermal diffusion experiment, the skin samples from the diffusion cells were recovered and wiped 3 times using a cotton cloth immersed in PBS to remove excess terbinafine from the surface of the skin. The skin area (0.196 ± 0.002 SD cm^2) exposed to the different terbinafine hydrochloride formulations were taken with a biopsy, cut into smaller sections, positioned in a glass homogeniser and grounded in 1 ml methanol. The resulting solution was centrifuged for 10 min at 6500 rpm to eliminate suspended solids and impurities from the skin. The terbinafine were then extracted with 10 ml ethyl acetate and the extract was evaporated at 39°C (Schmook *et al.*, 2000). 1 ml methanol was added to the residues and the solution was subjected to analysis by LC/MS (Liquid Chromatography/Mass Spectrometry).

3.3.4.2 LC/MS determination of terbinafine

The samples collected from the acceptor compartments of the perfusion apparatus over the 2-h sampling intervals, were analysed using LC/MS. Determination was conducted under the following LC/MS conditions (Figs. 32-37):

LC: Waters 2695 Separation Module (Alliance)

MS: Water Micromass, Quatro Micro ES⁺ (Electrospray chemical ionisation).

Column: Xterra Rp (18⁵ μ m, 3 x 9 x 150 mm)

Flow rate: 0.9 ml/min

Mobile phase: Two solvents (50:50), A (MeOH) and B (0.1 % Formic H⁺ in deionised water).

Retention time: 4.3 min

LLOQ (lower limit of quantification): 0.0001 mg/ml

There were a 6.7 % variation on the chromatographic area and a 0.61 % variation on the retention time (RT).

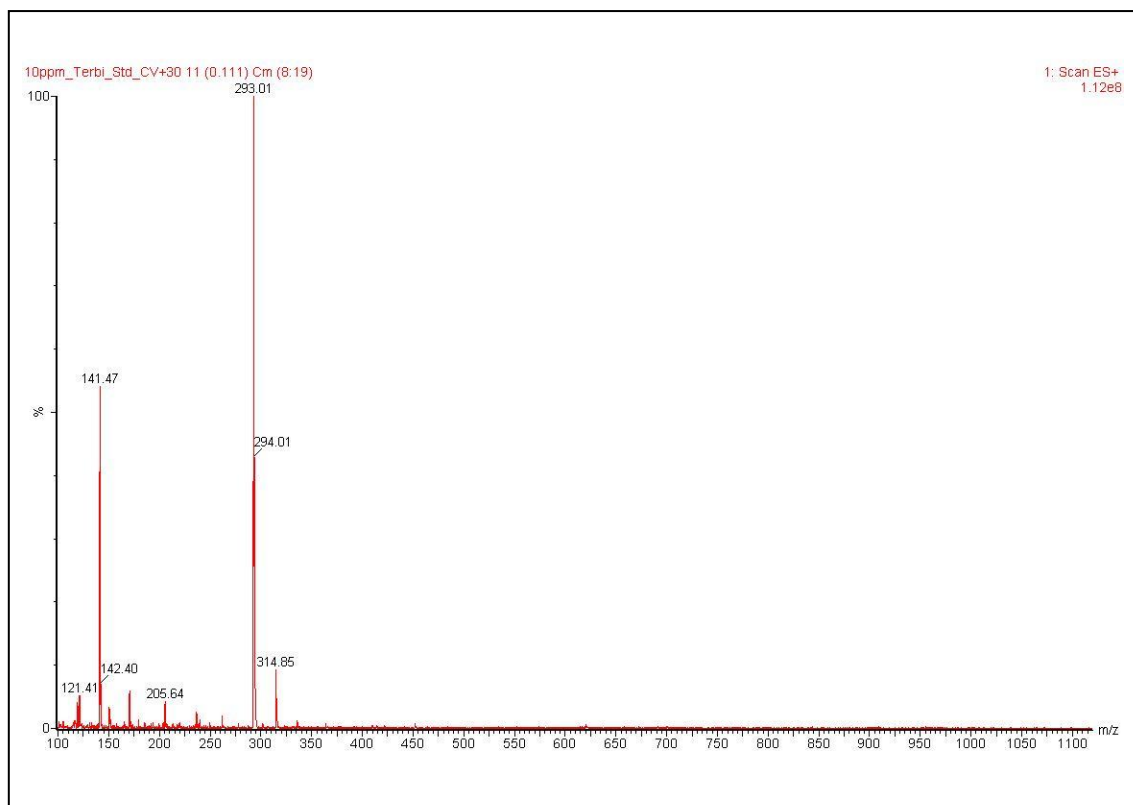


Fig. 32. Best cone voltage for terbinafine was at 30V

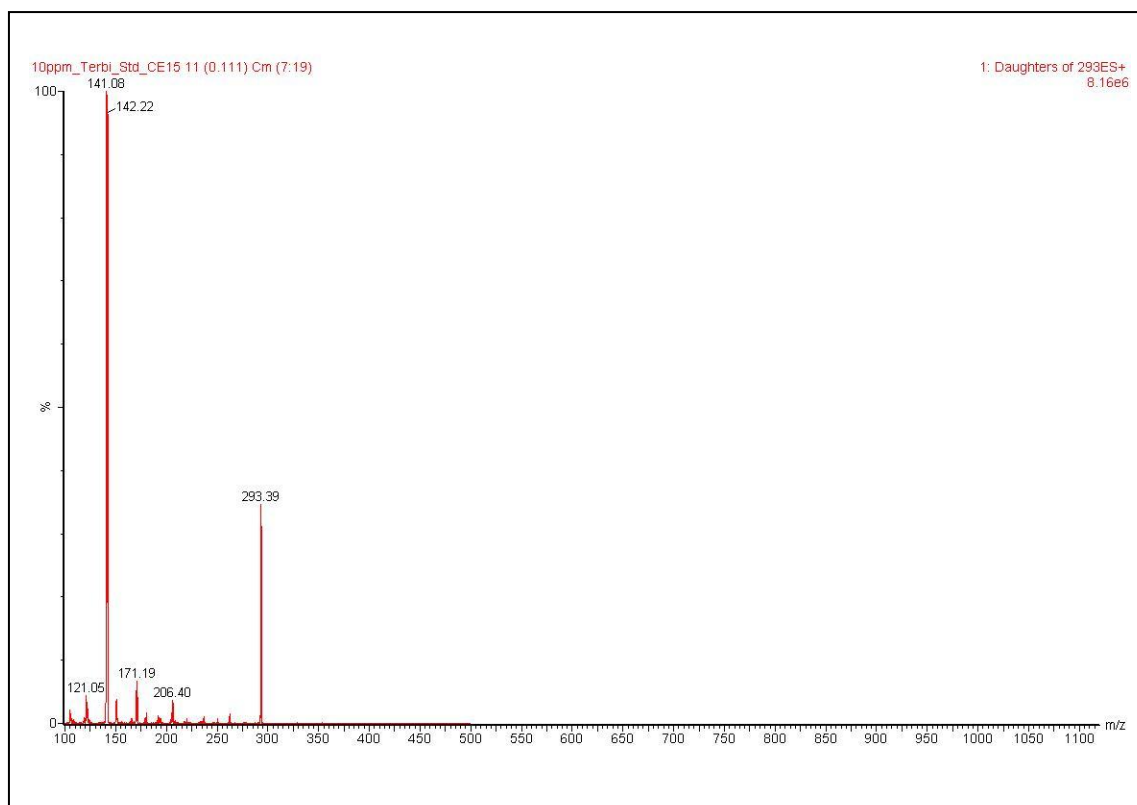
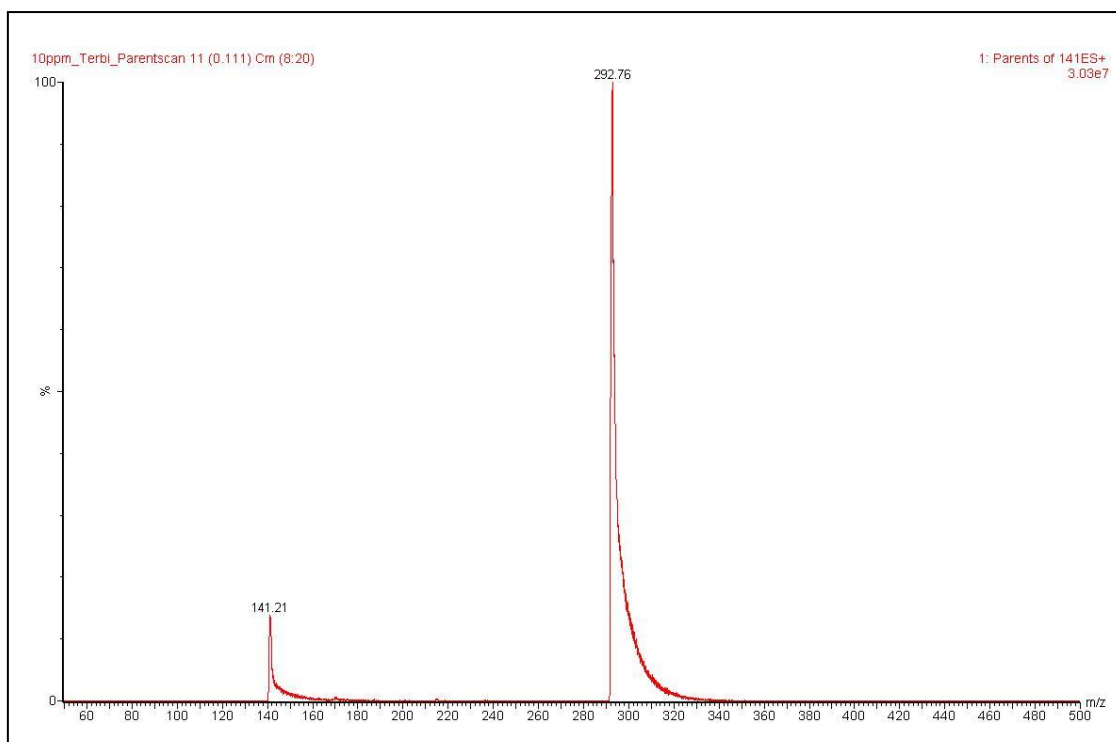
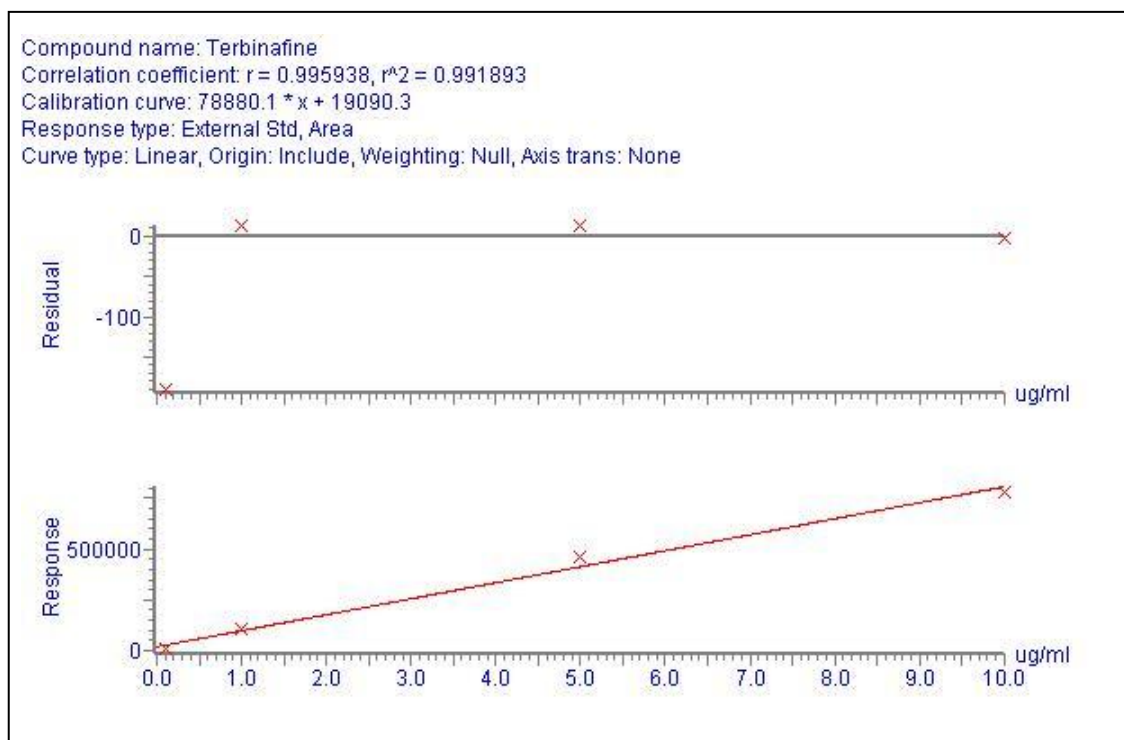


Fig. 33. Best product ion was 141.21 m/z at collision energy of 15

**Fig. 34. Parent scan of daughter ion****Fig. 35. Calibration curve 1**

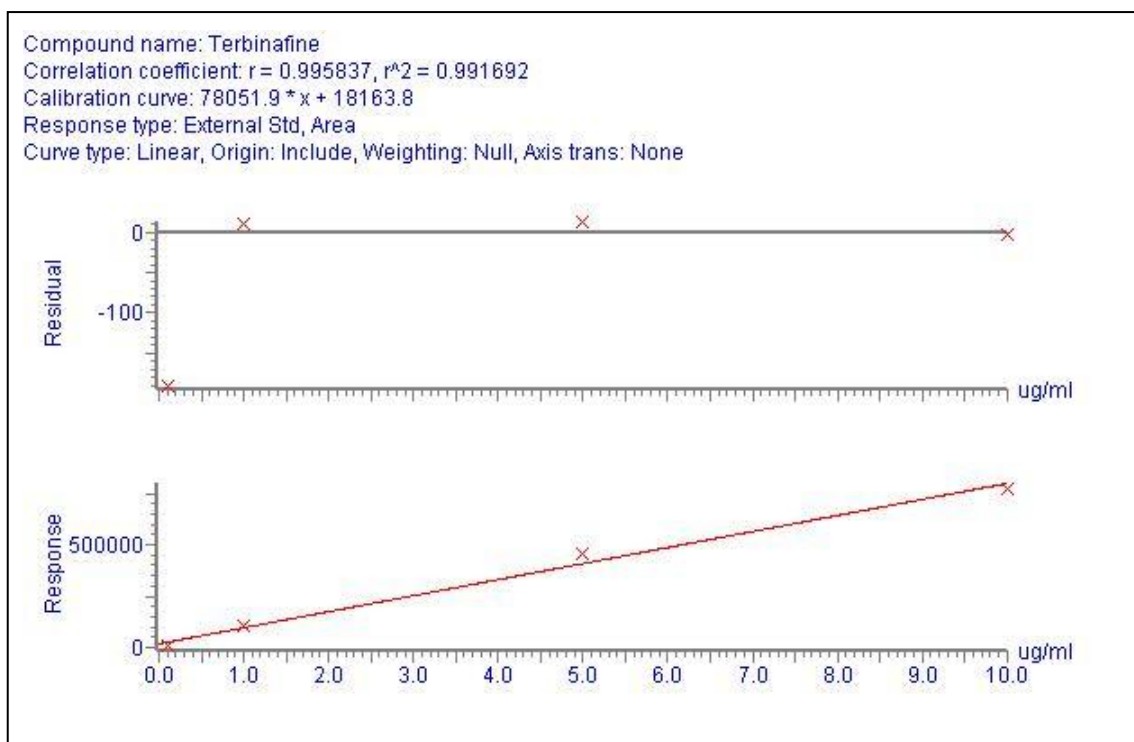


Fig. 36. Calibration curve 2

Injection Number	Area	RT
1	566769.375	3.66
2	535695.063	3.76
3	472059.406	3.75
4	455136.000	3.75
5	560435.938	3.73
6	568797.438	3.72
7	505724.938	3.74
8	505089.719	3.72
9	522681.625	3.74
10	541672.563	3.73
11	547673.125	3.74
12	547160.000	3.72
13	568252.563	3.73
14	561737.875	3.72
15	561984.188	3.73
Mean	534724.654	3.73
SD	35799.897	0.02
RSD	0.0670	0.0061
% CV	6.70	0.61

Fig. 37. Terbinafine Repeatability Analysis (5ppm)

3.3.4.3 Calculation of flux values

Flux (J) values of $^3\text{H}_2\text{O}$ across skin were calculated by means of the relationship: $J = Q/(A \times t)$ ($\text{dpm} \times \text{cm}^{-2} \times \text{min}^{-1}$), where Q = quantity of $^3\text{H}_2\text{O}$ crossing skin (dpm), A = skin area exposed (cm^2) and t = time of exposure (min). The trapezoid method of obtaining area under curve (AUC) was used.

3.3.4.4 Steady state kinetics and statistical analysis of data

Repeated measure ANOVA over all time periods and a Bonferroni multiple comparison procedure was used to determine steady state. If at consecutive time periods p-values exceeded 0.05, these flux values do not differ significantly, therefore indicating steady state. Non-linear regression analyses (third order polynomials) were performed using a GraphPad Prism, version 4, 2003 computer programme. A F-test was used to compare entire curves (Harwood and Mishell, 2001). A significant level of 5 % was used for all tests and comparisons.

3.4 Results

3.4.1 Terbinafine hydrochloride

No flux values for any of the terbinafine hydrochloride formulations (Topical1, Topical2, Topical3 or terbinafine hydrochloride solutions) through skin into the acceptor fluid were found. Topical3 showed a significantly ($p < 0.05$, ANOVA) higher mean drug concentration ($4.219 \mu\text{g/ml}$) in the skin than Topical2 ($1.590 \mu\text{g/ml}$). The mean terbinafine concentration in the skin after exposure to Topical3 was higher than the skin exposed to Topical1 ($3.589 \mu\text{g/ml}$), but this difference was not significant ($p > 0.05$). The mean terbinafine concentrations in the skin after 24 h exposure to Topical1, Topical2 and Topical3 were 3.589 , 1.590 and $4.219 \mu\text{g/ml}$ respectively (Fig. 38). Topical1 led to a significantly ($p < 0.05$, ANOVA) higher mean terbinafine concentration in skin than Topical2. The mean terbinafine concentrations in the skin after 24 h of exposure to the terbinafine hydrochloride solutions (PBS/Methanol 1:1) of 10, 20 and 30 mg/ml were 42.640 , 77.34 and $62.541 \mu\text{g/ml}$ respectively (Fig. 39). The mean drug concentration of the skin exposed to 10 mg/ml terbinafine hydrochloride solution (PBS/Methanol 1:1) was 11.880 times higher than the mean drug concentration of the skin exposed to Topical1 (10 mg/ml) and 10.107 higher than the mean drug concentration of the skin exposed to Topical3 (10 mg/ml) (Fig. 40). The mean drug concentration of the skin exposed to 20 mg/ml terbinafine hydrochloride solution was almost 2 times (1.814) and 1.237 times higher than the mean drug concentration of the skin exposed to the 10 and 30 mg/ml solutions respectively.

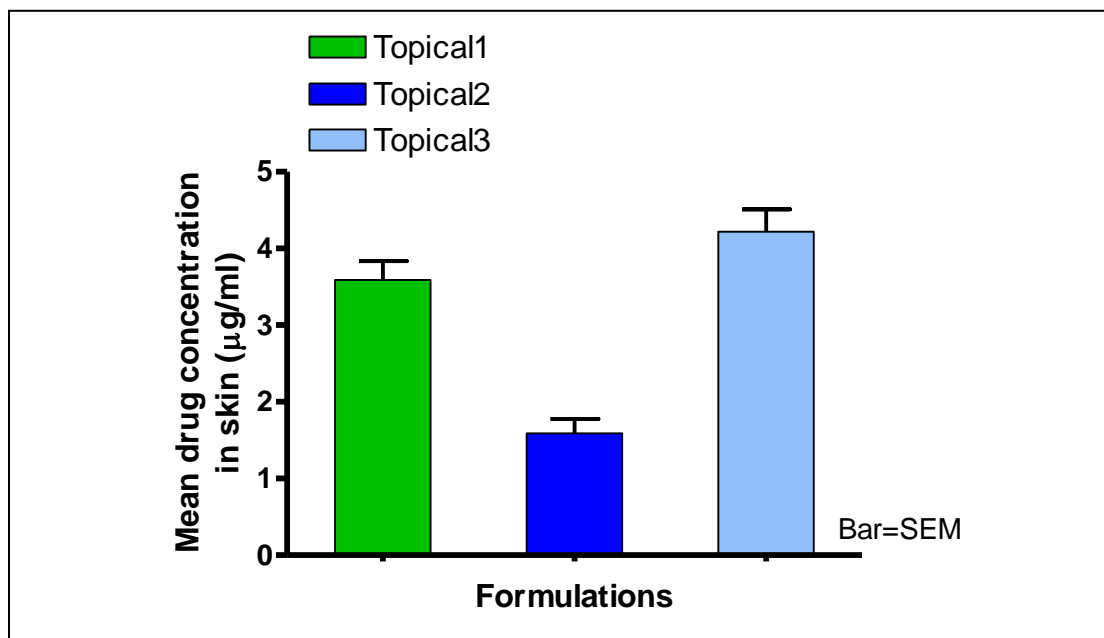


Fig. 38. Mean terbinafine concentrations in the skin after 24 h exposure to Topical1, Topical2 and Topical3

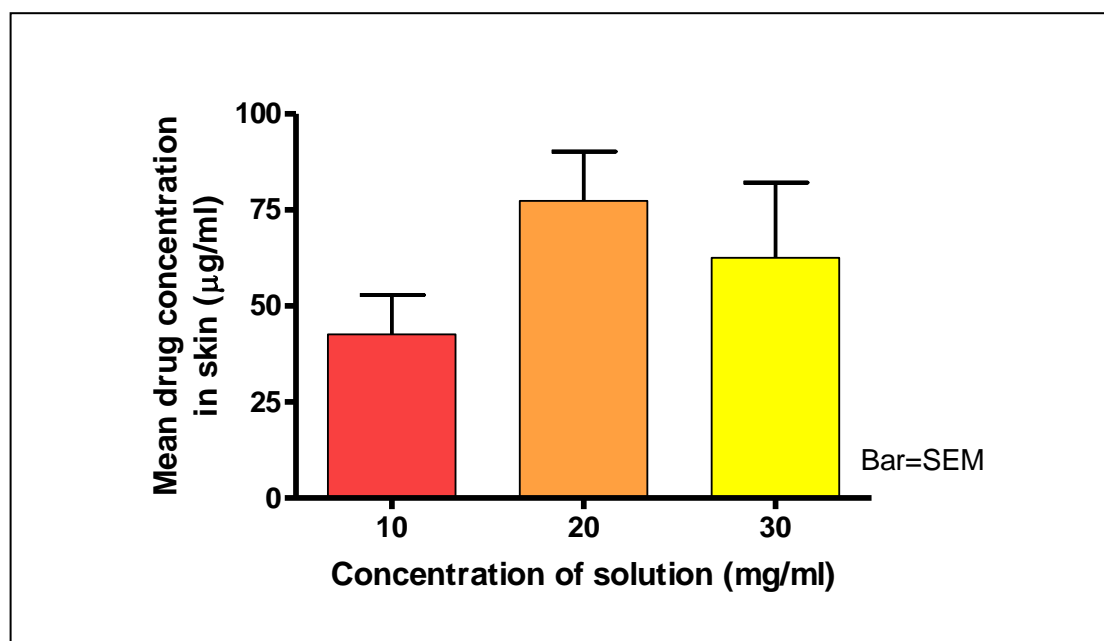


Fig. 39. Mean terbinafine concentrations in the skin after 24 h exposure to terbinafine solutions of 10 mg/ml, 20 mg/ml and 30 mg/ml

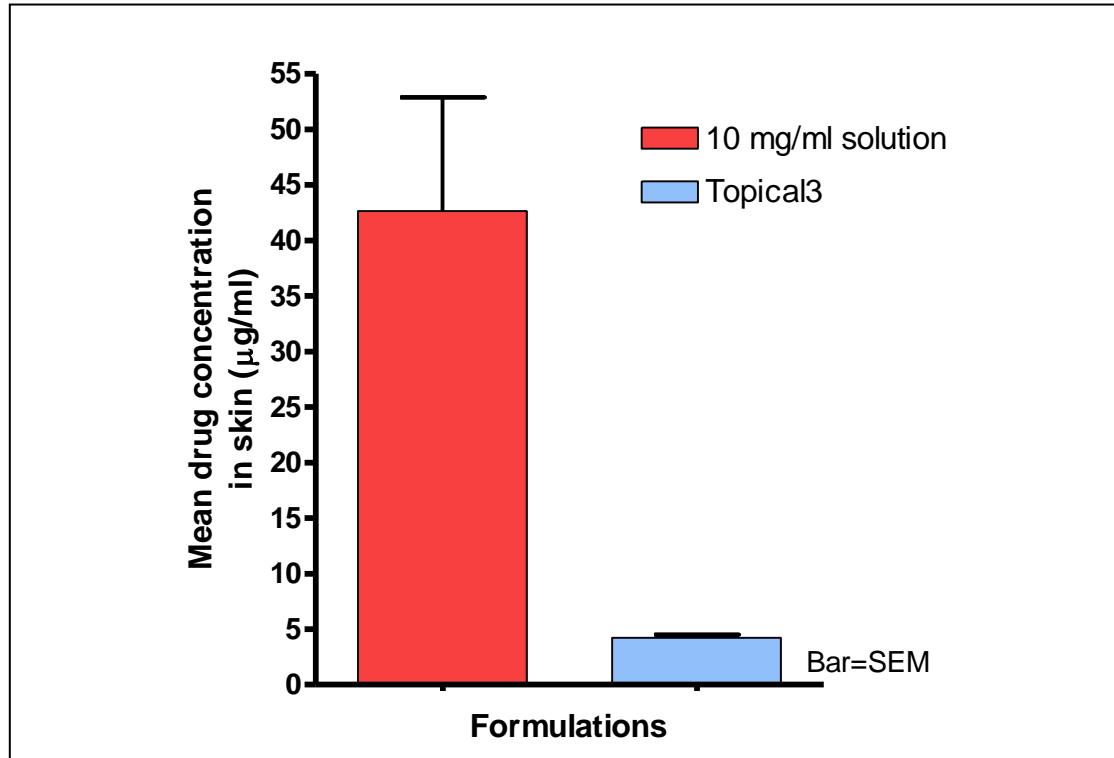


Fig. 40. Comparison between the mean terbinafine concentrations in the skin after 24 h exposure to a 10 mg/ml terbinafine hydrochloride solution and Topical3 (10 mg/ml)

3.4.2 Tritiated water

The results of this temperature study clearly demonstrated the temperature-dependency of water flux rates across skin tissue. The overall mean flux values of water across human skin at 32 °C and 37 °C are shown in Fig. 41. The increase of temperature caused the lag time to decrease from 16 to 12 h (Fig. 41b). An increase of 5 °C caused a significant ($p < 0.05$) increase in flux values of tritiated water across skin. The AUC of the overall mean flux values of tritiated water across skin at 37 °C were 2.0 times that of the overall mean flux values of tritiated water across skin at 32 °C (Fig. 41).

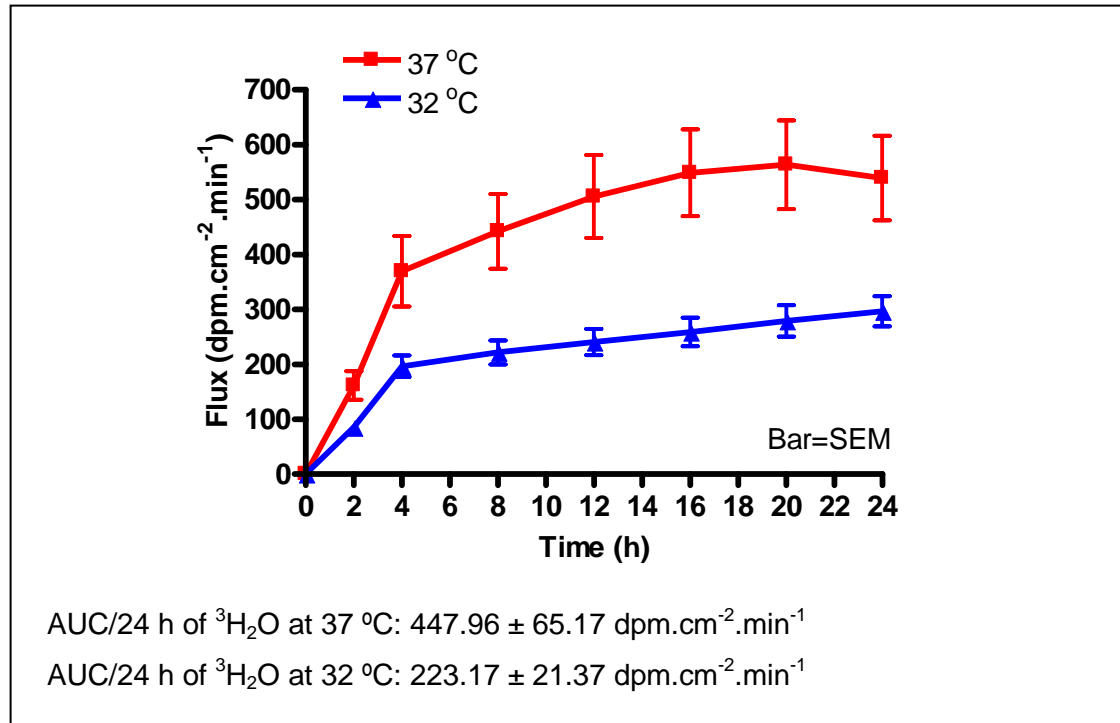


Fig. 41. Overall mean flux values of ³H₂O across human skin at 32 °C and 37

Bonferroni test; variable DV_1 (32 °C)								
Error: Within MS = 3615.8, df = 198.00								
Cell No.	Time (h)	{1}	{2}	{3}	{4}	{5}	{6}	{7}
		85.456	196.5	221.8	240.74	258.98	279.12	296.68
1	2		0	0	0	0	0	0
2	4	0		1	0.05754	0.0006	1E-06	0
3	8	0	1		1	0.24213	0.00246	1.4E-05
4	12	0	0.05754	1		1	0.1924	0.00353
5	16	0	0.0006	0.24213	1		1	0.21968
6	20	0	1E-06	0.00246	0.1924	1		1
7	24	0	0	1.4E-05	0.00353	0.21968	1	

Bonferroni test; variable DV_1 (37 °C)								
Error: Within MS = 31322., df = 246.00								
Cell No.	Time (h)	{1}	{2}	{3}	{4}	{5}	{6}	{7}
		161.57	369.32	442.18	505.56	548.85	563.61	539.48
1	2		4E-06	0	0	0	0	0
2	4	4E-06		1	0.0105	0.00012	0.00002	0.00033
3	8	0	1		1	0.12977	0.03926	0.26017
4	12	0	0.0105	1		1	1	1
5	16	0	0.00012	0.12977	1		1	1
6	20	0	0.00002	0.03926	1	1		1
7	24	0	0.00033	0.26017	1	1	1	

Fig. 41b. $^3\text{H}_2\text{O}$ reached steady state at 12 h (temperature 37 °C)

The intra-experiment variations for terbinafine and $^3\text{H}_2\text{O}$ were respectively 30.1 and 26.2 % and the inter-experiment variations were respectively 27.4 and 20.9 %.

3.5 Discussion

3.5.1 Terbinafine hydrochloride

In vitro human skin studies to measure drug diffusion and metabolism provide the most accurate absorption data, as compared with animal skin studies. Therefore one would like to use human skin, as done in the present study, to evaluate penetration properties of new candidate drugs (Schmook *et al.*, 2000; Collier *et al.*, 1991).

There is relatively little information available in the literature about the concentration of terbinafine in tissues. A possible reason could be due to a lack of availability of an analytical method (Yeganeh and McLachlan, 2000). This prompted us to use the flow-through diffusion system to study the permeation of terbinafine hydrochloride across human skin and to determine the concentration of the drug in skin tissue.

From the results of the present study, it was clear that terbinafine diffused into the skin along a concentration gradient, but interestingly, no flux values were found for terbinafine through skin into the acceptor compartment of the flow-through diffusion system. This may be explained as follows. Terbinafine hydrochloride is a highly lipophilic (octanol/water partition coefficient=3.3) and keratophilic compound. It has a relatively small molecular weight (327.9 g/mol) and it therefore readily diffused from its vehicle into the skin, but then accumulated in the skin ("reservoir effect") rather than to diffuse into the hydrophilic acceptor compartment through which PBS was pumped. This reservoir effect is in agreement with other authors (Tatsumi *et al.*, 2002; Leyden, 1998; Uchida and Yamaguguchi, 1993).

The extensive absorption of terbinafine into the skin and the maintenance of high concentrations in the skin for a considerable time after cessation of treatment, together with its fungicidal mode of action are of major relevance to its ability to achieve rapid anti-mycological and clinical response (Faergemann *et al.*, 1995; Hill *et al.*, 1990). These findings are in agreement with the results` obtained in other *in vivo* and *in vitro* studies.

The findings of this study concur with data from a previous *in vitro* skin study, in which a comparison of the penetration properties of human skin or epidermis models with human

and animal skin was conducted. The flux rate of terbinafine through human skin was below the detection limit of the study and therefore had the lowest flux rate of the four test compounds used in the study, but interestingly the skin concentration ($\mu\text{g/g}$) of terbinafine was the second highest of the four compounds (Schmook *et al.*, 2001).

Other authors demonstrated in an *in vivo* study that only 3-5 % of terbinafine (10 mg/ml cream) is absorbed into the systemic circulation after topical administration. The skin penetration was a slow process, with the peak amount of substance appearing as metabolites in the urine only 2 to 3 days after administration (Jones, 1990; Hill *et al.*, 1992). The results of the mentioned *in vivo* study may also explain why no terbinafine was detected in the acceptor compartment of this experiment, since in this study permeability of terbinafine hydrochloride was only studied over a 24 h period due to the degradation of the *in vitro* skin.

Even though terbinafine undergoes first pass effect in the liver, it is unknown whether or not there is significant skin metabolism of topically applied terbinafine hydrochloride (FDA website). Future studies with the flow-through diffusion could be used to evaluate the metabolism of drugs by skin tissue.

An *in vivo* study of multiple-dose oral terbinafine (250 mg, 1x/day) showed a peak plasma concentration of $1.70 \pm 0.77 \mu\text{g/ml}$ occurring 1.2 h post administration. Terbinafine distributed extensively to the peripheral body fluids and tissue, but had a slow redistribution back to the central plasma compartment. The fungicidal concentrations in the skin were maintained for weeks to months after a course of treatment. In the mentioned *in vivo* study terbinafine levels in the stratum corneum was $1 \mu\text{g/ml}$ (Koavarik *et al.*, 1995). The findings of this *in vivo* study could be used to put the concentration of terbinafine found in skin exposed to Topical1, Topical2 and Topical3 (3.589, 1.590 and $4.219 \mu\text{g/ml}$ respectively) into clinical perspective and illustrate the reservoir effect of terbinafine once again.

The reservoir effect of terbinafine in skin may have significant clinical value, since the skin is the site of action for topical terbinafine. It is therefore important that after application of this antifungal drug, terbinafine reaches its minimum inhibitory concentration (MIC) in the skin and not necessary in the systemic circulation. The

extensive absorption of terbinafine into the skin and the maintenance of high concentrations in the skin for a considerable time after cessation of treatment, together with its fungicidal mode of action are of major relevance to its ability to achieve rapid mycological and clinical response (Faergemann *et al.*, 1995; Hill *et al.*, 1990). Diffusion of terbinafine mainly into the skin and not the systemic circulation may decrease unnecessary side effects and limit drug interactions with especially cytochrome P450 enzyme inhibitors/inducers e.g. cimetidine, rifampicin and also with oral contraceptives, terfenadine, caffeine, cyclosporine (In: South African Medicines Formulary, 2003). It may also be advantageous for patients breast-feeding and patients with gastrointestinal or hepatic dysfunction. Topical administration and minimal drug plasma levels may therefore lead to greater patient convenience and possibly improving drug adherence.

Budimulja *et al.* (2000) conducted an *in vivo* study in adult patients with tinea corporis/cruris. The patients were treated with 1 % Lamisil Cream® once daily for 7 days. Rapid mycological cure were achieved and maintained for up to 7 weeks after completion of treatment. Hill *et al.* (1992) also investigated the effect pharmacokinetics of 1 % Lamisil Cream®. Treatment for 7 days also resulted in terbinafine still being detectable 7 days after cessation of therapy at fungicidal concentrations, showing the highly lipophilic and keratophilic properties of the drug.

The administration of Topical3 led to a higher ($P > 0.05$) mean skin concentration (4.219 µg/ml) than Topical1 (3.589 µg/ml) and a significantly ($P < 0.05$) higher mean skin concentration than Topical2 (1.590 µg/ml) (Fig. 38). The mean skin concentration after 24 h exposure to Topical3 was respectively 1.2 and 2.7 times higher than Topical1 and Topical2. All three commercial formulations used in this study had a concentration of 10 mg/ml, but Topical3 contained 0.5 % (m/m) benzyl alcohol as well as 10 % ethanol (96 %, v/v) as preservatives, where as Topical1 and Topical2 contained 1.0 % benzyl alcohol as a preservative. The finding that Topical3 led to greater terbinafine penetration into the skin may be attributed to the combination of benzyl alcohol (0.5 %) and ethanol (10 %) in the gel formulation of Topical3, which enhanced the absorption of terbinafine into the skin more efficiently than the 1.0 % benzyl alcohol cream formulation of the other two commercial formulations. These results correlate well with an *in vivo* study done by Cramer *et al.* (2006), where the authors compared the skin penetration of two different commercial terbinafine hydrochloride formulations. The gel formulation that contained

the combination of benzyl alcohol and ethanol as preservatives showed a significantly higher skin penetration than the cream formulation that contained only benzyl alcohol.

In another *in vivo* study, single daily application of two 10 mg/ml terbinafine topical products with similar formulations to Topical 1, Topical2 and Topical3 were used and the skin concentrations of the respective formulations were compared using skin stripping. The topical formulation similar to Topical3 showed significantly faster skin penetration than the topical formulation similar to Topical1 and 2 (138 ± 16 vs. 121 ± 16 ng/cm²/min) (James *et al.*, 2007). The ratio between the *in vivo* skin concentrations achieved after 1 day application of the two different formulations was 1.2. Interestingly, the skin concentration ratio achieved in this *in vitro* study after 24 h application of Topical 1 and 3, was also 1.2.

Both Topical1 and Topical2 were 10 mg/ml cream formulations that contained 1.0 % benzyl alcohol as preservative, but the 24 h skin exposure to these two formulations led to a significantly higher mean terbinafine concentration in the skin exposed to Topical1. This interesting result may be attributed to the fact that the two formulations are manufactured by different pharmaceutical companies and may contain different ingredients, even though they are from similar pharmaceutical category. The results demonstrate the importance of formulation for percutaneous absorption of topical drug preparations, as the quantity of applied terbinafine penetrating the skin varied significantly between the two formulations. Different ingredients and solvents used in the formulation of drugs may change the partitioning and diffusion behaviour of the drugs into the stratum corneum, which is the main barrier of the skin (Rosado *et al.*, 2003; Kai *et al.*, 1990).

With the exception of topical corticosteroids, the only means a generic company has to demonstrate bioequivalence of topical dermatologic product to an innovator's product is through comparative clinical trials with a bioequivalence endpoint (Sha *et al.*, 1998). These clinical trials are time and money consuming. In the case of topical corticosteroids, the demonstration of bioequivalence may be conducted by using the FDA vasoconstrictive protocol (FDA, 1995). *In vitro* models to study the effects of different formulations and solvents on percutaneous drug absorption could improve the prediction of skin absorption in the pharmaceutical industry and may therefore save time

and resources. The *in vitro* flow-through diffusion model used in the present study has shown that the formulation of topically applied terbinafine hydrochloride can have a considerable impact on the transdermal penetration of this antifungal compound.

The results of the study are in correlation with Fick's law; the skin absorption rate of a penetrant in a mixture is proportional to the penetrant concentration (Dugard, 1977). As expected, the mean drug concentration of the skin exposed to 20 mg/ml terbinafine hydrochloride solution was almost 2 times (1.814) higher than the mean drug concentration of the skin exposed to the solution with a lower concentration (10 mg/ml). This finding supports the well-established diffusion theory, but the 20 mg/ml solution also had a slightly higher (1.237 times) drug concentration than the tissue exposed to the 30 mg/ml terbinafine hydrochloride solution (Fig. 39). The lower drug concentration found in the skin exposed to a higher terbinafine hydrochloride solution may be explained by the saturated solubility of the terbinafine hydrochloride in the 30 mg/ml terbinafine hydrochloride solution, the instability of the saturated solution, the subsequent decreased thermodynamic activity, the formation of crystals on the exposed skin surface and saturation of the exposed skin tissue. The absorption of a drug is invariant with concentration above the solubility limit of the specific drug (Hadgraft, 2001b). It may therefore be assumed that the solubility limit of terbinafine hydrochloride in PBS/Methanol (1:1) is approximately 20 mg/ml.

Chemicals that reduce the skin's ability to perform its barrier function are collectively known as penetration enhancers. These substances cause the skin to become more permeable and they allow drug molecules to cross the skin at a faster rate. A wide selection of chemical structures has been identified as penetration enhancers, of which methanol is one. Methanol interferes with the skin's barrier function by exerting an effect on the stratum corneum's lipids and proteins (Hadgraft, 2001a; Goates and Knutson, 1994). It is known to cause rapid and irreversible damage to stratum corneum. The extent of damage increases with the contact time (Nangia *et al.*, 1998; Dugard *et al.*, 1984). Ethanol is also commonly used as a solvent and permeation enhancer, but is a much milder solvent than methanol (Femenia-Font *et al.*, 2005; López-Pinto *et al.*, 2005; Ho *et al.*, 2004). This may explain why the mean drug concentration of the skin exposed to 10 mg/ml terbinafine hydrochloride solution (PBS/Methanol 1:1) was 10.107 times

higher than the mean drug concentration of the skin exposed to Topical3 (10 mg/ml, 1 % benzyl alcohol, 10 % ethanol) (Fig. 40).

For dermatophytes the minimum inhibitory concentration (MIC) range for terbinafine in *in vitro* skin is 0.001 to 0.01 µg/ml, ≤ 0.05 to 2.0 µg/ml for dimorphic fungi, 0.05 to 1.56 µg/ml for aspergilla, 0.1 to 0.4 µg/ml for sporothrix schenckii, 0.2 to 0.8 µg/ml for malasseziafurfur and it also exerts good activity against yeasts (MIC range: 0.1 to >100 µg/ml) (Balfour and Faulds, 1992; Petranyi *et al.*, 1987). The *in vitro* MIC for some Candida species is 0.25 to 1 µg/ml and Trichophyton species 0.0039 to 0.078 µg/ml (Hazen, 1998). Terbinafine displays a primary fungicidal action against dermatophytes, other filamentous fungi and sporothrix schenckii. The concentrations of terbinafine found in skin exposed to Topical1, Topical2 and Topical3 were 3.589, 1.590 and 4.219 µg/ml respectively that are all above the mentioned *in vitro* mentioned MICs and may put the drug concentrations found in this study into clinical perspective.

Choosing the appropriate vehicle/formulation for a specific drug plays a critical role in the clinical efficacy of a dermatological topical drug. The formulation has to exhibit qualities such as optimised release of the drug and at the same time facilitates drug transport across or into the skin. *In vivo* assessment of topical drug bioavailability may be expensive and may not always be easily accomplished. A reliable *in vitro* model to assess preclinical formulations will therefore be very beneficial. Such an *in vitro* model may serve as a tool to evaluate whether the presence of certain vehicle components may enhance percutaneous absorption (Alberti *et al.*, 2001a).

3.5.2 Tritiated water

The results of the study clearly show a significant increase in flux rate of water across skin tissue when the normal temperature of skin is elevated from 32 °C to 37 °C (Fig. 41). The AUC/24 (average flux over 24 h) of tritiated water across skin at 37 °C were double that of the overall mean flux values of tritiated water across skin at 32 °C.

The enhancement in percutaneous absorption due to the temperature increase is consistent to that reported by other authors (Clarys *et al.*, 2001; Ogiso *et al.*, 1998; Ohara *et al.*, 1995). These authors attributed the enhancement to an increase in the fluidity of stratum corneum lipids which might also lead to an associated increase

(expansion) in intercellular space, thus leading to the increase in epidermal permeability. Many of the earlier studies employed rat skin and/or donor solutions/gels containing penetration enhancers such as ethanol, propylene glycol and terpenes, which are known to disturb the intercellular lipid arrangement. This does not allow the effect of temperature on skin permeation to be studied independently, making it difficult to differentiate the effect of temperature and/or chemical enhancers on either the polar and/or non polar pathways across the skin. At low temperatures, the lipids are much more rigid and closely packed and this hinders the diffusion of the penetrant molecules across the stratum corneum. With the aid of differential scanning calorimetry (DSC), thermal transitions of stratum corneum lipids has been shown to occur near 40, 65-70 and 80-85 °C (Lawson *et al.*, 1998; Tanojo and Bouwstra, 1994; Knutson *et al.*, 1985).

The results of the present study are in agreement with a previous *in vitro* skin study where a two-fold increase in flux was observed for every 7-8 °C rise in receptor temperature for all penetrants investigated (Tanojo *et al.*, 1997).

This calls for further investigation into the effect of increases in physiologically acceptable temperature on percutaneous absorption and dermal retention of a wide range of permeants. This is required especially when the regulatory authorities are becoming increasingly concerned about the possibility of toxicity from active agents or vehicle excipients, used in topical semisolids as a result of exposure to elevated temperature/UV radiation at the site of application. Increased temperatures may also be of importance in patients with fever.

Chapter 4: Small intestine mucosa

4.1 Summary

The oral route still remains the most popular route to administrate new drugs, despite the extensive research on other routes of drug delivery. In mammals/humans the jejunum is generally the major absorbing site for most drugs. Approximately 90 % of all absorption of nutrients and drugs takes place in the small intestine. This part of the small intestine also has the largest surface area and it is the site of the most active carrier-mediated transport in the gastrointestinal tract. It was therefore decided to use jejunum mucosa for evaluating the small intestine permeability.

Due to modern isolation technology and high throughput biological screen capability a reliable, high through put method for evaluating intestinal permeability is essential. Several methods have been proposed and investigated for *in vitro* evaluation and prediction of gastrointestinal permeability of drugs, but no official method is available at present due to certain limitations.

The permeability of excised human jejunum mucosa to 4 different oral drugs, using our flow-through diffusion system, was investigated. The 4 drugs were selected as representative model compounds of drug classes 1 (high solubility, high permeability) and 3 (high solubility, low permeability) according to the Biopharmaceutics Classification System (BCS). Permeability values from our *in vitro* diffusion model were compared with *in vivo* gastrointestinal drug permeability and the BCS permeability classification.

The flux rates of the 4 chosen test drugs were influenced by the length of the experiment. Between the time periods 2-4 h and 4-6 h, AZT's mean flux values were respectively 1.8 and 2.0 times higher than didanosine and 2.3 and 2.2 times higher than enalapril. At these same time periods propranolol's mean flux values were respectively 1.2 and 1.4 times higher than didanosine and 1.6 times higher than enalapril during the mentioned time periods. Class 1 drugs showed a significantly higher flux rate across the jejunal mucosa compared to the class 3 drugs and these results are in line with their BCS classification. The *in vitro* model has proved to be reliable to predict permeability of the class 1 and 3 drugs and also showed correlation with human *in vivo* data.

The results of this study show that the permeability values of gastrointestinal mucosa obtained with the developed flow-through diffusion system are good predictors of BCS permeation classification and concurred with other *in vitro* studies. Moreover, the proposed method allowed obtainment of good results for a wide variety of compounds/drugs.

4.2 Introduction

4.2.1 Oral administration of drugs

Regardless of the tremendous research on drug delivery methods in the last few decades, the oral route remains the most preferred route of administration of new drugs (Balimane *et al.*, 2000). More than 60 % of marketed drugs are oral products, despite some shortcomings (Masaoka *et al.*, 2006). The oral administration route is preferred due to its convenience, high patient compliance, less stringent production conditions and lower costs. It is also considered safe, efficient and easily accessible, with minimal discomfort to the patient compared to other routes of administration. Tremendous growth in the field of genomics and combinatorial chemistry, combined with advances in technological innovations led to vast numbers of potential drug candidates. The screening of potential compounds for permeability/absorption, solubility, stability and biological activity is now one of the rate limiting processes in drug discovery. The implementation of appropriate screening models is therefore essential for the design of an oral preparation. These models must be able to accurately predict *in vivo* permeability, dissolution and absorption and must be cost-effective and exhibit a high capacity (Balimane *et al.*, 2000).

4.2.2 Small intestine

4.2.2.1 Anatomy

The human small intestine is approximately 2-6 m long and is divided into three areas namely the duodenum, jejunum and ileum. These three sections comprise respectively 5, 50 and 45 % of the length of the GIT (Ganong, 1995). The small intestine is the principle site for absorption of nutrients and drugs. Approximately 90 % of all absorption in the gastrointestinal tract occurs in the small intestinal region and the upper part of the small intestine is considered to have the higher capacity for drug absorption. In mammals/humans the jejunum is generally the major absorbing site for most drugs. The jejunum has the largest surface for absorption and is the site of the most active carrier-mediated transport (Lennernäs, 2007). The duodenum is the shortest segment and starts at the pyloric sphincter of the stomach and extends approximately 25 cm until it merges with the jejunum. Duodenum means “twelve”, since it is roughly 12 fingers breadth in length. The jejunum is approximately 2.5 m long and extends to the ileum.

Jejunum means “empty”, since this part of the small intestine is usually found empty at death. The last portion of the small intestine is the ileum. The ileum means “twisted”, measures approximately 3.6 m and joins the large intestine at the ileocecal sphincter.

The small intestine fills much of the peritoneal cavity and its position is stabilised by the mesentery proper, a broad mesentery attached to the dorsal body wall. The stomach, the large intestine, the abdominal wall and the pelvic girdle restrict movement of the small intestine during digestion. Blood vessels, lymphatic vessels and nerves reach the segments of the small intestine within the connective tissue of the mesentery. The primary blood vessels are branches of the superior mesenteric artery and the superior mesenteric vein (Martini, 2006; Tortora and Grabowski, 1993).

4.2.2.2 Histology

The walls of the small intestine are composed of four layers of tissue: mucosa, submucosa, muscularis externa and the serosa (Fig. 42).

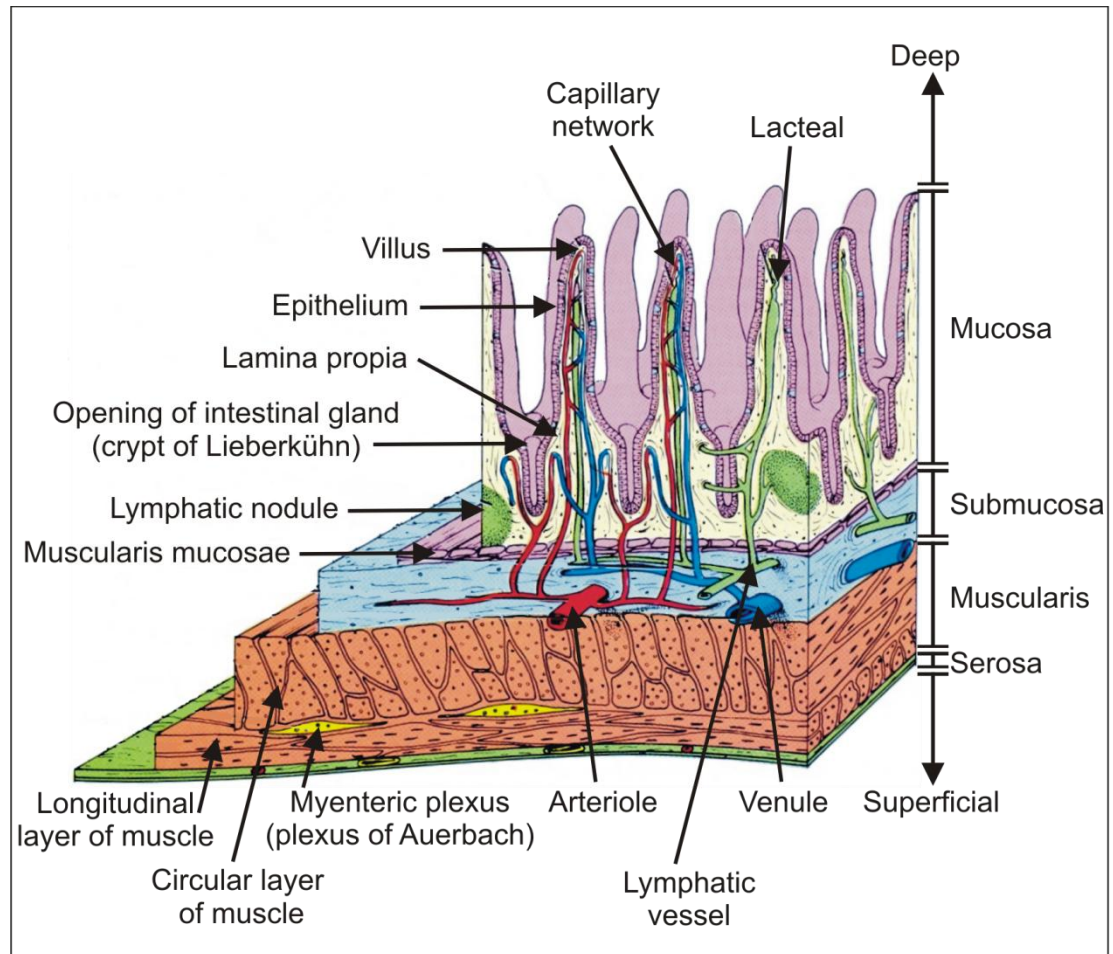


Fig. 42. Sectional views of layers of small intestine (Tortora and Grabowski, 1993)

The mucosa is composed of the intestinal epithelium, lamina propria and muscularis mucosae. The mucosa has a characteristic villous form in the whole of the small intestine, with short glands known as crypts of Lieberkühn between the villi, extending down to the muscularis mucosae. The villi are finger-like projections of the lamina propria that is the connective tissue layer of the mucosa. The villi tend to be longest in the duodenum and become shorter towards the ileum. The mucosa and submucosa are thrown up into circularly arranged folds, called plicae circulares or valves of Kerkring, which are particularly numerous in the jejunum. Plicae circulares are only found in the jejunum and ileum (Fig. 42).

The epithelium of the mucosa consists of simple columnar epithelium or enterocytes, mucous/goblet cells, Paneth cells, stem cells and endocrine cells. The enterocytes are

responsible for digestion and absorption and cover the villi and crypts. The mucous/goblet cells, Paneth cells, stem cells and endocrine cells are dispersed between the enterocytes and their numbers and distribution vary in the different zones of the small intestine. The proportion of goblet cells in the epithelium increases distally. Numerous microvilli are present at the luminal surface of the enterocytes and since the microvilli project from the epithelium's free border like the bristles on a brush, these epithelial cells are said to have a brush border. The connection tissue of the lamina propria contains an arteriole, a venule, a capillary network and a lacteal, which is a lymphatic vessel (Fig. 43). Nutrients and drugs pass through the epithelial cells covering the villus and then pass through the capillary walls and the lacteal to enter the blood and lymphatic fluid. The entire epithelium of the intestine is replaced every 3 to 5 days. Another prominent feature of the small intestine is lymphoid aggregations known as Peyer's patches within the lamina propria. These structures are associated with the function of the immune system (Martini, 2006; Tortora and Grabowski, 1993). The muscularis mucosa, a layer of circular smooth muscle fibres, lies immediately beneath the mucosal crypts and separates the mucosa (lamina propria) from the submucosa. The submucosa is vascularised loose connective tissue containing larger blood vessels, lymphatic vessels and adipose cells as well as submucosal (Meissner's) plexuses. It extends into and forms the core of the plicae circulares. Beneath the mass of submucosal glands is the muscularis externa, which is composed of an inner circular layer and an outer longitudinal layer of smooth muscle. These two muscle types are responsible for peristaltic activity. Between the two layers of smooth muscle is the myenteric plexus (plexus of Auerbach). The peritoneal aspect of the muscularis externa is invested by the loose collagenous serosal, which is lined on its peritoneal surface by mesothelium, that is identical in appearance to the mesothelium lining of the pleura.

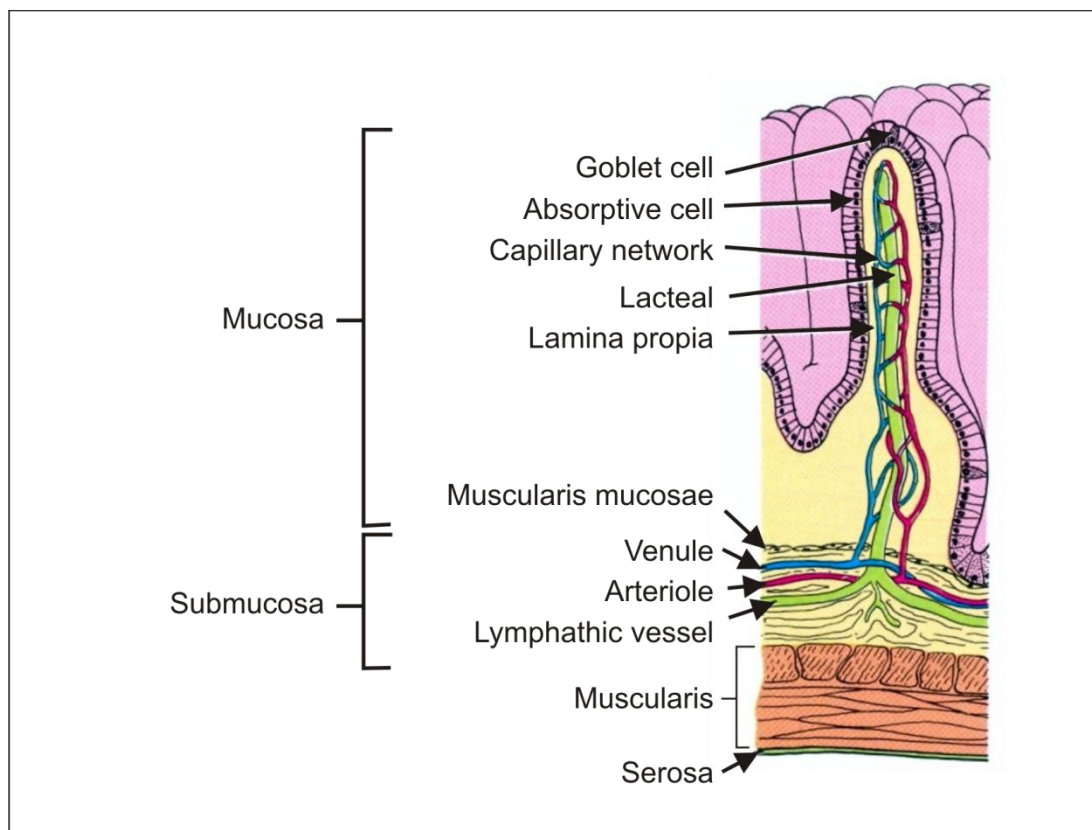


Fig. 43. Enlarged villus showing lacteal and capillaries (Tortora and Grabowski, 1993)

If the small intestine was a simple tube with smooth walls it would have had a total area of roughly 3300 cm^2 , but the surface area for digestion and absorption is increased to a factor of more than 600 (2 million cm^2) due to the plicae and the forest of villi and microvilli (Martini, 2006; Tortora and Grabowski, 1993).

4.2.3 Functions of the GIT

The GIT has several important functions such as the absorption and metabolism of nutrients and drugs, secretion and it acts as an efficient barrier to digestive enzymes and potentially hazardous bacteria and toxins. The enterocytes and colonocytes of the epithelial layer of the GIT maintain a balance between absorption and protection and this subsequently affects the permeability of drugs, while the gut-associated lymphoid tissue (GALT) is the largest immunological tissue in the body.

The human body plays host to 10^{14} bacteria of which most are located in the GIT. The microflora in the lumen of the GIT forms a complex ecosystem and plays an important role in the normal physiology of the body. Different regions of the GIT contain different compositions and concentrations of microflora e.g. the stomach and jejunum contain few bacteria due to the lower pH and inhibitory action by bile/pancreatic secretion. The colon contains a much higher concentration of bacteria due to a higher pH, the abundance of nutrients and decreased peristalsis (Lennernäs, 2007).

4.2.3.1 Metabolism

The small intestine plays a significant role in the metabolism of orally administered drugs, although it is regarded as an absorptive organ (Lin *et al.*, 1999). Metabolic pathways include both phase I and II reactions (Kaminsky and Zhang, 2003). Most drug-metabolising enzymes that are present in the liver are also found in the small intestine, but at lower levels (Lin *et al.*, 1999). The importance of the small intestine's metabolising enzymes arises from their location in the epithelial cells (enterocytes) and may result in the reduced systemic uptake of drugs. Examples of the enzymes include aminopeptidase A, alkaline phosphatase, cytochrome P-450 and UDP-glucuronosyltransferase (Bohets *et al.*, 2001). Clinical studies have shown that the small intestine contributes substantially to the overall first-pass cytochrome P450-mediated metabolism of drugs. In addition to oxidative metabolism by cytochrome P450, hydrolysis is also an important phase I reaction in the biotransformation of ester-containing drugs. Carboxylesterase is involved in the hydrolysis of a variety of ester- and amide-containing endogenous compounds. Carboxylesterases are important in both the inactivation of drugs and in the activation of prodrugs and are widely distributed in many tissues, including the intestines (Zhang *et al.*, 2002; Satoh and Hosokawa, 1998).

4.2.3.2 Absorption

4.2.3.2.1 Site of absorption

Drugs are absorbed throughout the GIT. Absorption of drugs throughout the GIT varies with location. The stomach is the first site for drug release and absorption. The pH of the stomach in the fasted state usually lies in the range of pH 1-3. After a meal the pH, increases to values up to 7 due to the buffering effects of the food. For drugs that are

highly soluble at a gastric pH, complete dissolution can occur in the stomach, but for weak acids little dissolution will occur in the stomach, while high dissolution will occur in the small intestine due to this region's higher pH. Poorly soluble neutral compounds have slow dissolution in the gastric region and often dissolution is not completed before the drug reaches the first absorptive sites in the small intestine. For poorly soluble weak bases, solubility is likely to be higher in the stomach than elsewhere in the GIT. This may result in supersaturation, but precipitation of the drug is hindered by bile components as the drug moves out of the stomach into the higher pH of the small intestine (Dressman *et al.*, 2007). The pH of the small intestine is 7-7.8 (Bajpai *et al.*, 2003). In the fasted state, it may be below neutral (pH 6-6.5) (Dressman *et al.*, 1990). The proximal small intestine's pH is influenced more by food intake than the pH of the distal regions.

Generally, the upper part of the small intestine is considered to have a higher capacity for drug absorption than the lower part. This is due to an increase in tightness of the epithelium in the distal intestinal area, as well as a decrease in total surface area. The luminal pH of the different areas also plays a role. In mammals/humans the jejunum is generally the major absorbing site for most drugs. It has the largest surface area and it is the site of the most active carrier-mediated transport in the gut (Kasim *et al.*, 2004; Sun *et al.*, 2002). The villous structures in the upper intestine enlarge the surface area of the intestine membrane. Villous structures of the jejunum amplify the area four-fold compared to the colon and two-fold compared to the ileum. It seems that this area is relatively leaky and therefore contributes to the high permeability of hydrophobic drugs in the upper intestinal membrane. However, some drugs have been reported to exhibit high permeability even to the lower part of the intestine (ileum and colon) (Balimane *et al.*, 2000). Generally, the permeability of the colonic membrane is much lower than that of the small intestine, but some drugs are known to be absorbed from the colon.

4.2.3.2.2 Absorption and solubility

The epithelial cells lining the gastrointestinal tract are the major barrier to absorption of many orally administered drugs (Jackson, 2005). They are a heterogeneous population of cells that include enterocytes/absorptive cells, goblet cells that secrete mucin, endocrine cells, Paneth cells, M cells, tuft cells and cup cells (Madara and Trier, 1987; Carr and Toner, 1984). Enterocytes make up 90 % of the cells in the epithelium and are

responsible for the majority of the absorption of both nutrients and drugs in the small intestine. The epithelial membrane controls the transport of both low and high permeability compounds regardless of the transport mechanism *in vivo* (Lennernäs, 2007). It is highly polarised with distinct apical and basolateral membranes that are separated by tight junctions (Balimane *et al.*, 2000). The basolateral surface faces the bloodstream and the apical surface faces the intestinal lumen. The membranes around the intestinal cells are lipid bilayers containing proteins (e.g. receptors and carrier molecules).

The mucus layer at the surface of the intestinal epithelium exhibits a rate-limiting barrier, especially against the absorption of highly permeable drugs. The thickness of the mucus layer varies in different luminal segments (Winne and Verheyen, 1990). The mucous gel layer consists of a three-dimensional network of glycoproteins and the extracellular matrix comprises tight junctions, which function is regulated by different transmembrane and intracellular proteins (Ho *et al.*, 2004).

The small intestine's primary source of solubilisation is the bile components. The bile salt conjugates, phospholipids and cholesterol combine (additionally with lipolysis products in the fed state) to create mixed micelles that can solubilise lipophilic molecules and correlations have been established for solubilisation by mixed micelles as a function of logP for neutral compounds (Dressman *et al.*, 2007).

The rate and extend of drug absorption from a solid dosage form during its transit through the small and large intestine depends on several factors: drug release and dissolution kinetics, stability and binding properties in the lumen, gastrointestinal transit time and intestinal permeability (Lennernäs, 2007). Permeation of compounds across the intestinal membrane is a complex and dynamic process. Two fundamental processes determine oral drug absorption: the drug's solubility or dissolution rate in gastrointestinal fluids and its gastrointestinal permeability through the lipid bilayers of the epithelial cells lining the intestinal wall. However, the permeability of a drug is not a simple two-step process of solubilisation and diffusion, but rather consists of a variety of complex molecular events (Pleuvry, 2005).

4.2.4 Different types of permeation routes

Most drugs have to cross membranes to exert their pharmacological effect. It is generally accepted that the main barrier to drug absorption is formed by the intestinal epithelium. Several routes can be followed to cross the epithelium and the type of route depends on the physicochemical properties of the drug. Drugs can cross the lipophilic membrane of the GIT by mechanisms such as passive diffusion (paracellular and transcellular), carrier-mediated process (facilitated and active) and endocytosis. The two passive transport routes entail either the transport of a compound through the cell membranes of the enterocytes (transcellular) or *via* the tight junctions between the enterocytes (paracellular) (Balimane *et al.*, 2000). 80-95 % of commercial drugs are absorbed primarily by passive diffusion (Mandagere *et al.*, 2002; Steinberg *et al.*, 1996).

4.2.4.1 Lipophilic and transcellular

When a drug follows passive transcellular transport, it diffuses through the highly lipophilic membranes of the GIT epithelium. This is the predominant route for lipophilic compounds (Lennernäs *et al.*, 1997). The diffusion processes are governed by Fick's law of diffusion (Martinez *et al.*, 2002). Transcellular passive diffusion is the most significant transport mechanism for intestinal drug absorption and the rate of transport is determined by the physicochemical properties of the epithelium, as well as the properties of the drug (lipophilicity) (Taub *et al.*, 2002).

4.2.4.2 Paracellular and hydrophilic

Generally hydrophilic molecules that are not recognised by a carrier are not able to partition into the lipophilic intestinal membrane and thus permeate the membrane through the paracellular route. Hydrophilic molecules permeate the intestinal wall through the paracellular route located at the tight junctions. The available surface area for paracellular intestinal absorption has been estimated to be about 0.01 % of the total surface area of the small intestine (Madara and Pappenheimer, 1987).

Paracellular permeation is mainly determined by the size and the number of pores at the tight junctions between the cells. Permeation is also influenced by the size and the charge of the drug (Mälkiä *et al.*, 2004). There are aqueous channels through which

small water-soluble molecules can pass. The ordered lipid bilayers make it impossible for these aqueous channels to be wide enough to admit drug molecules larger than approximately 200 Da or 0.4 nm (Martinez *et al.*, 2002; Balimane *et al.*, 2000; Pade and Stavchansky, 1997). Tight junctions restrict the transport of hydrophilic molecules *via* the paracellular pathway. The intestinal epithelium is composed of intercellular junctional complexes that varying tightness along the length of the gastrointestinal tract. The tightness of the intercellular junctional complex can be characterised in a leaky to tight epithelium by measuring transepithelium electrical resistance (TEER). The epithelium in the jejunum is relatively leaky, whereas the epithelium in the colon is tighter.

Larger compounds consequently take the transcellular route, which is also the main transport route for hydrophobic molecules. Therefore, drugs intended for oral administration must be sufficiently hydrophilic to dissolve in gastrointestinal fluid, but they must also possess sufficient hydrophobic character to undergo passive transport across the intestinal epithelia. It has been suggested that molecules limited to the paracellular route have lower absorption characteristics, whereas those using transcellular routes have better absorption characteristics (Pade and Stavchansky, 1997).

4.2.4.3 Active and other transport processes

Active transport processes require specific binding of the drug to a transporter protein and may entail transport into either the cells or efflux out of the cells (Mälkiä *et al.*, 2004). A few larger molecules are transferred across the intestinal membrane by a process called pinocytosis. During pinocytosis the cell membrane invaginates to form a vesicle around the extracellular molecule and transport it into the cell or to the other side (Pleuvry, 2005). Other influx and efflux mechanisms (*via* carriers) also exist.

4.2.5 Physicochemical properties of permeants

Factors such as the log P, hydrogen bonding, solute charge, electrostatics of lipid bilayers, solute size and order of the partition phase describe and influence drug partitioning into cell membranes. The permeability of the drugs varies as function of surface area to volume ratio and regional pH effects on ionisation (Mälkiä *et al.*, 2004).

The driving force for diffusion across the apical and basolateral membranes of the enterocytes is the soluble drug concentration gradient and for ionisable drugs this varies with the pKa, the pH and the pH profile between the intestinal compartments (Hendriksen *et al.*, 2003).

4.2.6 Predicting intestine permeation of compounds

The pharmacokinetic profile of a drug is dependent on the drug's ability to cross biological membranes. The permeability characteristics of compounds therefore affect its absorption, distribution and elimination (Mälkiä *et al.*, 2004). It is estimated that approximately 40 % of the failures in drug development programs during clinical phases are due to problems in pharmacokinetics and drug delivery (Kennedy, 1997).

The absorption of an orally administered compound depends on many different parameters, such as the chemical structure of the drug, permeability of the intestine brush border membrane (BBM), intestinal motility, gastrointestinal transit times, fluid volume, bile salt composition, enzyme systems, food and the physiological state of the human intestinal tissue. It is therefore clear that the absorption of a drug by the intestine is a complex process (Oulianova *et al.*, 2007; Martinez *et al.*, 2002).

It is important that the limiting factor to intestinal absorption can be modelled when using *in vitro* models to predict *in vivo* performance. Release of the active compound and absorption must occur within the available transit time. The drug must be released before reaching the absorptive site of the GIT and must be stable in the luminal fluids (Dressman and Reppas, 2000). The physicochemical properties of an orally administered drug are generally the major determinants of intestinal permeability. This includes molecular size and shape, pKa, lipophilicity (log P/log D), charge/ionisation and hydrogen bonding properties. Solubility of the compound in the intestinal tract is an extremely important factor that dictates the dissolution characteristics of the drug and eventually influences the bioavailability of the compound. Even though the underlying driver for solubility in the gastrointestinal fluids is the aqueous solubility of the drug, the solubility of the drug in the GIT may be influenced by pH profile, solubilisation *via* naturally occurring surfactants and food components, as well as complex formation with food and native components of the gastrointestinal contents (Dressman *et al.*, 2007).

4.2.6.1 *In vitro* methods

Good oral availability is dependent on maximum intestinal permeability and maximum solubility/dissolution of the drug at the site of absorption. The extent of *in vivo* absorption can therefore be predicted based on *in vitro* permeability and solubility measurements. The research of intestinal permeability represents an essential part in the prediction of the oral bioavailability of any new drug candidate (Trapani *et al.*, 2004). Due to the multiple processes involved in drug absorption in the intestine, it is often difficult to use just one *in vitro* model to accurately predict the *in vivo* permeability characteristics (Balimane *et al.*, 2000). Different *in vitro* methods exist to assess the intestinal permeability of potential drug candidates. Each *in vitro* method has its distinct advantages and disadvantages. General advantages of *in vitro* techniques for assessment of permeability are that it is less labour and cost-intensive compared to *in vivo* animal studies.

The successful application of *in vitro* models to predict drug absorption across the intestinal mucosa depends on how closely the *in vitro* model mimics the *in vivo* intestinal epithelium characteristics. Since it is very difficult to develop a single *in vitro* system that can simulate all the conditions existing in the human intestine, various *in vitro* systems are presently used as decision-making tools in early drug discovery.

4.2.6.1.1 Animal tissue

Excised animal tissue models have been used since the 1950s to explore the mechanism of absorption of nutrients from the intestine. Animal intestinal tissues consist of essentially the same kind of endothelial cells than humans. Various animal species are used for screening the permeability of newly discovered drugs, even though there are marketed interspecies differences across animal species such as *in vivo* drug solubility, gastric transit time, intestinal permeability, diet, pH differences and species-by-formulation interactions (Martinez *et al.*, 2002; Quastel, 1961).

4.2.6.1.2 Everted gut technique

Wilson and Wiseman (1954) introduced the everted gut technique in the 1950s to study the transport of sugars and amino acids from the mucosal to serosal side of the intestine. Everted sacs are usually prepared from rat intestine by quickly removing the intestine

from the decapitated animal, flushing it with a saline solution, everting it over a glass rod, filling it with fresh oxygenated culture medium and then dividing it into sacs (2-4 cm). The sacs (mucosal side outside) are submerged in a culture medium containing the drug of interest and accumulation of the drug in the inner compartment is measured. Under optimal conditions the sacs remain viable for up to 2 hours.

Advantages of this technique include that the sample volume on the serosal side is relatively small and drugs therefore tend to accumulate faster, it is relatively inexpensive and regional differences in drug absorption can be compared. The small volume inside the sac (serosal side) may however be a disadvantage, since sink conditions may be lost with well-absorbed chemicals. Other disadvantages include that compounds have to cross all the layers (including the muscle layer) of the small intestine instead of just the intestinal mucosa, the lack of active blood and nerve supply that can lead to a rapid loss of viability and everting the intestinal tissue may lead to morphological damage, causing misleading results (Bohets *et al.*, 2001; Wilson and Wiseman, 1954).

4.2.6.1.3 *In vitro* transport across intestinal segment

This *in vitro* method was first demonstrated by Ussing and Zerahn, using an Ussing chamber and it has been proved useful for investigations of *in vitro* transport and metabolism. This method engages the isolation of intestinal tissues, cutting it into strips of appropriate size and clamping it on a suitable device. The permeability of a compound is measured based on the appearance of drug in the serosal side of the chamber, rather than the disappearance of drug at the mucosal side (Ungell *et al.*, 1998; Ussing and Zerahn, 1951).

4.2.6.1.4 Isolated membrane vesicles

Membrane vesicles were first used in transport studies reported by Hopfer *et al.* (1973) and since then it has been used extensively in transport characterisation studies (Waclawski and Sinko, 1996; Sinko *et al.*, 1995; Madara and Trier, 1987). Brush border membrane vesicles can be isolated and prepared from humans and various animal species by either conducting intestinal scrapings or from isolated enterocytes.

The membrane vesicles prepared from intestinal tissues provide the flexibility of examining the interaction of drugs to a specific membrane of interest (e.g. brush border

membrane vs. basolateral membrane of enterocytes). Vesicles offer an opportunity to study the properties of drugs, nutrient transport at cellular level and allow a complete manipulation of solute environment both inside and outside of the vesicle. It is possible to isolate the transporter proteins that are specifically expressed on either the brush border or the basolateral side of the membranes.

Unfortunately, prepared vesicles are not 100 % pure and generally contain other membrane or organelle fragments. The process of isolation of vesicles often leads to damage of the transporter proteins and enzymes. A sensitive analytical method is required for this method of permeability study, since the volume of the vesicles is extremely small (Balimane *et al.*, 2000).

4.2.6.1.5 Artificial membranes

Artificial membranes, such as the Parallel Artificial Membrane Permeability Assay (PAMPA) system, mimics passive diffusion of the intestine and offer a potentially high throughput approach for the assessment of drug absorption potential, since the majority of drugs are mainly absorbed through passive transport. It consists of a filter-supported lipid membrane in a 96-well microtitre plate format and has gained acceptance by several pharmaceutical industries. Each well is divided into a donor and acceptor chamber, separated by a microfilter disc. Unfortunately, it is impossible to maintain sink conditions during this type of permeation experiments, due to the very small volume of the donor and acceptor compartments. It is a static method and, may reduce drug permeation rate and lead to its underestimation. It is therefore continuously subjected to new studies for further improvement and optimization (Corti *et al.*, 2006).

4.2.6.1.6 Cell-based

Cell culture models for drug absorption are based on the assumption that passage of drugs across the intestinal epithelium (a monolayer of cells) is the main barrier for drugs to reach the portal circulation. Varieties of cell monolayer models that mimic *in vivo* intestinal epithelium in humans have been developed e.g. Caco-2, HT-29, T-84, MDCK and LLC-Pk1 cells (Taub *et al.*, 2002; Balimane *et al.*, 2000).

Caco-2 cells are the most advanced and frequently used cell model to predict small intestine drug permeability and absorption (Borchardt, 1995; Artursson *et al.*, 1994;

Rubas *et al.*, 1993). Caco-2 cells are derived from human colon cancer cells (adenocarcinoma) that undergo spontaneous enterocytic differentiation in culture and can be cultivated to spontaneously differentiate into a monolayer of polarised cells. Although they are derived from a colon cancer, they acquire many features of absorptive intestinal cells during culture. They are grown on a semipermeable porous filter, and when they reach confluency the cell polarity, tight junctions, transporters, enzymes and microvilli are established. For transport experiments, test compounds are typically added to the apical side of the monolayer and appearance in the basolateral compartment is measured.

During the last 5 to 10 years there has been a tremendous growth in the use of Caco-2 cells for the screening of intestinal drug absorption and it has found wide acceptance as an intestinal permeability model. The predictability and utility of this model have been demonstrated many times; by many investigators and recently this permeability screening tool have been automated (Chong *et al.*, 1997; Artursson and Karlsson, 1991). A fully automated Caco-2 cell system allows much greater throughput (500 to 2000 compounds studies per month), without a proportional increase in resources. The use of 24-well monolayer (cell surface area 0.33 cm²) coupled with the use of LC/MS significantly reduced the amount of compound (no more than 50 µg) required to perform permeability experiments with this model (Balimane *et al.*, 2000).

However, its use as a high throughput-screening tool is limited by several factors. Caco-2 monolayers are considered a good model for compounds that are transported *via* the transcellular pathway, which usually include compounds with moderate lipophilicity. Therefore the permeation of more hydrophilic compounds that typically follow the paracellular route is often underestimated. Artursson *et al.* (1997) reported that the expression of the paracellular transport route, active transport routes and efflux systems is different in Caco-2 cell lines compared to the human intestine. Paracellular passage is not modelled well with low molecular weight, hydrophilic compounds (e.g. ranitidine, atenolol, furosemide, hydrochlorothiazide, etc.). This type of drugs shows poor diffusion in this cell model despite adequate absorption (greater than 50 % of dose) in humans. A great percentage of new drug candidates with poor aqueous solubility cannot be evaluated in this model, since the use of organic cosolvents is limited in this model. The integrity of tight junctions is easily compromised by commonly used organic solvents

(e.g. methanol, ethanol, PG, PEG, etc.) even at a minute concentration (more than 1-2 % v/v). Other shortcomings include the possibility of microbial contamination, being labour intensive, high costs, relatively high inter-experiment and inter-laboratory variations and long cell growth cycles (Corti *et al.*, 2005). The preparation of a fully functional cell monolayer generally requires a 3-week cell culture period with eight to nine laborious cell feedings. To increase experimental efficacy alternative cell lines have been introduced, requiring less than a week of culture by modifying both the coating material and growth media (Balimane *et al.*, 2000; Lentz *et al.*, 1998; Chong *et al.*, 1996).

Caco-2 cells are also known to over express the P-glycoprotein efflux system and this may result in the underestimation of the absorption of some compounds. Nonspecific drug binding to plastic devices may further lead to this underestimation of permeability. Huge variations in the results obtained from Caco-2 cell studies are documented in the literature. This variability may be attributed to factors such as cell passage number, culture time, type of support and medium (Trapani *et al.*, 2004). Despite these limitations and drawbacks, Caco-2 cell model is the most widely used intestinal cell culture models at present and it is providing valuable information to the decision making process in early drug discovery (Balimane *et al.*, 2000).

Tavelin *et al.* (1999) recently developed a new cell model that originates from foetal rat intestine. The cell line is called 2/4/A1 and exhibits similar passive absorption (passive transcellular and paracellular drug transport) than the human jejunum. This immortalised cell line forms viable, differentiated monolayers with tight junctions, brush border membrane enzymes, as well as the transporter proteins. Since the tight junctions in the Caco-2 cell line appear unrealistically tighter than the tight junctions in the endothelial cells in human intestine, the 2/4/A1 cells were proposed as a better model to study passively transported compounds *via* the paracellular route. Due to its lack of functional expression of active transport and efflux systems, the 2/4/A1 cell line only holds promise for characterisation of the passive absorption of drugs (Mälkiä *et al.*, 2004).

Madin-Darby canine kidney (MDCK) cells are another cell type used as a model to evaluate the intestinal transport characteristics of compounds. MDCK cell lines are derived from dog kidney. The distal tubular part of the nephron are used to differentiate

into columnar epithelial cells and to form tight junctions (like Caco-2 cells) when cultured on semipermeable membranes (Irvine *et al.*, 1999; Cho *et al.*, 1989). The advantage of these cells is that they form tight junctions after a culture time of only 3 days (Bohets *et al.*, 2001).

4.2.6.2 *In situ* methods

In situ perfusion involves the use of intestinal segments/sacs of animals (rats, rabbits, pig, dog, frog and monkey) and sometimes humans, to study the permeability and absorption kinetics of drugs (Trapani *et al.*, 2004). *In situ* experiments for studying intestinal drug absorption involve the perfusion of a drug solution through isolated cannulised intestinal segments. The hydrodynamics of the buffer flow through the intestinal segments may influence the absorption characteristics of the drugs. This technique uses the difference in inlet and outlet flow concentration to calculate the permeability. Absorption is assessed based on the disappearance of drug from the intestinal lumen. Drug absorption will therefore be overestimated if the drug extensively accumulates in the intestinal tissue or if significant drug metabolism occurs (Balimane *et al.*, 2000).

The advantages of the *in situ* system compared to *in vitro* techniques include the presence of an intact blood and nerve supply in the experimental animals and the barriers that compounds have to cross to reach the portal blood circulation are identical in the *in situ* and *in vivo* situation (Balimane *et al.*, 2000). Drug absorption in different regions of the small intestine can also be evaluated. Various modifications of the perfusion technique exist: single pass perfusion (Amidon *et al.*, 1981), recirculating perfusion (Tsuji *et al.*, 1978), oscillating perfusion and the closed loop method (Doluisio *et al.*, 1969). This methodology is found to be highly accurate for predicting the permeability of passively transported compounds, however the use of a scaling factor has been recommended for predicting permeability of carrier-mediated compounds (Lennernäs, 1998).

Despite its advantages, the use of single pass perfusion method is severely limited due to high costs, as it requires a large number of animals to get statistically significant absorption data and relatively high amounts of test compounds are required to perform

studies (>10 mg), which is not feasible in early drug discovery. It therefore shows low potential for high throughput screening strategies. Furthermore, the surgical manipulation of intestine combined with anaesthesia may cause significant changes in the blood flow to the intestine and may have an effect on the absorption rate of the test compounds (Balimane *et al.*, 2000; Uhing and Kimura, 1995).

4.2.6.3 *In vivo* methods

In vivo methods for evaluation of drug absorption are commonly used, even though it is extremely time and resource intensive due to experimental animal work and bioanalysis of a large number of samples. It is based on the similarity of the composition of epithelial cell membranes of mammals and the fact that absorption is essentially an interaction between the drug and the biological membrane. These studies should be performed with caution due to potential species differences that may affect the drug absorption such as pH, GI motility, transit time and differential distribution of enzymes and transporters. Comparison of AUC (plasma concentration vs. time curve) values after intravenous, oral and intraportal (or intraperitoneal) administration can often indicate the absolute extent of *in vivo* drug absorption. This method is able to differentiate well absorbed compounds from reduced bioavailable compounds (Balimane *et al.*, 2000).

4.2.6.4 *In Silico* methods

In silico methods are based on computational or virtual screenings of physico-chemical properties (such as lipophilicity, H bonding capacity, molecular size, polar surface area (PSA) and quantum properties) of drugs to predict their oral absorption potential. A variety of computational models exists, due to the increase in computer resources over the past decades. The *in silico* predictive models minimise the time consuming steps of synthesis and save time by enabling screening of drug candidates prior to synthesis. These methods are very attractive, since intestinal absorption can be predicted before the synthesis of compounds, but at present their predictive power is unsatisfactory compared to experimental models (Corti *et al.*, 2006; Balimane *et al.*, 2000).

To develop reliable and valid computational methods it is important that the quantitative structure-activity relationship (QSPR) models should be based on experimental data that

were obtained from a diverse set of compounds with respect to their properties (e.g. physicochemical and pharmacological) and chemical structures. At present, most reports involve *in silico* modelling studies performed on compounds closely related in structure, thus making the model ineffective when applied to a wider structurally and diverse data set (Balimane *et al.*, 2000).

Lipinski and co-workers (2001) developed one of the best-known computational models to predict intestinal absorption from molecular properties. It is based on the physicochemical properties of 2200 drug candidates that had entered clinical phase II trials. They formulated a simple rule, taking into account hydrogen bonding, molecular weight and lipophilicity. This so-called rule-of-five (ROF) states that a compound is likely to have limited oral bioavailability due to low intestinal epithelial permeability or solubility if it generates two or more alerts. The ROF is produced if the number of H-bond donors or acceptors in a compound exceeds 5 and 10 respectively; the molecular weight exceeds 500, or the lipophilicity expressed as the logarithm of the octanol-water partition coefficient (log P) exceeds 5. Weaknesses of the ROF include that it does not apply to compounds that are subject to active transport processes. It is mainly built on compounds with a high intestinal absorption and it gives relatively rough and sometimes false predictions (Bohets *et al.*, 2001; Lipinski *et al.*, 2001).

Absorption is a very complex process that cannot be estimated accurately from structure alone. Several companies have therefore attempted to combine parameters that affect intestinal absorption in commercial available packages (Bohets *et al.*, 2001).

4.2.6.5 Summary

Using *in silico* methods to predict intestinal permeability is attractive because of their high throughput capacity, minimum usage of resources and manpower and reproducibility to predict passive drug transport. However, they are often limited in scope and do not take into account physiological factors such as drug-membrane interactions, that can completely override the physicochemical properties of potential drugs. This lack of real physiological conditions leads to predictions based only on the chemical properties of the drug and thus making it vulnerable to false predictions (Balimane *et al.*, 2000).

Due to modern isolation technology and high throughput biological screen capability a reliable, high throughput method for evaluating intestinal permeability is essential. Many researchers have attempted to predict the oral absorption of drugs by using animal studies and different *in vitro*, *in vivo* and *in situ* models for drug permeability (Oulianova *et al.*, 2007). These systems have presented a compromise either between high throughput with low predictive potential or low throughput with high predictive potential (Youdim *et al.*, 2003).

4.2.7 Biopharmaceutics Classification System (BCS)

BCS was introduced in the mid-1990s to function as a scientific framework for classifying active pharmaceutical ingredients, based upon their aqueous solubility and intestinal permeability. The Biopharmaceutics Classification System (BCS) classifies drugs in to four different classes according to their aqueous solubility and intestinal permeability characteristics (rate-limiting biopharmaceutical factors). The classes are as follows:

- Class 1: High solubility, high permeability
- Class 2: Low solubility, high permeability
- Class 3: High solubility, low permeability
- Class 4: Low solubility, low permeability (WHO website (b))

The dissolution of the active pharmaceutical ingredient (API) combined with the permeability and solubility properties of an API are the three major factors that govern the rate and extent of drug absorption from an immediate release (IR) solid dosage form. With respect to dissolution properties, IR dosage forms can be categorised as having “very rapid”, “rapid”, or “not rapid” dissolution characteristics (WHO website (a); HHS/FDA Guidance for Industry, 2000). Depending on the classification, the oral availability of the compound may be expected to rely heavily on the formulation and manufacturing method, e.g. class 2 (low solubility, high permeability) or mostly dependent on the permeability properties, e.g. class 3 (highly solubility, low permeability).

The FDA issued a guidance for the industry in 2000 on waivers of *in vivo* bioavailability and bioequivalence studies based on the BCS (HHS/FDA Guidance for Industry, 2000). These industry guidelines classify under which circumstances bioavailability and/or bioequivalence studies may be replaced/waived (not considered necessary for product

approval) by *in vitro* bioequivalence testing. On the basis of solubility and permeability of the API and dissolution characteristics of the dosage form, the BCS approach therefore provides an opportunity to waive *in vivo* pharmacokinetic bioequivalence testing for certain categories of IR products. A biowaiver is allowed for drugs with high solubility and high permeability (class 1 compounds) and that are formulated in solid IR oral formulations. These IR compounds are candidates for *in vitro* assessment of bioequivalence, as long as they are stable in the gastrointestinal tract and have a wide therapeutic index (Lennernäs, 2007). Instead of conducting expensive and time consuming *in vivo* studies, an *in vitro* test could therefore be adopted as the surrogate basis for the decision as to whether the two pharmaceutical products are equivalent. This can reduce time and costs during the development process and reduce unnecessary drug exposure to healthy volunteers in bioequivalence studies (FDA Guidance for Industry, 2000).

4.2.7.1 Class boundaries

4.2.7.1.1 High solubility

A drug is considered highly soluble when the highest dose recommended by World Health Organisation (WHO) (if the drug appears on the WHO Model List of Essential Medicines) or highest dose strength available on the market as a oral solid dosage form (if the drug does not appear on the WHO Model List of Essential Medicines) is soluble in 250 ml or less of aqueous media over the pH range of 1.2 to 6.8. The pH-solubility profile of the API should be determined at $37 \pm 1^\circ\text{C}$ in aqueous media. A minimum of three replicate determinations of solubility at each pH condition is recommended. Initial recommendations in the BCS Guidance suggested that the solubility should be measured over a pH range of 1.2 to 7.5. Successive scientific discussions and publications suggested that a pH range of 1.2 to 6.8 is more appropriate (HHS/FDA Guidance for Industry, 2000).

4.2.7.1.2 High permeability

A drug is considered highly permeable when its extent of absorption in humans is 85 % or more based on a mass balance determination, or in comparison to an intravenous comparator dose. Initial recommendation in the BCS Guidance suggested an absorption value of >90 % as a prerequisite for classification as highly permeable. However, successive scientific discussions and scientific publications suggested lowering the criteria to 85 % absorption. Acceptable test methods for permeability determination of the drug substance include:

- 1) *in vivo* intestinal perfusion in humans, or
- 2) *in vitro* permeation, using excised human or animal intestinal tissue

When one of these two alternative methods is used for permeation studies, suitability of the methodology should be demonstrated, including determination of permeability relative to the permeability of a reference compound whose fraction dose absorbed has been documented to be at least 85 %. Supportive data can be provided by the following additional test methods:

- 1) *in vivo* or *in situ* intestinal perfusion, using animal models, or
- 2) *in vitro* permeation across a monolayer of cultured epithelial cells (e.g. Caco-2), using a method validated, using APIs with known permeabilities

In vivo, *in situ* animal models or *in vitro* models (cell lines) are only considered appropriate for passively transported drugs. The data from either of the two latter methods would not be considered acceptable on a standalone basis (HHS/FDA Guidance for Industry, 2000; WHO website (a)).

A considerable amount of human permeability data exist. This database has played an important role in classifying drugs in specific BCS categories, which can be used to set bioequivalence standards for drug product approval. Since these permeability data are expensive and time consuming, alternative models for prediction of drug absorption and permeability are needed and explored. The BCS Guidance lists *in vitro* permeation using excised human intestinal tissue as one possible permeability classification tool.

4.2.7.1.3 Rapidly dissolving

A drug product is considered to be rapidly dissolving when ≥ 85 % of the labelled amount of drug substance dissolves within 30 minutes using United States Pharmacopeia (USP)

apparatus I (basket, 100 rpm) or II (paddle, 50 rpm) in a volume of ≤ 900 ml buffer solutions (HCl or simulated gastric fluid, pH 4.5 buffer and pH 6.8 buffer or simulated intestinal fluid) (HHS/FDA Guidance for Industry, 2000).

4.2.8 Drugs

Examples of drugs that are already BCS classified were obtained from Aspen Pharmacare (South Africa), for the permeability study (Fig. 44):

Class 1 (high solubility, high permeability):

- Zidovudine
- Propranolol HCl

Class 3 (high solubility, low permeability):

- Didanosine
- Enalapril Maleate

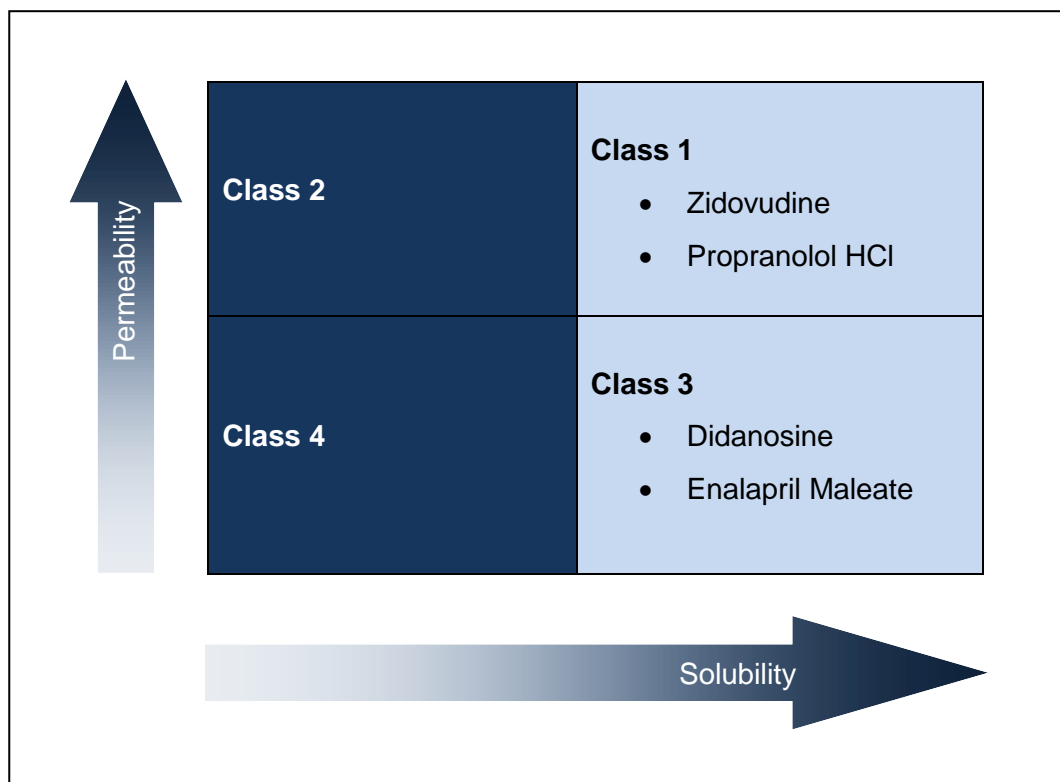


Fig. 44. Drugs obtained from Aspen Pharmacare (South Africa), with their BCS classification

Class 1 and 3 drugs were selected, since dissolution is rate limiting to absorption in the case of class 2 and 4 drugs. Official apparatus for *in vitro* drug dissolution testing are already available and formal guidelines already exist, which provide useful recommendations for their correct use (FDA Guidance for Industry, 1997). Solubility screening is relatively routine and techniques ranging from simple turbidity-based measurements to true equilibrium solubility measurements are well established (Ingels and Augustijns, 2003; Saha and Kou, 2002; Krishna *et al.*, 2001). It is well documented that low dissolution/solution issues can be surmounted by the incorporation of cosolvents, surfactants, or complexation agents into the transport buffer to aid solubilisation (Takahashi *et al.*, 2002; Yamashita *et al.*, 2000). The earlier studies on vaginal and skin permeation showed that the permeability barriers of biological membranes can be overcome by formulation, chemical enhancers and increased temperature. It was therefore beyond the scope of this study to examine class 2 and 4 drugs. The absorption of class 1 drugs is rapid and complete, with extents of absorption ≥ 85 %. Permeation across the intestinal membrane is the rate limiting step of the absorption process for class 3 drugs.

4.2.8.1 Zidovudine (AZT)

Zidovudine or azidothymidine (AZT, also called ZDV) was the first approved antiretroviral drug and is still widely used for treatment against HIV and human T-cell immunodeficiency virus (HIV-I and -II). Its chemical formula is $C_{10}H_{13}N_5O_4$ (3'-azido-3'-deoxythymidine) and it has a molecular mass of 267.24 g/mol (Figs. 45 and 49).

Like other reverse transcriptase inhibitors, AZT works by inhibiting the action of reverse transcriptase, the enzyme that HIV uses to make a DNA copy of its RNA. The viral double-stranded deoxyribonucleic acid (DNA) is subsequently spliced into the DNA of a target cell, where it is called a provirus. The azido group increases the lipophilic nature of AZT, allowing it to easily cross cell membranes by diffusion and thereby to cross the blood-brain barrier. Cellular enzymes convert AZT into the effective 5'-triphosphate form. Previous studies have shown that the termination of the formed DNA chains is the specific factor in the inhibitory effect (Brunton *et al.*, 2006).

The main limitation to therapeutic effectiveness of AZT is its dose dependent haematological toxicity. It is rapidly absorbed from the gastrointestinal tract with a peak plasma concentration of 1.2 µg/ml, at 0.8 h. It is also rapidly metabolised to the inactive glucuronide, with a mean elimination half-life ($t_{1/2}$) of 1 h, thus necessitating frequent administration of large doses to maintain therapeutic drug levels (Thomas and Panchagnula, 2002). Since AZT has a narrow therapeutic index, side effects occur frequently. AZT is absorbed rapidly and almost completely, which leads to very high initial plasma concentrations and consequently high incidences of toxicity.

Huang *et al.* previously reported that zidovudine permeates the intestine by a Na⁺/nucleoside carrier (Huang *et al.*, 1994). An *in vitro*, brush border membrane vesicle (BBMV) study by Oulianova *et al.* (2007) confirmed these results.

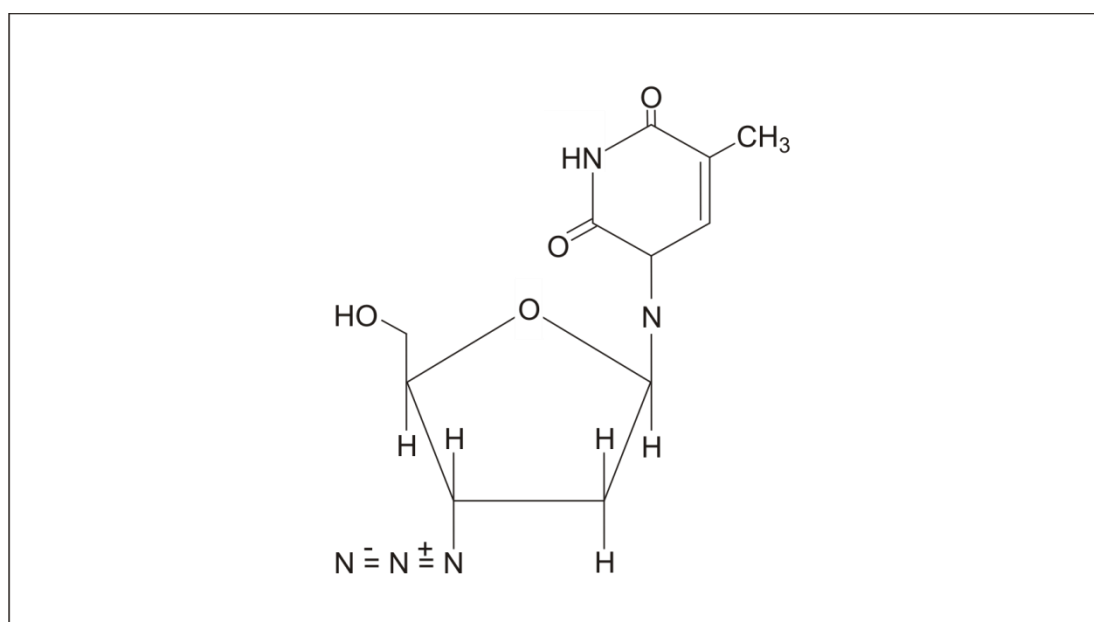


Fig. 45. Zidovudine (The Merck Index, 2001)

4.2.8.2 Propranolol hydrochloride

Propranolol hydrochloride is a commonly used non-selective β -blocker that is prescribed in the management of hypertension, myocardial infarction, angina pectoris, phaeochromocytoma and cardiac arrhythmias. Its chemical formula is C₁₆H₂₁NO₂.HCl and it has a molecular mass of 295.81 g/mol (Figs. 46 and 49).

Propranolol is almost completely absorbed after oral administration (>90 %). Peak plasma concentrations are reached within 1-2 h after administration of a single dose. Propranolol is almost completely metabolised in the liver and the main metabolites are metabolised by cytochrome P450. The half-life is about 4 h. The pKa value of propranolol is approximately 9.5 and it is highly soluble at a pH of 7.2. It has a solubility of 0.1 g/ml water and has two polymorphic forms (Brunton *et al.*, 2006).

Propranolol is predominantly absorbed by a passive diffusion (transcellular) mechanism and has a high partition coefficient (Trapani *et al.*, 2004; Yamashita *et al.*, 1997). Solubility will therefore not be the rate-limiting step in the absorption process in the GIT. Factors influencing permeability *in vitro* versus *in vivo* will be limited, since propranolol is a highly lipophilic and since it is transported transcellularly. The high permeability is in line with the reported high oral absorption (>90 %) (Vogelpel *et al.*, 2004).

It is often used as a marker in permeability studies, because of the foregoing properties. It has neither affinity for cellular efflux or influx systems and is highly soluble in aqueous buffers (Motz *et al.*, 2007).

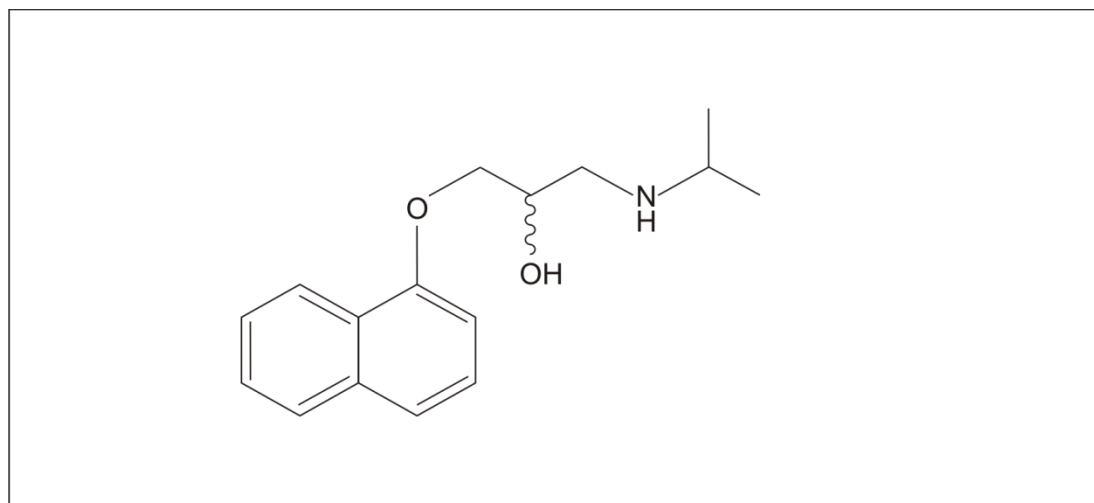


Fig. 46. Propranolol (The Merck Index, 2001)

4.2.8.3 Didanosine

Didanosine (2'-3'-dideoxyinosine, ddi) is a reverse transcriptase inhibitor and used in combination with other antiretroviral drug therapy as part of highly active antiretroviral

therapy (HAART). Its chemical formula is $C_{10}H_{12}N_4O_3$ and has a molecular mass of 236.23 g/mol (Figs. 47 and 49).

Didanosine is a nucleoside analogue of the purine nucleoside inosine. It differs from other nucleoside analogues, because it does not have any of the regular bases. Instead, it has hypoxanthine attached to the sugar ring. Within the cell, ddl is phosphorylated to the active metabolite 2',3'-dideoxyadenosine-5'-triphosphate (ddATP) by cellular enzymes. The active metabolite inhibits viral reverse transcriptase and acts as chain terminator of the proviral DNA. Like other anti-HIV nucleoside analogs, it acts as a chain terminator by incorporation and it inhibits viral reverse transcriptase by competing with natural dATP (Brunton *et al.*, 2006).

Its oral bioavailability is 40 %, but it is variable and dose-dependent. Didanosine has a $t_{1/2}$ of 1.4 ± 0.3 h and its metabolic pathways have not been fully evaluated in humans. The water solubility of didanosine is approximately 30 mg/ml when the pH is below the pKa of 9.1 and the solubility increases to >400mg/ml when the pH exceeds 10 (Anderson *et al.*, 1988a). Even though didanosine has good solubility, poor intestinal permeability may hinder oral absorption of the drug. It has weak acid stability and is easily damaged by the stomach acid. Therefore, the original formula approved by the FDA was chewable tablets that included an antacid buffering compound to neutralize stomach acids (Brunton *et al.*, 2006).

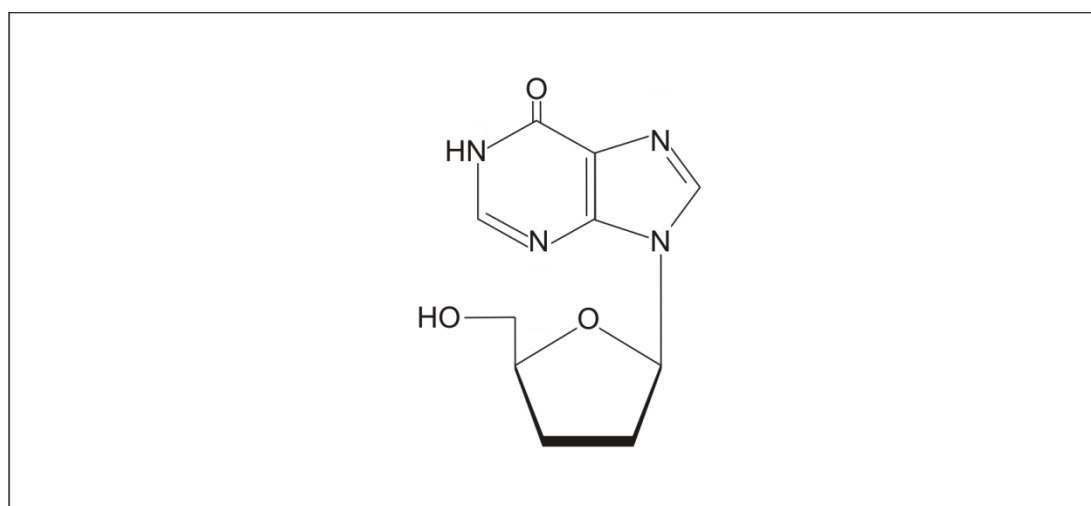


Fig. 47. Didanosine (The Merck Index, 2001)

4.2.8.4 Enalapril Maleate

Enalapril (L-proline, 1-[N-(ethoxycarbonyl)-3-phenylpropyl]-L-alanyl-(S)) maleate is an angiotensin converting enzyme (ACE) inhibitor used in the treatment of hypertension and some types of chronic heart failure. Enalapril maleate is the maleate salt of enalapril, the ethyl ester of a long-acting angiotensin converting enzyme inhibitor, enalaprilat. Its chemical formula name is $C_{20}H_{28}N_2O_5 \cdot C_4H_4O_4$ and molecular mass is 492.52 g/mol (Figs. 48 and 49).

Enalapril maleate was the first member of the group of ACE inhibitors known as the dicarboxylate-containing ACE inhibitors and can be administered orally and intravenously. The drug acts by inhibiting the conversion of angiotensin I to angiotensin II (a powerful vasoconstrictor).

Enalapril maleate has an oral bioavailability of about 60 % (not reduced by food). Enalapril has a half-life of only 1.3 hours, but enalaprilat has a plasma half-life of about 11 hours, due to the tight binding to ACE. As a prodrug, enalapril is metabolised through hepatic metabolism to the active form enalaprilat by various esterases. Enalaprilat is the active metabolite responsible for the therapeutic action. It is a dipeptide with a proline peptide bond and can exist as rotamers. The interconversion between cis- and trans-configurations may lead to poor chromatographic properties and prevents generation of a single sharp peak for quantitative analysis (Lee *et al.*, 2003).

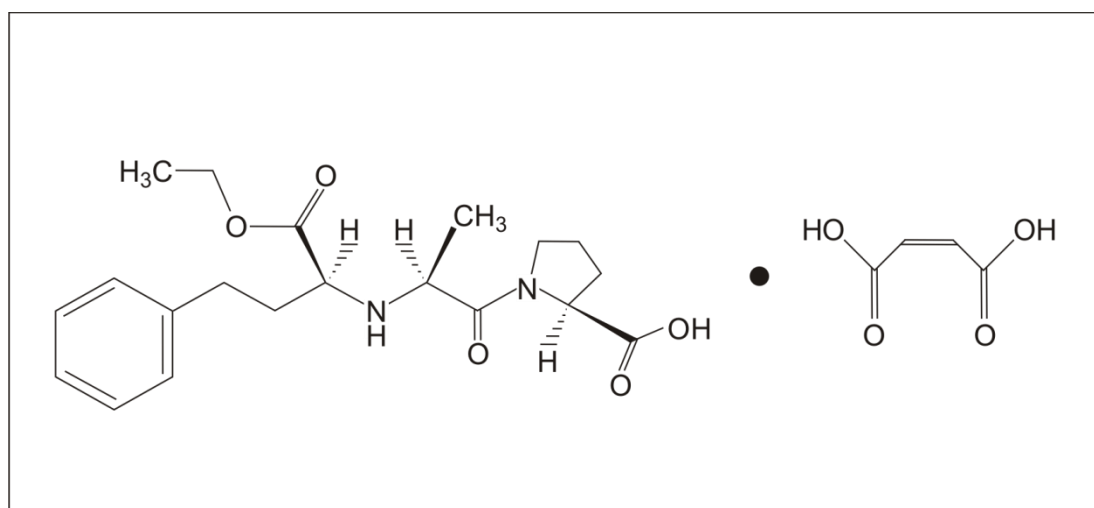


Fig. 48. Enalapril maleate (The Merck Index, 2001)

Drugs	AZT	propranolol HCl
therapeutic category	antiviral	anti-hypertensive, antianginal, antiarrhythmic
octanol/water partition coefficient	1	3.26
pKa	9.68	9.5
solubility	<ul style="list-style-type: none"> ▪ water (1 in 40) ▪ alcohol (1 in 14) 	<ul style="list-style-type: none"> ▪ water (0.1 g/ml) ▪ alcohol (1 in 20) <p>Practically insoluble in:</p> <ul style="list-style-type: none"> ▪ ether, benzene, ethyl acetate
MW (g/mol)	267.24	295.81

Drugs	didanosine	enalapril maleate
therapeutic category	antiviral	anti-hypertensive
octanol/water partition coefficient	0.043	<ul style="list-style-type: none"> ▪ Cis isomer: -0.46 ▪ Trans isomer: -0.65
pKa	9.1	pKa ₁ 3.0 pKa ₂ (25°) 5.4
solubility	<ul style="list-style-type: none"> ▪ water (1 in 40) ▪ alcohol (1 in 1) 	<ul style="list-style-type: none"> ▪ water (high: 25 mg/ml) ▪ alcohol (80 mg/ml)
MW (g/mol)	236.23	492.52

Fig. 49. Physicochemical characteristics of AZT, propranolol HCl, didanosine, enalapril maleate (Brunton *et al.*, 2006; The Merck Index, 2001; Dollery *et al.*, 1999)

4.2.9 Aim

Due to modern high-throughput technologies, large numbers of compounds are produced by parallel synthesis and combinatorial chemistry. The pharmaceutical industry therefore requires rapid and accurate methods for screening drugs leads for intestinal permeability potential in the early stages of drug discovery (Hamalainen and Frostell-Karlsson, 2004). Approximately 50 % of all investigational new drugs fail in pre-clinical and clinical phases of development due to inadequate absorption, distribution, metabolism, excretion and/or toxicity (Hamalainen and Frostell-Karlsson, 2004). This may be decreased by applying efficient *in vitro* screening methods earlier in the discovery process. It is important to decide as early possible on the most promising compound and physical formulation for the intended route of administration. Reliable *in vitro* models can be applied to determine permeation of the test compounds, which will help avoid the wasting of valuable resources on the development of drugs that are destined to fail in preclinical and clinical phases due to insufficient absorption properties. Several methods have been proposed and investigated for *in vitro* evaluation and prediction of gastrointestinal permeability, but no official method is available at present due to certain limitations (Corti *et al.*, 2006). The BCS allows a biowaiver for solid, IR, oral formulations with high solubility and high permeability (class 1 compounds). These IR compounds are candidates for *in vitro* assessment of bioequivalence, as long as they are stable in the gastrointestinal tract and have a wide therapeutic index (Lennernäs, 2007).

The aim of the study was to investigate the permeability of excised human small intestine mucosa to 4 different oral, dosage drugs using our flow-through diffusion system. The 4 drugs were selected as representative model compounds of drug classes 1 (high solubility, high permeability) and 3 (high solubility, low permeability) according to the Biopharmaceutics Classification System (BCS). Furthermore, the objectives of the study were to determine the suitability of our *in vitro* diffusion model to evaluate and predict *in vivo* gastrointestinal drug permeability and to compare it with the BCS permeability classification. Also to establish a standard procedure of determining drug permeability class that may potentially be used for the establishment of drug product bioequivalence standards.

4.3 Materials and Methods

4.3.1 Small intestine mucosa

Six human jejunal specimens were obtained from excess tissue removed from 2 female and 4 male patients, mean age 43 ± 9 SD (range:33-54) yr, following various surgical procedures at Tygerberg Hospital, Bellville, South Africa. No specimens were obtained where there was clinical evidence of any disease that might have influenced the permeability characteristics of the intestine.

All jejunal specimens were immediately placed in a transport fluid after removal and kept at 4 °C. The transport fluid consisted of a stock solution of Eagle's Minimum Essential Medium (MEM) without L-glutamine and sodium bicarbonate (Gibco, Paisley, Scotland), to which the latter as well as an antibiotic (penicillin/streptomycin, 100 IU/ml) and an antimycotic (amphotericin-B, 2.5 µg/ml) were added prior to using it for the transport of tissue specimens. The intestine tissue was transferred to our laboratory within 2 hours. At the laboratory the serosal, muscularis externa and excess connective tissue were trimmed away using a forceps and scalpel. Specimens from each patient were snap-frozen in liquid nitrogen and stored at -85 °C. The frozen mucosal specimens were equilibrated in PBS (pH 7.4) for 30 min at room temperature to thaw completely prior to the start of each experiment, after which they were carefully cut into 5 X 5 mm specimens, so as not to damage the epithelial surfaces.

The Ethics Committee of Stellenbosch University and the Tygerberg Academic Hospital approved the study. Project number: 95/019 (resubmitted and approved on 28/7/03).

4.3.2 Drugs

Examples of drugs that are already classified (BCS) as high and low permeability drugs (class 1 - high solubility, high permeability and class 3 - high solubility, low permeability) were supplied by Aspen Pharmacare (South Africa):

Class 1:

- Zidovudine
- Propranolol HCl

Class 3:

- Didanosine
- Enalapril Maleate

4.3.3 Permeability experiments

The specimens of jejunal mucosa were mounted in flow-through diffusion cells (exposed circular areas 0.039 cm^2) and each permeation study was performed on 7 tissue replicates from each patient (Van der Bijl *et al.*, 2003). The mucosal tissue was mounted between the donor and acceptor compartments and the epithelial/mucosal side faced towards the donor compartment. Tissue disks were equilibrated for 10 min in PBS (pH 7.4) at 37 °C in both the donor and acceptor compartments of the diffusion cells before each permeability experiment started. This step was followed by the removal of the PBS from the donor compartment and the addition of 0.5 ml of PBS containing 5 mg/ml zidovudine, propranolol hydrochloride, didanosine or enalapril maleate. The drug-containing PBS solutions were prewarmed to 37 °C.

The donor compartments were covered with adhesive tape to prevent evaporation. Some patients' samples were investigated from 0-6 h, while other patients' tissues were used from 6-24 h. The reasons for this division were mainly to save consumables and money. PBS at 37 °C was pumped through the acceptor chambers at a rate of 1.5 ml/h and collected by means of a fraction collector, at 2-h intervals for the 6 h experiment and at 6-h intervals for the 6-24 h experiment. The total time of the 2 experiments was 24 h. The zidovudine, propranolol, didanosine or enalapril in the acceptor chambers was quantified by means of LC/MS analysis.

Intra- and inter-experiment variations were calculated for the AZT, propranolol, didanosine and enalapril experiments ($\text{SD/mean} \times 100$).

4.3.4 LC/MS detection of zidovudine, propranolol, didanosine and enalapril

Permeant-containing effluent samples, collected from the acceptor compartment of the perfusion apparatus over the 2-24 h sampling intervals were analysed using a LC/MS. Determination was conducted under the following LC/MS conditions:

LC: Waters 2695 Separations Module (Alliance)

MS: Waters API Quattro Micro, electrospray positive

The standards of the four selected drugs were dissolved in the same PBS as the drug containing samples collected from the flow-through diffusion system to prevent matrix suppression. Fresh or new standards were made for each experiment and all the samples were analysed on one day. Standards were also injected before and after the sample analyses to monitor variation between different days.

The coefficient of determination was better than 0.99 for all the analytes (R^2) and the reproducibility of 6 injections from standards from 6 different vials were better than a % RSD of 2 % for all analytes.

4.3.4.1 Zidovudine (AZT)

Column: Waters Xbridge C18 3.5 μ m, 2.1 x 50 mm

Mobile phase: Solvent A: 0.01 % trifluoro acetic acid, Solvent B: acetonitrile

Injection volume: 10 μ l; Retention time: 5.03 min; Flow rate: 0.35 ml/min

Gradient: Start at 100 % solvent A, hold for 0.5 min, followed by a linear gradient to 100 % B over 7.5 min, hold at 100 % B for 1 min followed by re-equilibration to initial conditions.

MS conditions: Two MRM transitions were monitored, the primary at 268.1>127 at a collision energy of 20 eV and Cone voltage of 15 V, and the second 268.1>142.1 at a collision energy of 10 eV and Cone voltage of 15 V conditions. The calibration curve and chromatogram of AZT are shown in Figs. 50 and 51.

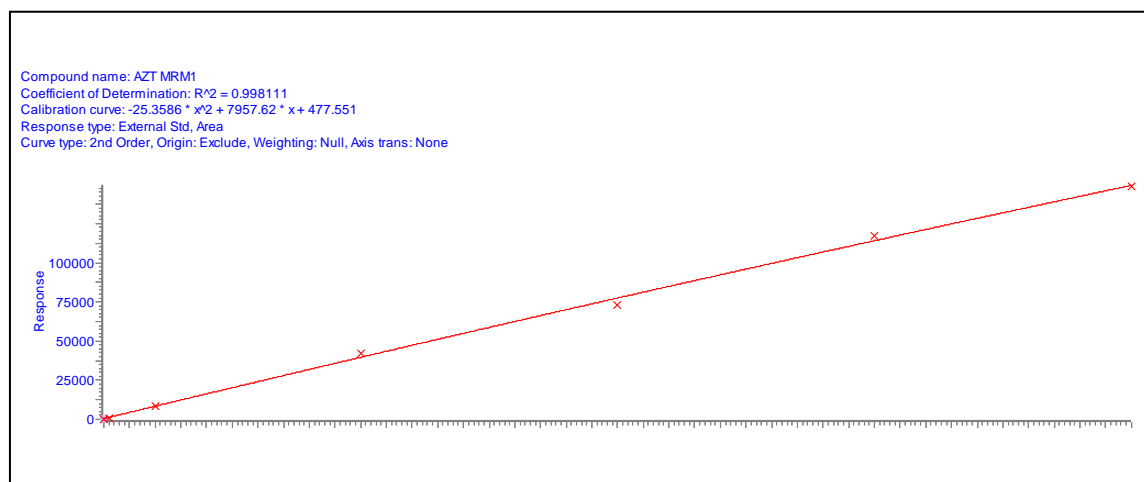


Fig. 50. Calibration curve of AZT

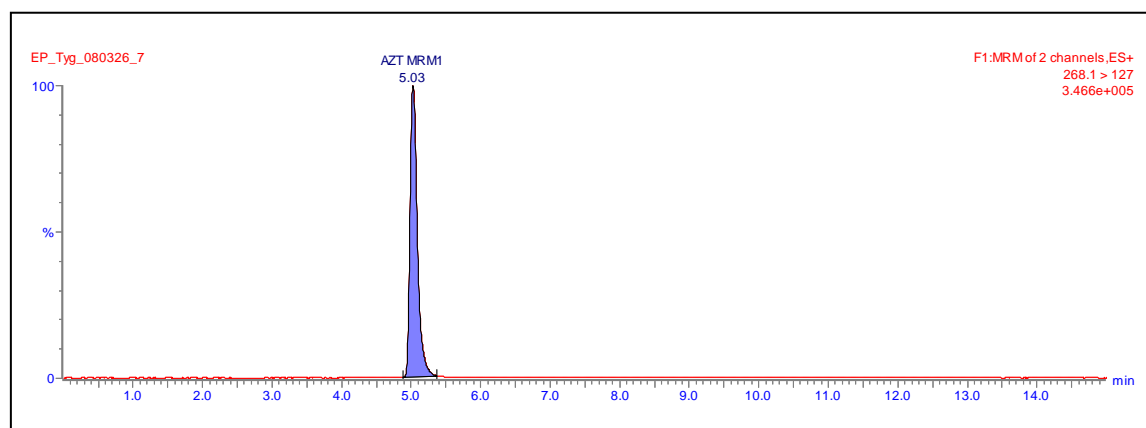


Fig. 51. Chromatogram of AZT

4.3.4.2 Propranolol

Column: Waters Atlantis dC18 3 μm , 2.1 x 150 mm

Mobile phase: Solvent A: 1 % formic acid, Solvent B: acetonitrile

Injection volume: 4 μl ; Retention time: 7.55 min; Flow rate: 0.2 ml/min

MS conditions: Two MRM transitions were monitored, the primary at 260.2>116 at a collision energy of 15 eV and Cone voltage of 18 V, and the second 260.2>183 under the same conditions.

HPLC Gradient: Start at 95 % solvent A, hold for 0.5 min, followed by a linear gradient to 100 % B over 1.5 min, hold at 100 % B for 2 min followed by re-equilibration to initial conditions. The calibration curve and chromatogram of propranolol are shown in Figs. 52 and 53.

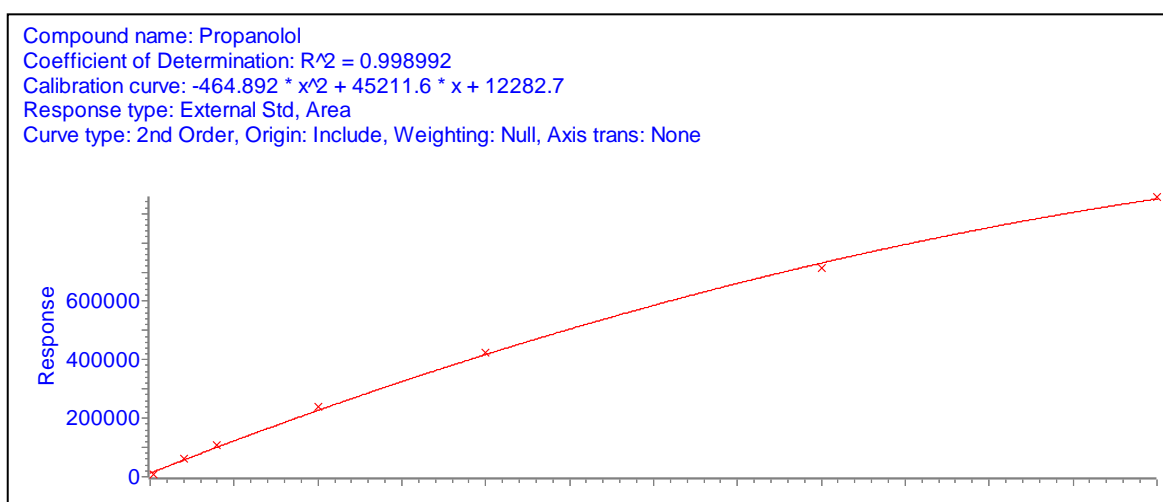


Fig. 52. Calibration curve of propranolol

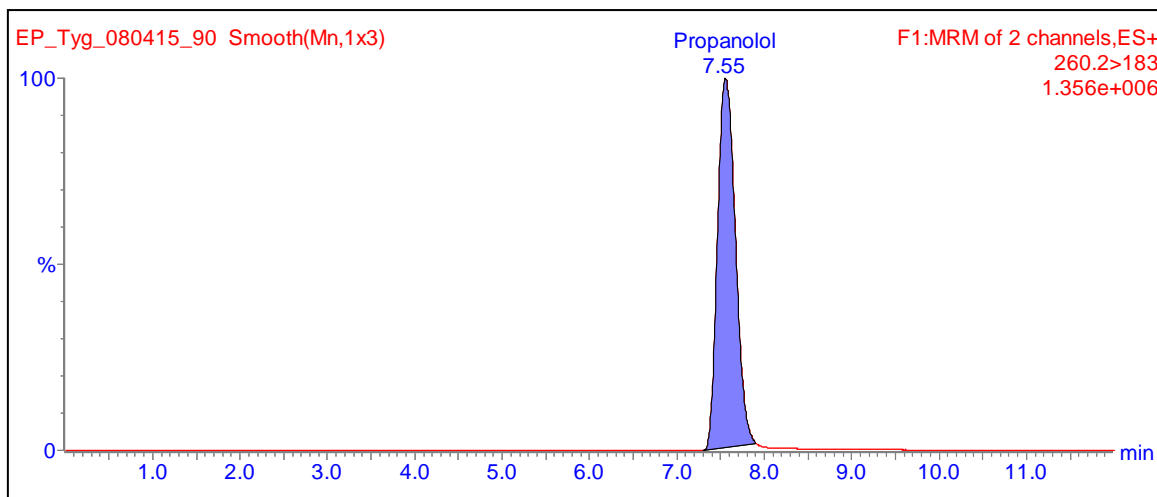


Fig. 53. Chromatogram of propranolol

4.3.4.3 Didanosine

Column: Waters Xbridge C18, 2.1 x 50 mm

Mobile phase: Solvent A: 0.01 % TFA acid, Solvent B: acetonitrile

Injection volume: 4 µl; Retention time: 4.69 min; Flow rate: 0.35 ml/min

Gradient: Start at 100 % solvent A, hold for 0.5 min, followed by a linear gradient to 20 % B over 3.5 min and another linear gradient to 100 % B over 1.5 min, hold at 100 % B for 0.5 min, followed by re-equilibration to initial conditions.

MS conditions: One MRM transition was monitored 237>137 a collision energy of 15 eV and Cone voltage of 15 V. The calibration curve and chromatogram of didanosine are shown in Figs. 54 and 55.

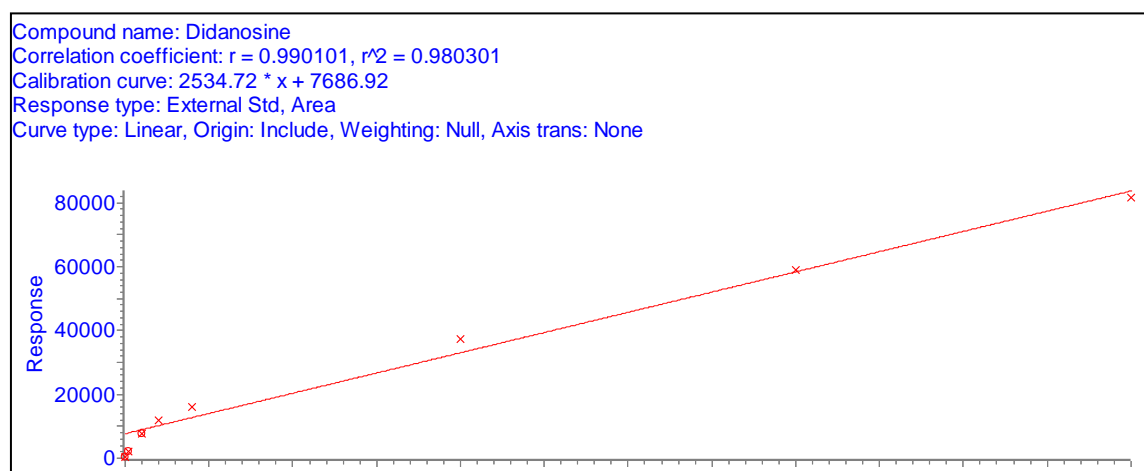


Fig. 54. Calibration curve of didanosine

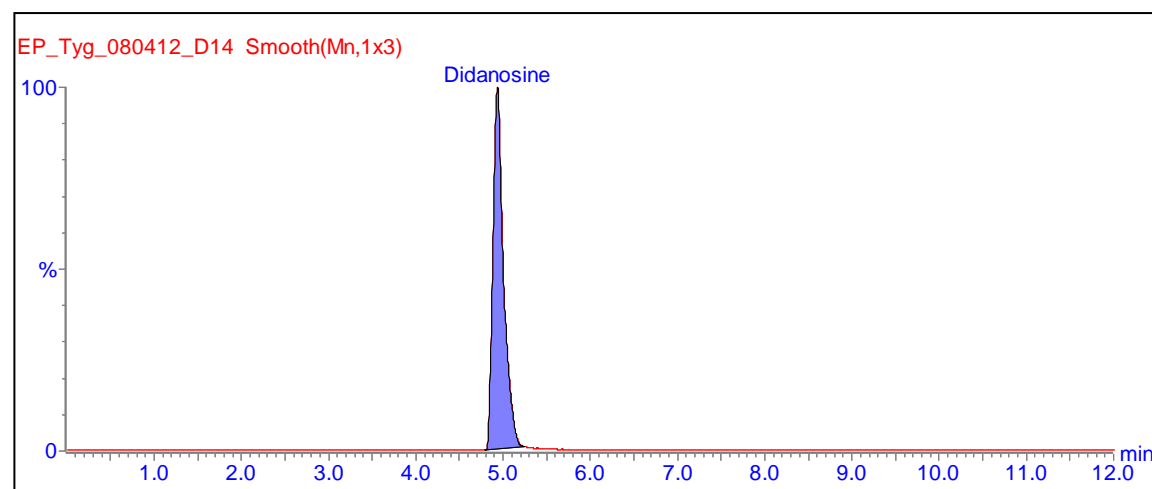


Fig. 55. Chromatogram of didanosine

4.3.4.4 Enalapril

Column: Waters Atlantis dC18 3 μ m, 2.1 x 150 mm

Mobile phase: Solvent A: 1 % formic acid, Solvent B: acetonitrile

Injection volume: 5 μ l; Retention time: 8.33 min; Flow rate: 0.2 ml/min

Gradient: Start at 95 % solvent A, hold for 0.5 min, followed by a linear gradient to 100 % B over 3.5 min, hold at 100 % B for 2 min, followed by re-equilibration to initial conditions.

MS conditions: Two MRM transitions were monitored, the primary at 376.9>234.1 at a collision energy of 20 eV and Cone voltage of 15 V, and the second 376.9>303.2 under the same conditions. The calibration curve and chromatogram of propranolol are shown in Figs. 56 and 57.

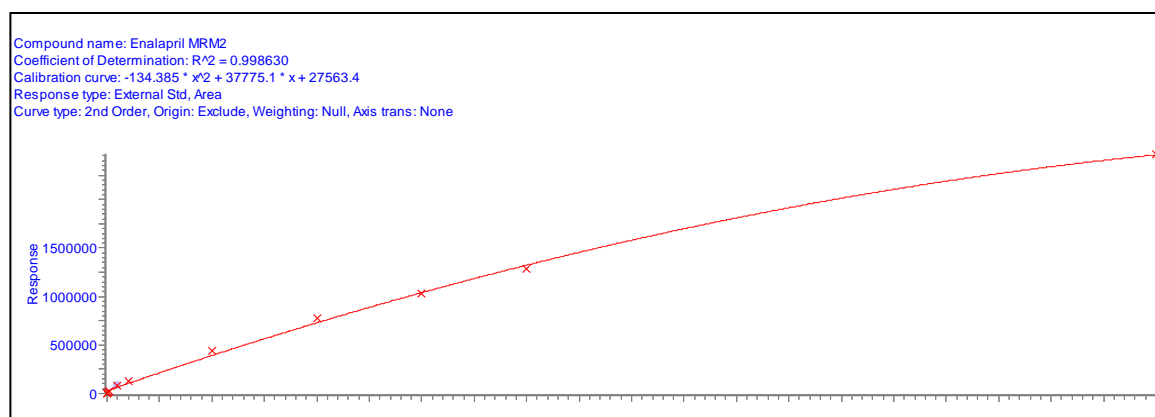


Fig. 56. Calibration curve of enalapril

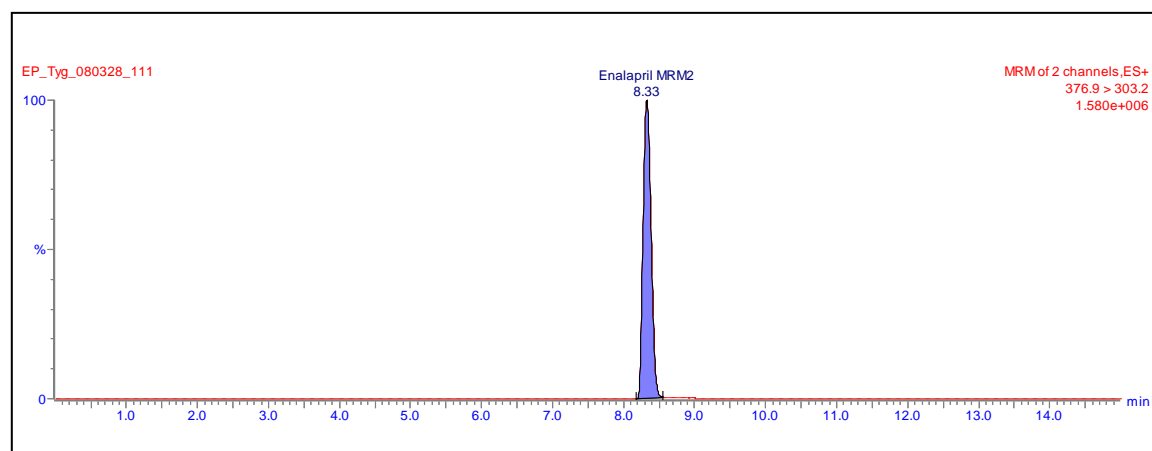


Fig. 57. Chromatogram of enalapril

4.3.5 Calculation of flux values

The flux values (J) across the membranes were calculated by means of the following relationship: $J = Q/(A \times t)$, where Q is the quantity of substance crossing membrane (in μg), A is the membrane area exposed (in cm^2) and t is the time of exposure (in min). The trapezoid method of obtaining area under curve (AUC) was used.

4.3.6 Steady state kinetics and statistical analysis of data

Repeated measure ANOVA over all time periods and a Bonferroni multiple comparison procedure was used to determine steady state. If at consecutive time periods p-values exceeded 0.05, these flux values do not differ significantly, therefore indicating steady state. Repeated measures ANOVA, Bonferroni multiple comparison procedures and a Bootstrap test were performed for comparative purposes. A significant level of 5 % was used for all tests and comparisons.

4.4 Results

The overall mean flux values of zidovudine, propranolol, didanosine and enalapril across human small intestine mucosa versus a 24 h period were investigated in two sections/time periods.

There are relative correspondences between the mean flux measures of the two groups at 6 h, but since these are essentially two groups, the analysis for each time period was done separately and not as one. During each time period a repeated measures ANOVA was done among the four different test drugs' (zidovudine, propranolol, didanosine and enalapril) mean flux values across intestine mucosa over time.

During the first time period (0-6 h) there were significant interactions ($F(9, 141)=3.553$ with $p=0.0005$) among the four drugs' flux values and time, indicating that the drugs caused different mean flux values across the mucosa over time (Fig. 58). A Bonferonni corrected multiple comparisons method was used to compare the interactions (e.g. mean flux values over time). The mean flux values for AZT were not significantly higher ($p>0.05$) than propranolol for time 2-6 h, but was significantly higher than didanosine and enalapril for time 2-6 h.

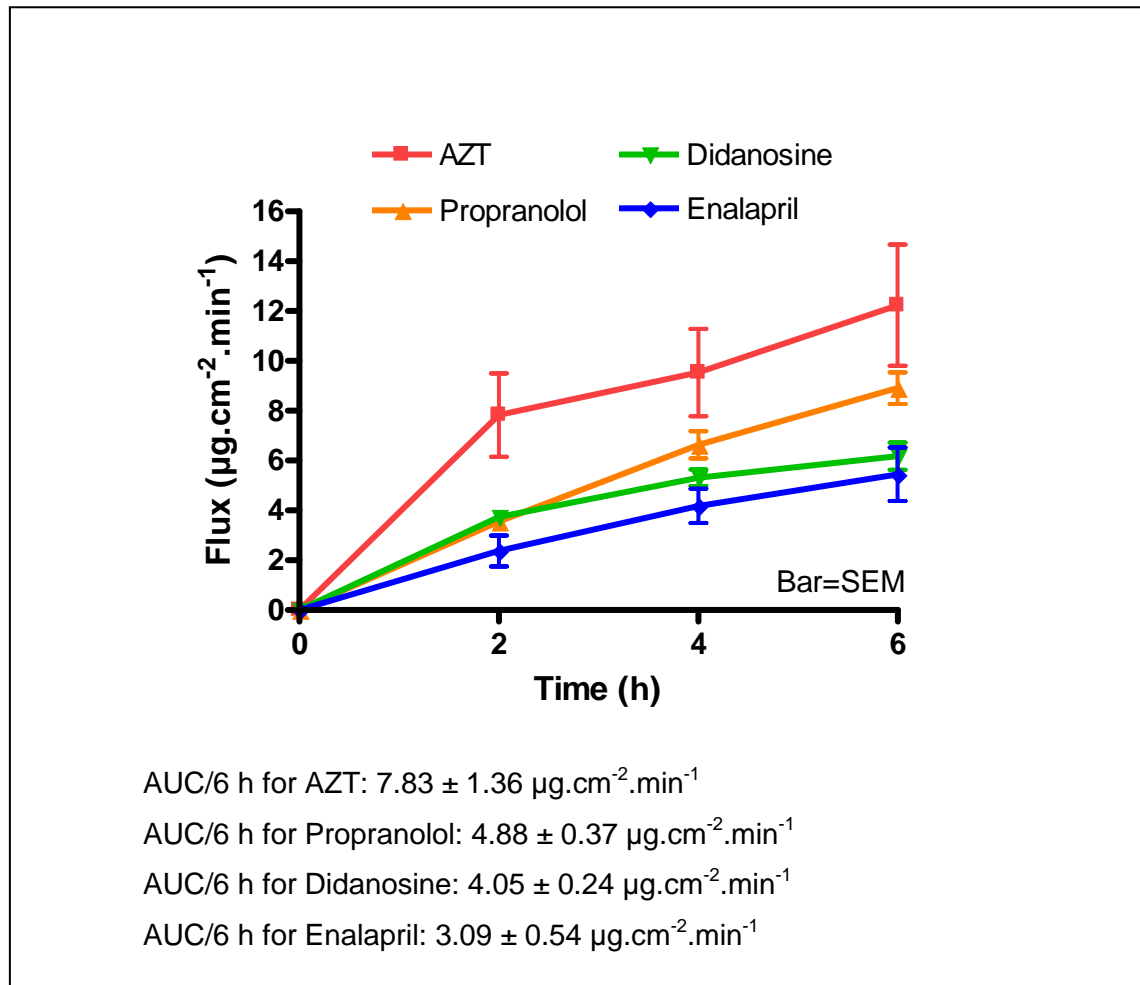


Fig. 58. Overall mean flux values of AZT, propranolol, didanosine and enalapril across the intestinal mucosa

Similar repeated measures ANOVA was done among the different drugs' flux values over time to compare the mean flux values across the intestine mucosa among the four drugs for time 6-24 h (Fig. 59). Over this time period there was also significant interaction ($F(9, 297)=3.2667$, $p=0.00083$), but none of the drugs' mean flux values was significantly different from the others at each time point using a Bonferroni correlated multiple comparisons procedure. AZT, propranolol and didanosine reached steady state at 6 h, whereas enalapril reached steady state at 12 h (Fig. 59b).

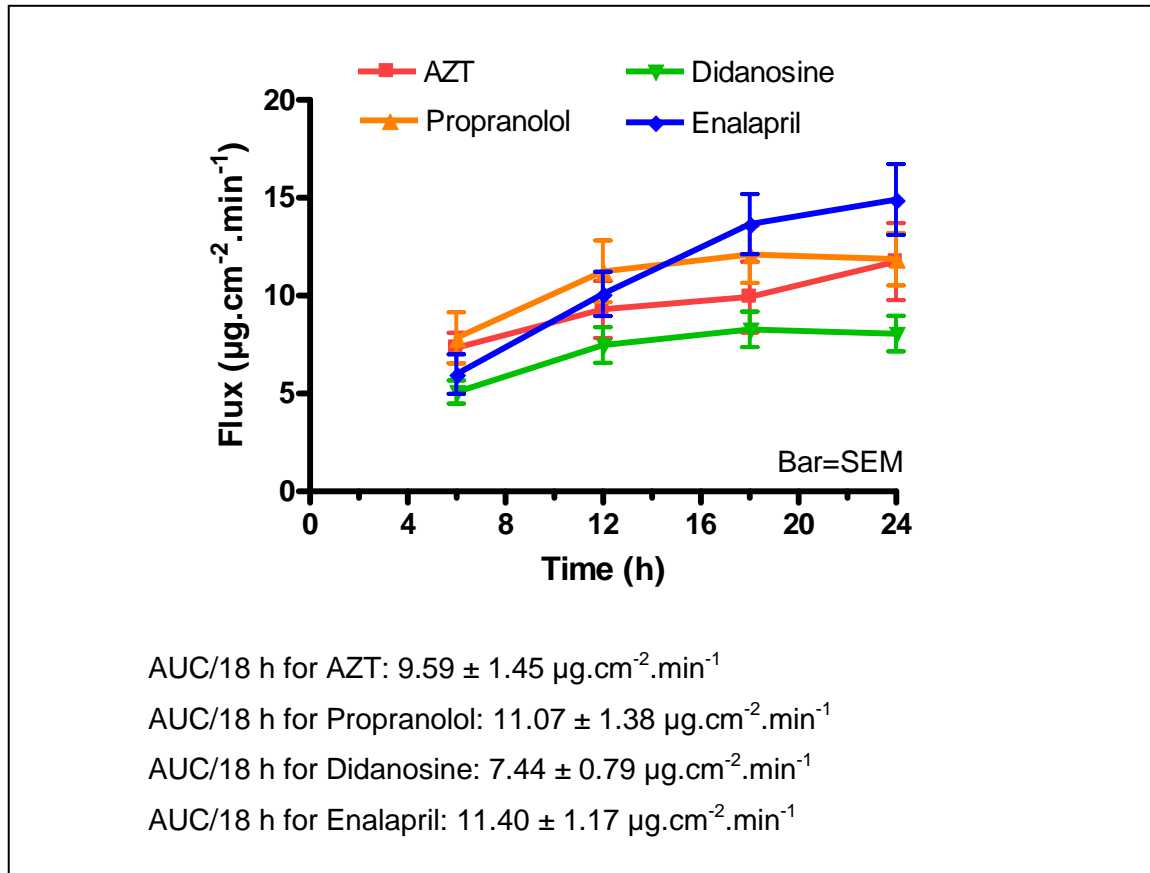


Fig. 59. Overall mean flux values of AZT, propranolol, didanosine and enalapril across the intestinal mucosa

Bonferroni test; variable DV_1 (AZT, Propranolol, Didanosine, Enalapril)							
Probabilities for Post Hoc Tests							
Error: Between; Within; Pooled MS = 63.088, df = 422.45							
Cell No.	TREATMENT	TIME	{1}	{2}	{3}	{4}	{5}
1	AZT	0		0.032	0.033	0.000	0.001
2	AZT	6	0.032		1.000	1.000	1.000
3	AZT	12	0.033	1.000		1.000	1.000
4	AZT	18	0.000	1.000	1.000		1.000
5	AZT	24	0.001	1.000	1.000	1.000	
	TREATMENT	TIME	{6}	{7}	{8}	{9}	{10}
6	Propranolol	0		0.002	0.000	0.000	0.001
7	Propranolol	6	0.002		1.000	1.000	1.000
8	Propranolol	12	0.000	1.000		1.000	1.000
9	Propranolol	18	0.000	1.000	1.000		1.000
10	Propranolol	24	0.001	1.000	1.000	1.000	
	TREATMENT	TIME	{11}	{12}	{13}	{14}	{15}
11	Didanosine	0		0.019	0.000	0.001	0.000
12	Didanosine	6	0.019		1.000	1.000	1.000
13	Didanosine	12	0.000	1.000		1.000	1.000
14	Didanosine	18	0.001	1.000	1.000		1.000
15	Didanosine	24	0.000	1.000	1.000	1.000	
	TREATMENT	TIME	{16}	{17}	{18}	{19}	{20}
16	Enalapril	0		0.041	0.000	0.000	0.000
17	Enalapril	6	0.041		0.411	0.000	0.000
18	Enalapril	12	0.000	0.411		1.000	1.000
19	Enalapril	18	0.000	0.000	1.000		1.000
20	Enalapril	24	0.000	0.000	1.000	1.000	

Fig. 59b. AZT, propranolol and didanosine reached steady state at 6 h, whereas enalapril reached steady state at 12 h

The mean flux values for AZT, propranolol, didanosine and enalapril across the intestinal mucosa are shown as column bars for the time periods 2-4 and 4-6 h in Fig. 60.

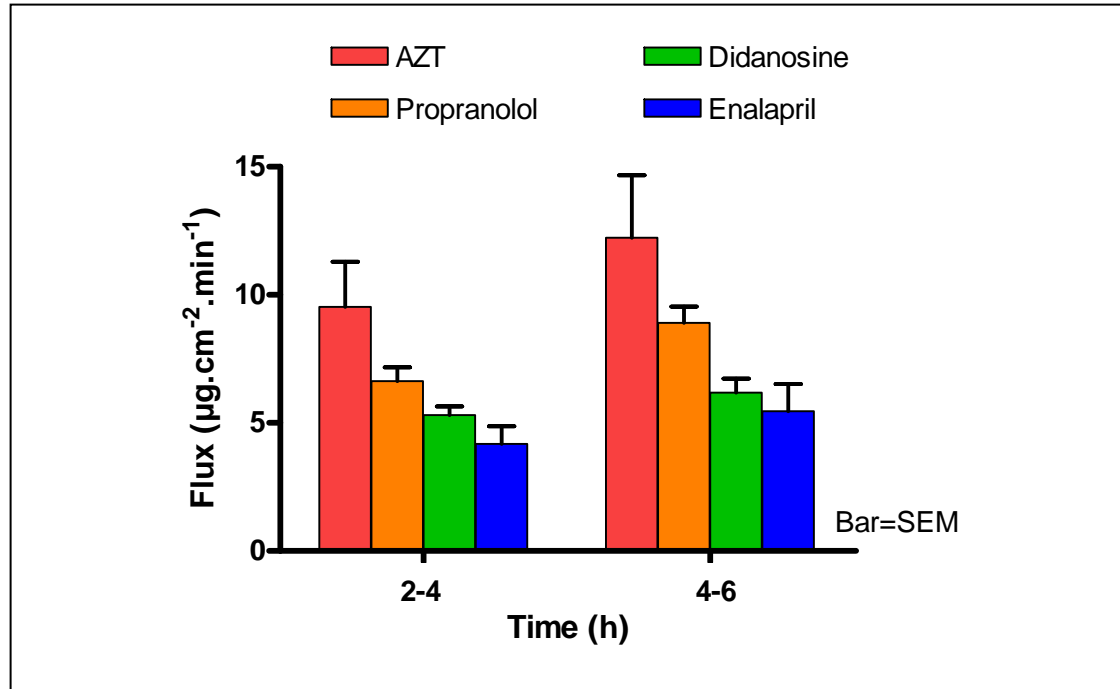


Fig. 60. Mean flux values of AZT, propranolol, didanosine and enalapril across the intestinal mucosa at different time periods

Between the time (h) periods 2-4 and 4-6 AZT's mean flux values were respectively 1.8 and 2.0 times higher ($p < 0.5$) than didanosine and 2.3 and 2.2 times higher ($p < 0.5$) than enalapril. Between both the time periods 2-4 and 4-6 h AZT's mean flux values were 1.4 times higher ($p > 0.5$) than propranolol and didanosine's mean flux values were respectively 1.3 and 1.1 times higher ($p > 0.5$) than enalapril during the mentioned time periods. Propranolol's mean flux values were respectively 1.2 and 1.4 times higher than didanosine and 1.6 higher than enalapril during both the 2-4 and 4-6 h time periods ($p > 0.05$).

Similar repeated measures ANOVA and a Bonferroni corrected multiple comparisons method as previously mentioned, as well as a Bootstrap test were used to compare the interactions (e.g. mean flux values over time) for the class 1 and 3 drugs (Fig. 61). The mean flux values of the class 1 drugs across intestine mucosa were significantly ($p < 0.05$) higher than for the class 3 drugs from time period 2-6 h.

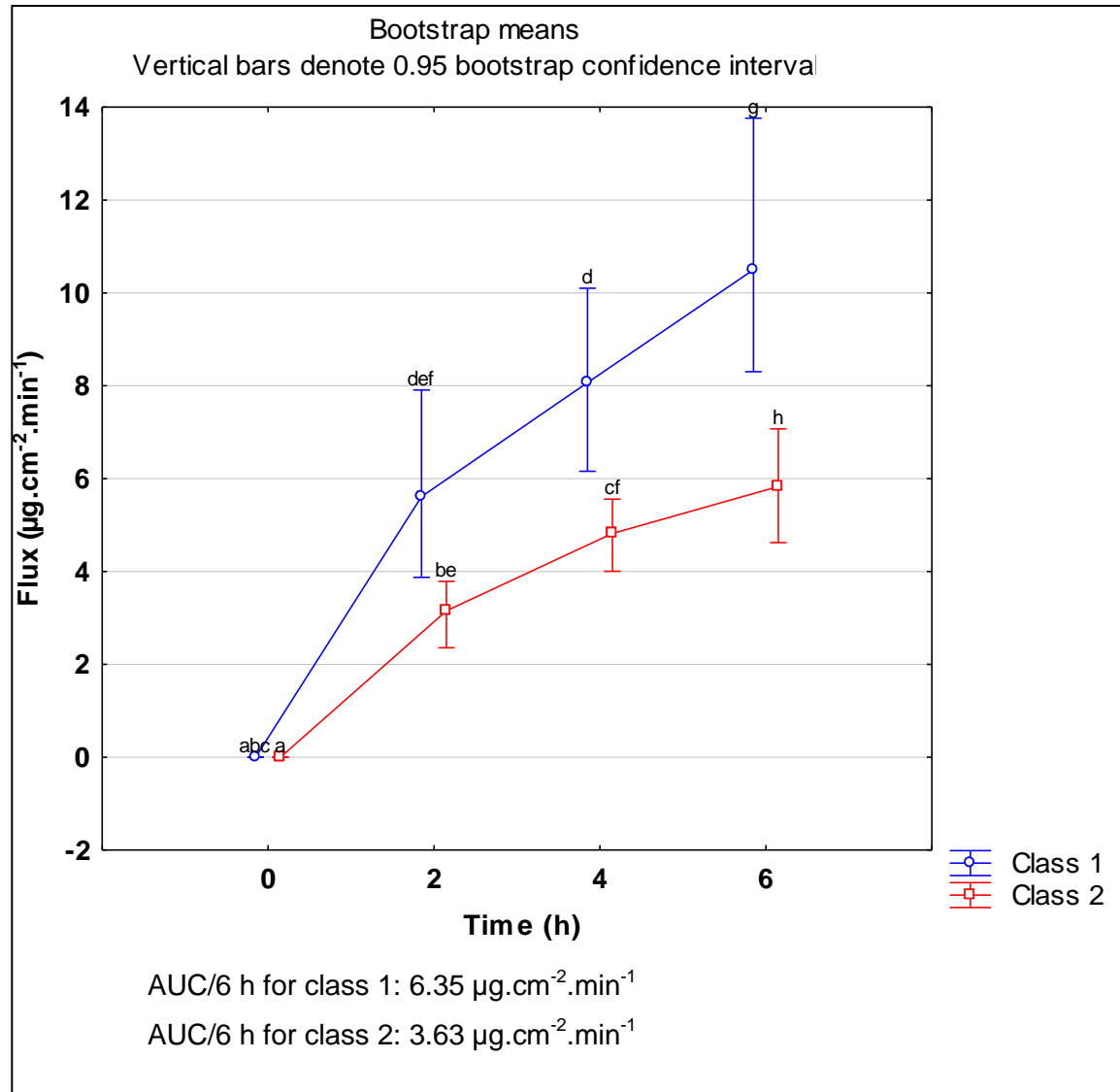


Fig. 61. Mean flux values over time (0-6 h) for the class 1 and 3 drugs

The intra-experiment variations for AZT, propranolol, didanosine and enalapril were respectively 57.9, 48.7, 46.4 and 56.0 % and the inter-experiment variations were respectively 41.0, 26.4, 22.2 and 20.6 %.

4.5 Discussion

Several methods have been proposed and investigated for *in vitro* evaluation and prediction of gastrointestinal permeability of drugs, but no official method is available at present due to certain limitations (Corti *et al.*, 2006).

The flux rates of the 4 chosen test drugs were significantly influenced by the length of the experiment. Steady state fluxes were obtained for all 4 permeants that diffused across the intestinal mucosa during the 6-24 h period. The steady state fluxes for AZT, propranolol and didanosine were obtained after 6 h and for enalapril it was reached after 12 h.

Even though didanosine has the smallest molecular weight (236.23 g/mol) of the 4 compounds, this compound had the second lowest AUC during the first 6 h (Fig. 58) and lowest AUC between 6-24 h (Fig. 59), which correlates well with its BCS classification as a class 3 drug. Although the oral bioavailable delivery of didanosine has been extensively studied, the exact reasons for its limited oral bioavailability remain unknown. Didanosine has weak acid stability and its *in vivo* bioavailability is incomplete and erratic, even when it is administered with antacids. The mean fraction available is 43 %, with a range of 16-54 % (Knupp *et al.*, 1991). Once-daily didanosine doses lead to significant reductions in bioavailability in comparison to the same amount given twice daily, suggesting the involvement of a saturable process (Drusano *et al.*, 1992). The low and variable absorption of didanosine can be partially attributed to the first-pass elimination of didanosine by the liver, presumably by a purine nucleoside phosphorylase (PNP) enzyme. Presystemic metabolism in the intestinal wall does not contribute significantly to the first-pass elimination of didanosine, but degradation of unabsorbed didanosine by the intestinal flora in the lower intestinal tract may possibly be involved (Bramer *et al.*, 1993). Consistent with this possibility, other authors have shown that the intestinal permeability of didanosine, that is a relatively hydrophilic molecule (Fig. 49), is low. The main route for diffusion of didanosine through gastrointestinal epithelium is *via* paracellular transport and it is regionally dependent, decreasing significantly in the terminal ileum and proximal ascending colon (Sinko *et al.*, 1997; Sinko and Hu, 1996; Sinko *et al.*, 1995). Although these studies suggest that slow absorption coupled with degradation in the lower intestine may play a role, the exact reason for the low and variable oral bioavailability of didanosine remains to be conclusively identified.

The mean flux values for AZT, propranolol, didanosine and enalapril across the intestinal mucosa are shown as column bars for the time periods 2-4 and 4-6 h in Fig. 60. It was decided to choose these specific time periods to compare mean flux values of the 4 chosen compounds, since the small intestinal transit time is generally considered to range between 3.5 and 4.5 h (Dressman and Krämer, 2005; Yu *et al.*, 1996). The mentioned time periods are before the four drugs reached steady state. AZT, propranolol and didanosine reached steady state after 6 h and enalapril after 12 h. AZT's mean flux values between the time (h) periods 2-4 and 4-6 were respectively 1.8 and 2.0 times higher ($p < 0.05$) than didanosine and 2.3 and 2.2 times higher ($p < 0.05$) than enalapril. AZT has a higher molecular weight (267.24 g/mol) compared to didanosine (236.23 g/mol), but it showed a higher mean flux rate than didanosine for both the 0-6 h and 6-24 h period experiments. Both AZT and didanosine reached steady state at 6 h. Huang *et al.* previously reported that AZT permeates the intestine by a Na⁺/nucleoside carrier (Huang *et al.*, 1994). An *in vitro*, brush border membrane vesicle (BBMV) study by Oulianova *et al.* (2007) confirmed these results. Sinko *et al.* (1997; 1995) ruled out a significant carrier-mediated absorption of didanosine through rat intestinal brush border membrane vesicles, even though transporters are known to play an important role in the disposition of didanosine in other tissues. The carrier system involved with AZT absorption, may therefore explain its higher permeability rate across the small intestine.

Both AZT and propranolol HCl belong to BCS class 1, but interestingly AZT's mean flux values between the time (h) periods 2-4 and 4-6 AZT's were during both time periods 1.4 higher ($p > 0.5$) than propranolol. AZT is absorbed by carrier mediated transport, whereas propranolol is predominantly absorbed by a passive diffusion mechanism.

The results of the present study concur with other *in vitro* and *in vivo* studies that showed that propranolol HCl has a high partition coefficient (Trapani *et al.*, 2004; Yamashita *et al.*, 1997). Factors influencing permeability of propranolol HCl *in vitro* versus *in vivo* will be limited, since propranolol HCl is transported transcellularly and neither has affinity to cellular efflux or to influx systems (Vogelpoel *et al.*, 2004; Pade and Stavchansky, 1998). The high permeability is in line with the reported high oral absorption (>90 %) and BCS classification as a class 1 drug (Vogelpel *et al.*, 2004). The results are in contrast with other authors that found that propranolol had a high affinity for negatively charged

components of tissue, due to its cationic structure and therefore had a low permeability *in vitro*. It seems that the flow-through diffusion system used in the present study were able to overcome this problem by having a constant flow of buffer and concentration gradient between the acceptor and donor compartments. It therefore does not exhibit the problem of an unstirred waterlayer.

During time periods 2-4 and 4-6 h the mean flux values of didanosine were very similar to enalapril. Didanosine's mean flux values were respectively 1.3 and 1.1 times higher ($p>0.5$) than enalapril during the mentioned time periods, which correlate well with the same BCS classification of these two test drugs as class 3.

Enalapril maleate is an amino acid ester prodrug and the transport of enalapril maleate in rats and Caco-2 cells has been shown to be a combination of both passive and active processes, involving a carrier-mediated peptide transport system (Li *et al.*, 2006; Friedman and Amidon, 1989a;b). From the results obtained in the 0-6 h period mean flux graph (Fig. 58), it was clear that the order of the AUC's of the 4 drugs studied during this time period were AZT > propranolol > didanosine > enalapril. Enalapril reached steady state last (Fig. 59b), but at the end of the 24 h period enalapril exhibited the highest mean flux rate across intestinal mucosa even though it has the highest molecular weight (492.52 g/mol) of the four test drugs. The high, mean end flux of enalapril despite its initial low mean flux rate, may be explained by the combination of passive and active processes.

The BCS Guidance considers the use of excised human intestinal tissue as an acceptable test method for determination of permeability of drugs. Supportive data can be provided by *in vitro* permeation across Caco-2 cells. It is considered by some to be the 'gold standard' technique (Hamalainen and Frostell-Karlsson, 2004). Caco-2 cells possess many qualities of enterocytes. Cell cultured monolayers tend to be "flat", whereas the human jejunum is highly folded. This difference does not appear to dramatically affect the correlation of drug transport for passively diffused compounds utilizing transcellular routes, since the brush-border region of enterocytes appears to be responsible for the vast majority of drug absorption (Press and Grandi, 2008). Similar to Caco-2 cell model the flow-through diffusion model used in the present study require small quantities (mg) of compound.

Although the Caco-2 cell culture model is commonly accepted and used for the screening of potential drug candidates to predict intestinal absorption, many shortcomings remain associated with this model. Compared to Caco-2 cells the present *in vitro* assay does not have the drawbacks such as long cell growth cycles, high costs, the absence of mucus, no cellular heterogeneity, tightness of epithelium (therefore less permeable than human intestinal epithelium), thickness of unstirred waterlayer and the possibility of microbial contamination (Ingels and Augustijns, 2003; Wikman *et al.*, 1998). Other advantages of the present *in vitro* model include that regional differences in intestinal absorption can possibly be studied, the collected samples are analytically clean and species differences with respect to intestinal absorption characteristics can also possibly be determined. The present model offers the possibility to study bidirectional transport and the effect of specific transport inhibitors. Generally drug candidates with poor aqueous solubility cannot be evaluated in the Caco-2 cell model, since the use of organic cosolvents is limited in this model. The integrity of tight junctions is easily compromised by commonly used organic solvents. The results from the previously described vaginal mucosa and skin tissue study show that the *in vitro* diffusion model used in the present study can overcome this problem and the effects of a chemical enhancer can successfully be studied using the present model. Other disadvantages of the Caco-cells include the absence of CYP3A4 and the variable expression of metabolic enzymes and transporter enzymes (Bohets *et al.*, 2001; Barthe *et al.*, 1999; Hidalgo, 1996). Future studies with the *in vitro* flow-through diffusion system should therefore investigate the exact presence and influence of CYP3A4, metabolic enzymes and transporter enzymes.

The mean flux values of the class 1 drugs (AZT and propranolol) over the 0-6 h periods of the experiment were significantly ($p < 0.05$) higher than the permeability results from class 3 drugs (didanosine and enalapril) (Fig. 61). By comparing the permeability results of this study with the BCS classification, it may be possible that the present *in vitro* system may have future usefulness for the permeability prediction of class 1 and 3 drugs in humans. The results of this study show that the permeability/flux values obtained with the developed flow-through diffusion method are good predictors of the small intestine permeability to passively and carrier mediated absorbed drugs in humans. The proposed method allowed obtainment of good results for both high permeability and low permeability compounds and drugs that are absorbed by passive transcellular diffusion

and by transport-mediated drug absorption. Other authors only found a correlation between human *in vivo* and *in vitro* permeability for drugs absorbed by passive transcellular diffusion and a considerable deviation for transport-mediated drug absorption (Usansky and Sinko, 2005; Sun *et al.*, 2002; Lennernäs *et al.*, 1997). The *in vitro* model has proved to be reliable to predict permeability and also showed correlation with human *in vivo* data. Such a model may potentially facilitate drug discovery and development, as well as regulatory standards for marketed products. This could reduce costs and time during the development process and reduce unnecessary drug exposure to healthy volunteers. However, only limited amount of experiments could be conducted due to financial restrictions. Further permeability studies with this model are therefore necessary to enlarge the set of flux values of already BCS classified drugs, so that clear flux value margins can be established for the specific BCS classes. These established flux value ranges will help to accurately classify the permeabilities of future test compounds. An in-depth investigation of pH influence, formulations for the solubilisation of the test compounds and an adequate further enlargement of the set of drugs will be necessary to better evaluate the actual permeability predictive power of the present flow-through diffusion system.

The BCS allows a biowaiver for solid, IR oral formulations with high solubility and high permeability (class 1). These IR compounds are candidates for *in vitro* assessment of bioequivalence as long as they are stable in the gastrointestinal tract and have a wide therapeutic index (Lennernäs, 2007). Instead of conducting expensive and time consuming *in vivo* studies, an *in vitro* test could therefore be adopted as the surrogate basis for the decision as to whether the two pharmaceutical products are equivalent. This can reduce time and resources during the development process and reduce unnecessary drug exposure to healthy volunteers in bioequivalence studies (FDA Guidance for Industry, 2000). In view of the above considerations and the results found in the present study, it can be concluded that the flow-through diffusion system may have potential usefulness for future *in vitro* BA/BE determinations.

Chapter 5: General discussion and conclusions

Human vaginal tissue was used to test the *in vitro* flow-through diffusion model's suitability to evaluate the permeability of vaginal mucosa to three different peptides and to evaluate its value to examine test conditions such as different temperatures and concentrations, removal of epithelium and the use of a surfactant. It has been demonstrated that the de-epithelialisation of the vaginal mucosa increased the permeability of all three peptides tested in the study. This supports the premise that the main epithelial barrier is located in the epithelium and may have possible implications of increased microbicide, as well as HIV uptake *in vivo*. Furthermore, the results of the study show the concentration- and temperature dependency of flux rates of peptides across vaginal mucosa. This should be taken into consideration for determining optimum diffusion conditions for *in vitro* permeability studies. Decreased mucosal permeability could possibly be offset by the addition of an appropriate surfactant-containing permeation enhancer. The results have demonstrated that the effects of concentration, temperature, de-epithelialisation and permeation enhancers on vaginal permeability can successfully be studied with the *in vitro* flow-through diffusion model of the present study.

Human skin tissue was utilised to assess the *in vitro* flow-through diffusion model's ability to evaluate the permeability of skin to terbinafine hydrochloride and tritiated water. The test conditions that were investigated included the use of different formulations and concentrations, and the effect of different temperatures on the permeability of skin to tritiated water.

Terbinafine from different formulations readily diffused into skin tissue, but no flux values through skin into the acceptor fluid were observed with any of the terbinafine hydrochloride formulations. This reservoir effect of terbinafine has clinical value, since the accumulation of terbinafine in the skin tissue reduces systemic absorption. The results of this *in vitro* study concur with other *in vivo* and *in vitro* studies. The *in vitro* model used in the study has predictive values for *in vivo* studies and has demonstrated that the uptake of terbinafine and potential other drugs by the skin can be determined by this model. It may therefore serve as a prospective method for assessing the bioequivalence of alternative (generic) vehicles or formulations containing the same drug and for the comparison of potential penetration enhancers. Predicting skin permeation

of compounds has many advantages for the pharmaceutical, cosmetic and agrochemical fields.

Furthermore, the results of the study show the concentration and formulation dependency of the absorption of terbinafine by skin tissue. This should be taken into consideration for determining optimum diffusion conditions for *in vitro* and *in vivo* permeability studies. *In vivo* assessment of topical drug bioavailability may be expensive and also is not always easily accomplished and a reliable *in vitro* model to assess preclinical formulations will be very beneficial.

Increasing the normal skin temperature from 32 to 37 °C significantly increased the permeated amount of tritiated water through human skin. The *in vitro* flow-through diffusion system has the potential to serve as a tool to study application of heat as a penetration enhancer and may potentially be used for predicting flux rates across human skin at higher temperatures.

The results from the gastrointestinal study show that the permeability values obtained with the flow-through diffusion system are good predictors of BCS permeability classification. This correlation existed for drugs that are absorbed by passive transcellular and paracellular diffusion, as well as by transport-mediated drug absorption.

The BCS allows a biowaiver for solid IR oral formulations with high solubility and high permeability (class 1). These IR compounds are candidates for *in vitro* assessment of bioequivalence as long as they are stable in the gastrointestinal tract and have a wide therapeutic index (Lennernäs, 2007). Instead of conducting expensive and time consuming *in vivo* studies, an *in vitro* test could therefore be adopted as the surrogate basis for the decision as to whether the two pharmaceutical products are equivalent. This can reduce time and costs during the development process and reduce unnecessary drug exposure to healthy volunteers in bioequivalence studies (FDA Guidance for Industry, 2000). In view of the above considerations and the results found in the present study, it can be concluded that our flow-through diffusion system has potential usefulness for future *in vitro* BA/BE determinations. The proposed method was tested on 4 drugs that belong to either BCS class 1 or 3. Considering the widely differing chemical and physical properties of drugs, further validation of the above *in vitro*

model using all 4 BCS classes, larger number of drugs and different formulations is warranted. Further permeability studies with this model are necessary to enlarge the set of flux values of already BCS classified drugs, so that clear flux value margins can be established for the specific BCS classes. These established flux value ranges will help to accurately classify the permeabilities of future test compounds.

Caco-2 cells are the most advanced and frequently used cell model to predict small intestine drug permeability and absorption, despite several disadvantages (Borchardt, 1995; Artursson *et al.*, 1994; Rubas *et al.*, 1993). Despite its frequent use and value to predict intestinal permeability the Caco-2 cell model has been shown to have high intra- and inter-experiment variations (>20 %). The *in vitro* flow-through diffusion system used in the present study also showed high variation (especially with the peptide studies), but despite of this, the model also showed that valuable information can be obtained from its usage. However, it must be remembered that the high intra- and inter-experiment variations are a shortcoming of the present *in vitro* flow-through diffusion system.

Generally drug candidates with poor aqueous solubility cannot be evaluated in the Caco-2 cell model, since the use of organic cosolvents is limited in the Caco-2 cell model. The integrity of tight junctions is easily compromised by commonly used organic solvents. The results from the previously described vaginal mucosa and skin tissue study show that the *in vitro* flow-through diffusion model used in the present study can overcome this problem and the effects of a chemical enhancer can be successfully studied using this flow-through diffusion model. Another drawback of the Caco-2 cells is that it cannot be used to evaluate the permeability of different intestinal regions, where as the flow-through diffusion system has the possibility to that. A further advantage of the present *in vitro* flow-through model is that the biological membrane's architecture is preserved and closely mimics the *in vivo* situation. Compared to the Caco-2 cells, the flow-through diffusion system has the same potential for automation and it does not have the disadvantages of poor paracellular passage modelling, labour intensity, high costs and long cell growth cycles. The flow-through diffusion system has the potential to develop in to a simple, rapid, economic and highly-reproducible permeability method.

The major advantages of all the above experiments are that because they were all performed in the same apparatus under standardised conditions, relative comparisons of

data were not subject to interlaboratory variations. The absorption of drugs by different intestinal regions can potentially be studied and it may also be a useful method to compare the permeability values across species. Since the flow-through diffusion system has a constant flow of buffer, it does not have the problem of an unstirred water layer in the acceptor and donor wells, like other *in vitro* models. The amounts of drug required for the study are relatively small (milligram quantity) and the collected samples are analytically clean, which facilitates quantitative analysis.

In conclusion, the results presented in this thesis show that valuable information can be obtained from the *in vitro* flow-through diffusion model, leading to the intelligent selection of suitable compounds for enhanced tissue permeability. The permeability values obtained with the developed flow-through diffusion system are good predictors of *in vivo* permeation and concurred with other *in vitro* studies. The results show that the continuous flow-through diffusion system can effectively be utilised to investigate different test conditions (de-epithelialisation of mucosal surfaces, chemical enhancers, temperature, permeant concentration and formulation) on the permeability of different compounds/drugs. It seems that the *in vitro* flow-through diffusion model used in the present study have the potential to overcome some of the problems and limitations demonstrated by other *in vitro* techniques. It may potentially serve as a future tool for pharmaceutical companies to predict the diffusion characteristics of new drugs and different formulations across different biological membranes and may serve as a prospective method for assessing the bioequivalence of alternative (generic) vehicles or formulations containing the same drug/compound.

Chapter 6: Future studies

Areas for future research include:

- Future studies with the *in vitro* flow-through diffusion system should investigate the exact presence and influence of metabolic enzymes and transporter enzymes. Furthermore, “apical to basal” studies must be compared with “basal to apical” transports to determine the role of carrier-mediated transport of carrier-mediated efflux mechanism involved with the present *in vitro* assay.
- It was beyond the scope of the study to look at the effect of pathophysiological conditions and drug treatment on membrane permeability and the transport systems, but it will be an important aspect to investigate in follow-up studies, since transporter systems are affected by these variables.
- Additional studies are necessary to determine the exact influence of different concentrations of the test compound on the flow-through diffusion system’s ability to predict permeability. These studies may include the utilization of a mathematical model.
- Further permeability studies with this model are necessary to enlarge the set of flux values of already BCS classified drugs, so that clear flux value margins can be established for the specific BCS classes. These established flux value ranges will help to accurately classify the permeabilities of future test compounds.
- The following method for the determination of steady state was used: Repeated measure ANOVA over all time periods and a Bonferroni multiple comparison procedure was used to determine steady state. If at consecutive time periods p-values exceeded 0.05, these flux values do not differ significantly, therefore indicating steady state. It was found in this study that flux values at steady state still increased (non-significantly) over the rest of the experiment for most of the test conditions. It is therefore suggested that future studies should be done with the same data to develop a mathematical model that will be able to predict the max flux rate for the studied compounds.

- Further research and more data sets are needed to define and optimize the investigated experimental conditions.

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