IN VITRO PASSAGE OF IBUPROFEN THROUGH SYNTHETIC AND BIOLOGICAL MEMBRANES

A Thesis Submitted to Rhodes University in Fulfilment of the Requirement for the Degree of

MASTER OF SCIENCE

by

Carryn Hamilton Purdon

August 2001

Faculty of Pharmacy
Rhodes University
Grahamstown

STUDY OBJECTIVES

Introduction

Ibuprofen is a non-steroidal anti-inflammatory agent with analgesic and antipyretic activity. It is indicated for rheumatoid arthritis, osteoarthritis, mild-to-moderate pain, primary dysmenorrhea, and reduction of fever. Ibuprofen is available in the form of tablets, capsules, injections, suspensions, suppositories, gels, creams and mousses.

Objectives

The objectives of this study were:

1. To develop and validate a suitable high performance liquid chromatographic method with the necessary sensitivity to accurately and precisely quantitate ibuprofen in aqueous solution.
2. To utilise an appropriate diffusion technique for the measurement of the amount of ibuprofen released from a topical formulation during *in vitro* testing.
3. To verify the usefulness, or otherwise, of shed snake skin as a biological diffusion membrane.
4. To assess the permeation of ibuprofen using shed snake skin as the biological animal membrane.
5. To conduct a comparative diffusion study on proprietary ibuprofen-containing topical preparations from three countries.

ABSTRACT

Ibuprofen is a non-steroidal anti-inflammatory drug with three major types of effect: anti-inflammatory, analgesic and antipyretic. Ibuprofen may be administered in a number of different forms *via* the oral as well as the topical route. Published evidence suggests that topical, unlike oral, non-steroidal anti-inflammatory drugs are associated with few systemic side effects as plasma concentrations are low compared to oral therapy.

In some countries it is particularly difficult to obtain human skin for *in vitro* experimentation and it is therefore important to have alternate biological or synthetic membranes which mimic human skin for diffusion experiments. Synthetic membranes serve as predictive models for topical drug release and in South Africa, shed snake skin is easily obtainable from the many snake parks present in the country.

The FDA guidelines were considered when choosing the apparatus to be used in the comparative diffusion study on proprietary ibuprofen-containing topical preparations from three countries and the verification of the usefulness, or otherwise, of shed snake skin as a biological membrane for the assessment of the permeation of ibuprofen. Two diffusion techniques were considered appropriate for the measurement of the amount of ibuprofen released from a topical formulation during *in vitro* testing. One was the Franz diffusion cell, as modified by Keshary and Chien (88,169) and the other was the European Pharmacopoeia diffusion cell (187).

High performance liquid chromatography was used as the analytical technique for the analysis of ibuprofen in aqueous solution using ultraviolet detection at 222 nm. The validated method was applied to the determination of the diffusion of ibuprofen from topical ibuprofen-containing formulations (gels, creams and mousse) through synthetic silicone membrane and shed snake skin biological membrane from four different species.

In a study of fifteen topical ibuprofen-containing formulations (gels, creams and mousse) from three countries (South Africa, United Kingdom and France) it was found that there was a trend of products from two countries consistently exhibiting superior diffusion characteristics as well as products from the same two countries consistently exhibiting the lowest diffusion of ibuprofen. Interpretation of the results of these studies demonstrated the

importance of employing a combination of statistical analyses and peak integration values when drawing conclusions regarding comparative diffusion characteristics.

Shed snake skin has been described as a 'model' membrane, *i.e.* a membrane which shows similar permeability to human stratum corneum. The results reported here show clearly that, for ibuprofen, the four species of snake produce shed skin with completely different diffusion characteristics when all other conditions are identical. It may well be that there is one particular species of snake which produces shed skin of identical permeability to human stratum corneum, but to describe shed snake skin in general as a model membrane seems incorrect. It is therefore important that if shed snake skin is used as a membrane, the species, skin site and orientation should be reported.

The European Pharmacopoeia diffusion apparatus was judged to be the better of the two diffusion techniques assessed for the measurement of the amount of ibuprofen released from a topical formulation during *in vitro* testing using silicone membranes and for the measurement of the amount of ibuprofen diffusing across the ventral outside orientation of shed skin during *in vitro* testing, whereas the Franz diffusion apparatus was judged to be better for the measurement of the amount of ibuprofen diffusing across the dorsal outside orientation of shed skin during *in vitro* testing. However, the choice of this diffusion apparatus must be weighed against the relatively poor reproducibility as compared with the European Pharmacopoeia diffusion apparatus.

ACKNOWLEDGEMENTS

I would like to sincerely thank the following people:

My supervisor, Professor J.M. Haigh, for his guidance, encouragement, concern and infinite patience during the research and writing of this thesis.

Mr Dave Morley and my father for their advice, assistance and technical expertise in the laboratory, and to Mrs Shirley Pinchuck, for her help with the scanning electron microscopy.

The Dean and Head, Professor Kanfer and the staff of the Faculty of Pharmacy for the use of departmental facilities.

My peers in the department for their friendship and support, particularly Claire Steiner.

My parents and friends for their encouragement during my undergraduate and postgraduate studies and to Chris for his help, support and technical advice.

TABLE OF CONTENTS

STUDY OBJECTIVES ii
ABSTRACT iii
ACKNOWLEDGEMENTS v
LIST OF TABLES xii
LIST OF FIGURES xiii

CHAPTER 1: IBUPROFEN MONOGRAPH **1**

1.1 **PHYSICOCHEMICAL PROPERTIES OF IBUPROFEN** 1

1.1.1 Description 1
1.1.2 Melting Range 1
1.1.3 Solubility 1
1.1.4 Dissociation Constant 2
1.1.5 Partition Coefficient and Permeability Coefficient 2
1.1.6 Maximum Flux 2
1.1.7 Dissolution 2
1.1.8 Molecular Structure 2
1.1.9 Ultraviolet Absorption Spectrum 3

1.2 **CLINICAL PHARMACOLOGY OF IBUPROFEN** 4

1.2.1 Preparations 4
1.2.2 Mode of Action 5
1.2.3 Indications 6
1.2.4 Adverse Reactions 6
1.2.5 Contraindications 8
1.2.6 Drug Interactions 9
1.2.7 Toxicity 10

1.3 **PHARMACOKINETICS OF TOPICAL IBUPROFEN** 11

CHAPTER 2: THEORY OF TRANSMEMBRANE DIFFUSION **14**

2.1 **TRANSMEMBRANE DIFFUSION** 14

2.1.1 Introduction 14
2.1.2 Kinetics of the Diffusion Process 14
2.1.3 Modification of the Diffusion Process 18
2.1.3.1 Hydration 18

2.1.3.2 Diffusant Concentration 19
2.1.3.3 Skin Damage 19
2.1.3.4 Partition Coefficient 19
2.1.3.5 Vehicle/Dosage Form 20
2.1.3.6 Penetration Enhancers 20
2.1.4 Diffusion Boundary Layer Formation 21

2.2 **MEMBRANES FOR USE IN *IN VITRO* DIFFUSION EXPERIMENTS** 23

2.2.1 Biological Membranes 23
2.2.1.1 Human Skin 23
2.2.1.2 Animal Models 23
2.2.2 Synthetic Membranes 27
2.2.2.1 Filter Membranes 27
2.2.2.2 Cellulose Media 27
2.2.2.3 Synthetic Polymers 28
2.2.3 Conclusions 29

2.3 **DIFFUSION CELL APPARATUS** 31

2.3.1 Introduction 31
2.3.2 Two-Chambered Cell 33
2.3.3 One-Chambered Cell 33
2.3.3.1 Flow-Through Diffusion Cell 34
2.3.3.2 Static Diffusion Cell 35

2.4 **FORMULATION ASPECTS** 40

2.4.1 Introduction 40
2.4.2 Vehicles 41
2.4.2.1 Liquid Preparations 42
2.4.2.2 Simple Suspensions 42
2.4.2.3 Gels 43
2.4.2.4 Emulsions 43
2.4.2.4.1 Oil-in-Water Emulsions 43
2.4.2.4.2 Water-in-Oil Emulsions 43
2.4.2.5 Aerosol Quick-Break Foams 44
2.4.3 Penetration Enhancers 44
2.4.4 Surfactants 45

CHAPTER 3: THE *IN VITRO* ANALYSIS OF IBUPROFEN 46

3.1 **ANALYTICAL METHOD DEVELOPMENT** 46

3.1.1 Introduction 46
3.1.2 Principles of High Performance Liquid Chromatography 47

3.1.3 Experimental ... 49
3.1.3.1 Reagents ... 49
3.1.3.2 High Performance Liquid Chromatographic System ... 49
3.1.3.3 Ultraviolet Detection ... 49
3.1.3.4 Column Selection ... 50
3.1.3.5 Mobile Phase Selection and Preparation ... 51
3.1.3.6 Guard Columns ... 53
3.1.3.7 Preparation of Stock Solutions ... 53
3.1.4 Optimisation of Chromatographic Conditions ... 54
3.1.4.1 Detector Wavelength ... 54
3.1.4.2 Choice of Column ... 55
3.1.4.3 Mobile Phase Composition ... 55
3.1.5 Chromatographic Conditions ... 56
3.1.6 Conclusion ... 56

3.2 **METHOD VALIDATION** ... 57

3.2.1 Introduction ... 57
3.2.2 Accuracy and Bias ... 58
3.2.3 Precision ... 58
3.2.3.1 Repeatability ... 59
3.2.3.2 Intermediate Precision (Ruggedness) ... 59
3.2.3.3 Reproducibility ... 60
3.2.4 Specificity ... 60
3.2.5 Limit of Quantitation and Limit of Detection ... 61
3.2.6 Linearity ... 61
3.2.7 Stability of the Molecule ... 62
3.2.8 Conclusion ... 62

CHAPTER 4: THE *IN VITRO* RELEASE OF IBUPROFEN ... 63

4.1 **EXPERIMENTAL METHOD DEVELOPMENT** ... 63

4.1.1 Introduction ... 63
4.1.2 *In Vitro* Release Testing ... 63
4.1.3 Choice of Apparatus ... 65
4.1.4 Diffusion Test Conditions ... 65
4.1.4.1 Membrane Selection ... 65
4.1.4.2 Receptor Medium Selection and Preparation ... 67
4.1.4.3 Temperature of the Receptor Medium ... 69
4.1.4.4 Number of Samples and Sampling Time ... 70
4.1.4.5 Sample Applications ... 70
4.1.5 Diffusion Profile Comparison ... 72

4.2 **ELECTRON MICROSCOPY** ... 73

4.2.1 Introduction ... 73
4.2.2 Scanning Electron Microscopy ... 73

4.2.3 Specimen Preparation for Scanning Electron Microscopy 74
4.2.3.1 Chemical Methods 74
4.2.3.2 Physical Methods 75
4.2.4 Scanning Electron Microscopy Instrumentation and Conditions 76

4.3 **THE DIFFUSION PROFILES OF PROPRIETARY IBUPROFEN-CONTAINING TOPICAL PREPARATIONS FROM THREE COUNTRIES.** 77

4.3.1 Introduction 77
4.3.2 Comparisons of Ibuprofen Creams, Gels and Mousses 78
4.3.2.1 Results Obtained Using the Franz Diffusion Apparatus 81
4.3.2.2 Results Obtained Using the European Pharmacopoeia Diffusion Apparatus 85
4.3.3 Discussion 89
4.3.3.1 Formulation Factors 90
4.3.3.1.1 Propylene Glycol 90
4.3.3.1.2 Ethanol/Isopropanol 91
4.3.3.1.3 Purified Water 91
4.3.3.1.4 Sodium Hydroxide 92
4.3.3.1.5 Surfactant 92
4.3.3.1.6 Effect of pH 92
4.3.3.2 Transport Factors 93
4.3.4 Comparison of Diffusion Cells 94
4.3.4.1 Discussion 98
4.3.4.2 Conclusion 99

4.4 **THE PERMEATION OF IBUPROFEN THROUGH SHED SNAKE SKIN AS THE BIOLOGICAL ANIMAL MEMBRANE** 100

4.4.1 Introduction 100
4.4.2 Results Obtained Using the Franz Diffusion Cell 101
4.4.3 Results Obtained Using the European Pharmacopoeia Diffusion Cell 107
4.4.4 Diffusion Through the Scale and Hinge Regions of Shed Snake Skin 113
4.4.5 Results of Scanning Electron Microscopy 115
4.4.6 Discussion 119
4.4.7 Conclusion 122
4.4.8 Comparison of the Diffusion Cells 123
4.4.8.1 Python sebae natalensis (African Rock Python) 123
4.4.8.2 Naja melanoleuca (Forest Cobra) 124
4.4.8.3 *Naja haje haje* (Egyptian Cobra) 125
4.4.8.4 *Naja nivea* (Cape Cobra) 126
4.4.8.5 Discussion 127
4.4.8.6 Conclusion 127

APPENDIX I 128
APPENDIX II 129
APPENDIX III 132
APPENDIX IV 136
REFERENCES 138

LIST OF TABLES

Table 1.1 Ibuprofen preparations 4

Table 1.2 Drug disease contraindications 8

Table 1.3 Liquid Chromatographic Methods

Table 3.1 Liquid Chromatographic Methods 48

Table 3.2 Silica-based column packing material for partition chromatography 50

Table 3.3 Effect of mobile phase on ibuprofen retention time 52

Table 3.4 Effect of wavelength on the peak area of ibuprofen 54

Table 3.5 Percent error obtained during the determination of blinded unknowns 58

Table 3.6 Repeatability with respect to four concentrations 59

Table 3.7 Intermediate precision with respect to four concentration levels 60

Table 4.1 Receptor media investigated 67

Table 4.2 Proprietary ibuprofen-containing topical formulations 77

Table 4.3 The release constants, lag times and r^2 values for ibuprofen-containing topical formulations, using the Franz diffusion cell apparatus 83

Table 4.4 The release constants, lag times and r^2 values for ibuprofen-containing topical formulations, using the European Pharmacopoeia diffusion cell apparatus 87

Table 4.5 The release constants, lag times and r^2 values for ibuprofen diffusing through four species of shed snake skin, using the Franz diffusion cell apparatus 106

Table 4.6 The release constants, lag times and r^2 values for ibuprofen diffusing through four species of shed snake skin, using the European Pharmacopoeia diffusion cell apparatus 112

Table 4.7 Membrane thickness 115

LIST OF FIGURES

Figure 1.1	Structure of ibuprofen	1
Figure 1.2	Stereospecific inversion of R(-) to S(+)-ibuprofen	3
Figure 1.3	Ultraviolet spectrum of ibuprofen	3
Figure 1.4	Metabolism of ibuprofen	13
Figure 2.1	Diagrammatic representation of the transverse section of shed snake skin	25
Figure 2.2	Schematic of a modified Franz diffusion cell assembly	36
Figure 2.3	European Pharmacopoeia diffusion cell	38
Figure 2.4	Schematic of a modified European diffusion cell assembly	39
Figure 3.1	Calibration curve	62
Figure 4.1	Effect of synthetic membrane on the diffusion of ibuprofen	66
Figure 4.2	Effect of receptor medium on the diffusion profile of ibuprofen	68
Figure 4.3	The effect of temperature on the diffusion profile of ibuprofen	69
Figure 4.4	The effect of mass on the diffusion profile of ibuprofen	71
Figure 4.5	The diffusion profiles obtained for ibuprofen formulations using the Franz diffusion cell	79
Figure 4.6	The diffusion profiles obtained for ibuprofen formulations using the European Pharmacopoeia diffusion cell	80
Figure 4.7	Ibuprofen diffusion profiles (range 286-318 $\mu g/cm^2/h^{1/2}$)	81
Figure 4.8	Ibuprofen diffusion profiles (range 115-241 $\mu g/cm^2/h^{1/2}$)	82
Figure 4.9	Ibuprofen diffusion profiles (range 28-36 $\mu g/cm^2/h^{1/2}$)	82
Figure 4.10	Ibuprofen diffusion profiles (range 283-369 $\mu g/cm^2/h^{1/2}$)	85
Figure 4.11	Ibuprofen diffusion profiles (range 181-242 $\mu g/cm^2/h^{1/2}$)	86
Figure 4.12	Ibuprofen diffusion profiles (range 55-80 $\mu g/cm^2/h^{1/2}$)	86
Figure 4.13	Ibuprofen diffusion profiles (range 286-369 $\mu g/cm^2/h^{1/2}$)	94
Figure 4.14	Ibuprofen diffusion profiles (range 115-283 $\mu g/cm^2/h^{1/2}$)	96
Figure 4.15	Ibuprofen diffusion profiles (range 28-80 $\mu g/cm^2/h^{1/2}$)	97

Figure 4.16 Ibuprofen diffusion profiles of four species of shed snake skin obtained using the Franz diffusion cell apparatus 101
Figure 4.17 Ibuprofen diffusion profile of each shed snake skin orientation obtained using the Franz diffusion cell apparatus 102
Figure 4.18 Ibuprofen diffusion profiles of four species of shed snake skin obtained using the European Pharmacopoeia diffusion cell apparatus 107
Figure 4.19 Ibuprofen diffusion profile of each shed snake skin orientation obtained using the European Pharmacopoeia diffusion cell apparatus 108
Figure 4.20 Ibuprofen diffusion profiles of the scale and hinge regions of shed snake skin 114
Figure 4.21 Transverse section of silastic silicone membrane 116
Figure 4.22 Transverse section of ventral forest cobra scale 116
Figure 4.23 Transverse section of dorsal forest cobra hinge 116
Figure 4.24 Dorsal Egyptian cobra hinge (inside surface) 116
Figure 4.25 Dorsal Cape cobra hinge (outside surface) 116
Figure 4.26 Dorsal African rock python scale (inside surface) 117
Figure 4.27 Dorsal forest cobra scale (inside surface) 117
Figure 4.28 Dorsal Egyptian cobra scale (inside surface) 117
Figure 4.29 Dorsal Cape cobra scale (inside surface) 117
Figure 4.30 Surface region of 0.4 μm filter membrane 117
Figure 4.31 Ibuprofen diffusion profiles of the African rock python 123
Figure 4.32 Ibuprofen diffusion profiles of the forest cobra 124
Figure 4.33 Ibuprofen diffusion profiles of the Egyptian cobra 125
Figure 4.34 Ibuprofen diffusion profiles of the Cape cobra 126

CHAPTER ONE
IBUPROFEN MONOGRAPH

1.1 PHYSICOCHEMICAL PROPERTIES OF IBUPROFEN

1.1.1 Description

Ibuprofen is (RS)-2-(4-isobutylphenyl)-propionic acid. It contains not less than 98.5% and not more than 101.0% of $C_{13}H_{18}O_2$, calculated with reference to the dried substance (1-4).

Ibuprofen is a non-steroidal anti-inflammatory drug (NSAID) used in painful and inflammatory conditions. It is a white powder or colourless crystalline solid with a slight odour and taste (1,2,4). Ibuprofen is non-hygroscopic and is physically and chemically stable in the dry state. It is prepared chemically and is marketed as the racemic mixture of its two enantiomers (4).

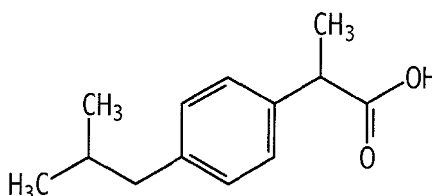


Figure 1.1 Structure of ibuprofen (molecular mass 206.3)

1.1.2 Melting Range

Ibuprofen melts in the range of 75°C to 78°C (2).

1.1.3 Solubility

Ibuprofen is practically insoluble in water but is soluble 1 in 1.5 of ethanol, 1 in 2 of ether, 1 in 1 of chloroform, and 1 in 1.5 of acetone. Ibuprofen is readily soluble in most organic solvents and is soluble in aqueous solutions of alkali hydroxides and carbonates. It is freely soluble in dichloromethane (2,4,5).

Solubility increases at pH values higher than the pKa. The approximate solubilities at pH 4, pH 6, and pH 7 are 1 in 35 000, 1 in 1 900, and 1 in 410 respectively (2).

1.1.4 Dissociation Constant

The dissociation constant (pKa) of ibuprofen is reported as 4.41 (35) and in a 60% ethanol solution as 5.2 (2).

1.1.5 Partition Coefficient and Permeability Coefficient

The octanol water partition coefficient (log *P*) is reported as 3.51. It has been shown that for maximum percutaneous absorption to take place log *P* should be in the range of 2-3. At low log *P*, the permeability coefficient is low but the aqueous solubility is high, while at high log *P*, the permeability coefficient is high but the aqueous solubility is low. The permeability coefficient is 4.36×10^{-2} cm/h for the un-ionised drug in an aqueous formulation (35,36).

1.1.6 Maximum Flux

The maximum flux through the skin is obtained by multiplying the permeability coefficient, for the un-ionised drug in aqueous formulation, and aqueous solubility. The maximum flux (Jmax) is reported as 0.61 $\mu g/cm^2/h$ (35).

1.1.7 Dissolution

The United States Pharmacopoeia (USP) specifies that for Ibuprofen Tablets USP, not less than 70% of the labelled amount of $C_{13}H_{18}O_2$ dissolves in 30 minutes. The dissolution medium is 900ml of phosphate buffer at a pH of 7.2, using apparatus 1 at 150 rpm (2).

1.1.8 Molecular Structure

Ibuprofen has a chiral centre in the propionic acid moiety and can exist in two enantiomeric forms. *In vivo* and *in vitro* studies indicate that only the S(+)-enantiomer of ibuprofen has clinical activity. On administration of the racemic mixture, the

pharmacologically inactive R(-)-enantiomer is converted to the active S(+)-enantiomer. A stereoselective assay is therefore necessary for pharmacokinetic studies (2,6).

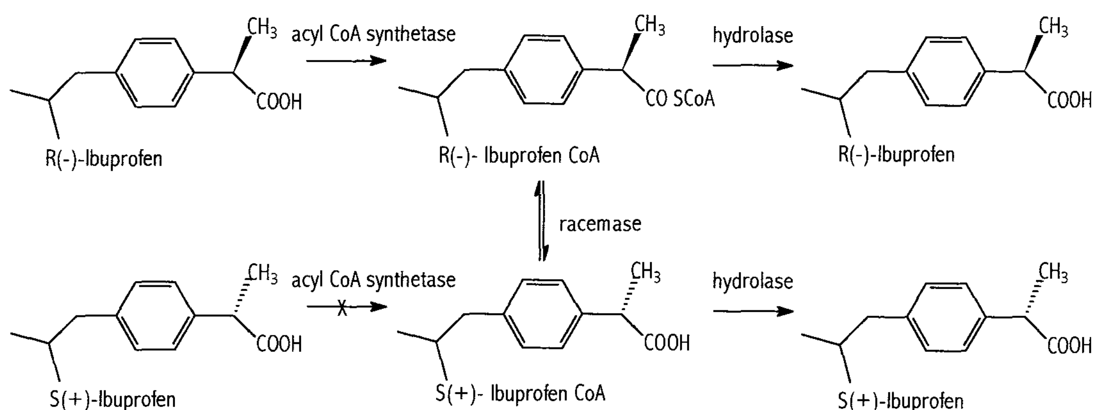


Figure 1.2 Stereospecific inversion of R(-) to S(+)-ibuprofen (37)

1.1.9 Ultraviolet Absorption Spectrum

The ultraviolet absorption spectrum of ibuprofen in 0.01M solution of sodium carbonate is depicted in Figure 1.3. The spectrum was obtained using a GBC UV/Vis 916 Spectrophotometer.

This spectrum is similar to that found in the literature (7).

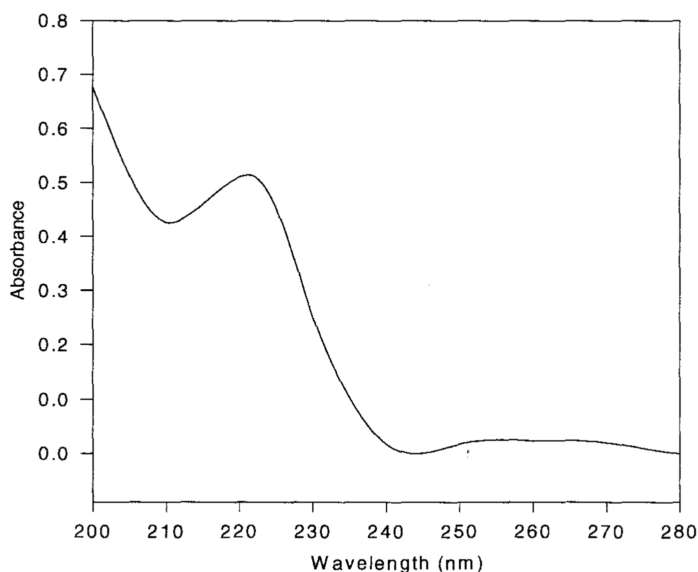


Figure 1.3 Ultraviolet spectrum of ibuprofen

1.2 CLINICAL PHARMACOLOGY OF IBUPROFEN

1.2.1 Preparations

Ibuprofen may be administered in a number of different forms (5) via the oral as well as the topical route. Published evidence suggests that topical, unlike oral, NSAIDs are associated with few systemic side effects as plasma concentrations are low compared to oral therapy.

ROUTE	FORM	STRENGTH
Oral	Liquid-filled capsules	200mg
	Suspensions	40mg/ml
		100mg/5ml
	Tablets	200 mg
		400mg
		600mg
		800mg
	Chewable tablets	50mg
		100mg
	Film-coated tablets	100mg
		200mg
		300mg
		400mg
		600mg
		800mg
Topical	Gel	5% m/m
	Cream	5% m/m
	Mousse	5% m/m

Table 1.1 Ibuprofen preparations

1.2.2 Mode of Action

Ibuprofen is a NSAID with three major types of effect:

- Anti-inflammatory effect
- Analgesic effect
- Antipyretic effect

Ibuprofen, as a NSAID, has the ability to alter the production of certain biochemical mediators, including prostaglandins and thromboxanes. Prostaglandins and thromboxanes are produced by the action of cyclo-oxygenase (COX) on arachidonic acid and are mediators of the inflammatory process (9,10). The primary action of ibuprofen is therefore the inhibition of arachidonate cyclo-oxygenase and thus inhibition of the production of prostaglandins and thromboxanes (11-14).

There are two types of cyclo-oxygenase, namely COX-1 and COX-2. COX-1 is a constitutive enzyme expressed in most tissues, including blood platelets, and is involved in cell-cell signalling, while COX-2 is induced in inflammatory cells when they are activated and is believed to be the enzyme that produces the prostanoid mediators of inflammation (11-14).

Inhibition of prostaglandin biosynthesis prevents their hyper-analgesic effect upon sensory nerves while inhibition of vasodilator prostanoid formation (prostaglandin E2) diminishes the vascularity and transudation of fluid which are two of the principal manifestations of inflammation.

Prostaglandins of the E and F series are potent uterotropic agents and their biosynthesis is normally increased in the hours before parturition, therefore ibuprofen, by inhibiting their formation, may slow the onset of labour. Inhibition of the formation of thromboxane A_2 by the platelets results in the platelets being less likely to aggregate in response to a thrombogenic stimulus (4).

1.2.3 Indications

Ibuprofen is used for its anti-inflammatory, analgesic and antipyretic effects in the management of conditions such as dysmenorrhoea, migraine, postoperative pain, ankylosing spondylitis, pericarditis, cystic fibrosis, osteoarthritis and rheumatoid arthritis including juvenile rheumatoid arthritis, and in other musculoskeletal and joint disorders such as sprains and strains (3-5,9,15).

In patients with primary dysmenorrhea, ibuprofen has reduced resting and active intra-uterine pressure and the frequency of uterine contractions, probably as a result of inhibition of prostaglandin synthesis (6).

In non-articular rheumatism which includes bursitis, fibrositis, lumbago, tenosynovitis, neck pain and myalgia of all types, ibuprofen is a suitable drug for management. Sports injuries and acute musculoskeletal trauma are other indications for ibuprofen (3,4,15).

Ibuprofen is one of the best tolerated NSAIDs for symptomatic relief of osteoarthritis and its effectiveness in achieving and maintaining control of pain coupled with its good tolerance, makes it a first line therapy for all forms of osteoarthritis (3,4,15).

Ibuprofen exhibits anti-inflammatory activity by reducing heat, tenderness, and swelling of joints in patients with rheumatoid arthritis resulting in an improvement in exercise tolerance, grip strength, and overall function. Oral ibuprofen is therefore recommended as first-line therapy in the majority of patients as it is effective, inexpensive and has the lowest reported incidence of side-effects, while topical ibuprofen is promoted on the basis that it diffuses rapidly and directly into joints resulting in high local and low plasma concentrations of the drug, thus giving a lower risk of systemic side effects than oral NSAIDs (9). Ibuprofen has been reported to be effective in the management of juvenile rheumatoid arthritis, particularly in patients who are unable to tolerate aspirin (3-5,9).

1.2.4 Adverse Reactions

The most frequent adverse effects occurring with ibuprofen are gastro-intestinal disturbances. Prostaglandins, particularly prostaglandin I2 and prostaglandin E2, are

synthesised by the gastric mucosa and seem to promote the secretion of cytoprotective mucus, therefore ibuprofen, by inhibiting the synthesis of these prostaglandins, may lead to gastro-intestinal disturbances (4,18). Reactions range from abdominal discomfort, nausea, vomiting, and abdominal pain to serious gastro-intestinal bleeding or activation of peptic ulcers (3-5,9).

Central nervous system related side effects include headache, dizziness, nervousness, tinnitus, depression, drowsiness and insomnia. Hypersensitivity reactions that occasionally occur include fever and rashes while hepatotoxicity and aseptic meningitis occur only rarely (3-5,19).

Ibuprofen has little effect on renal function in normal individuals but can precipitate renal failure in patients who depend upon the vasodilatory action of prostaglandin E2 or I2 to maintain renal blood flow. This occurs in hypertension, diabetes, cirrhosis of the liver and a number of other conditions (4). In cases such as these, ibuprofen and other non-steroidal anti-inflammatory drugs may cause cystitis and haematuria as well as acute renal failure, interstitial nephritis, and nephrotic syndrome (3,4,20-23). Ibuprofen may occasionally promote salt and water retention by interfering with the prostaglandin-induced inhibition of both chloride reabsorption and the action of anti-diuretic hormone (4).

Dermatologic effects which have occasionally occurred during ibuprofen therapy include urticarial, vesiculobullous, and erythematous macular rashes, erythema multiforme, exfoliative dermatitis, toxic epidermal necrolysis and photosensitivity reactions. Stevens-Johnson syndrome, flushes, alopecia, rectal itching and acne have also been reported (5).

Adverse haematologic effects include neutropenia, agranulocytosis, aplastic anaemia, haemolytic anaemia (with or without positive direct antiglobulin test results), and thrombocytopenia (with or without purpura). Although a causal relationship has not been established, bleeding episodes have been reported during therapy with the drug. Ibuprofen can inhibit platelet aggregation and may prolong bleeding time (5,16).

1.2.5 Contraindications

MOST SIGNIFICANT
• Acetylsalicylic acid allergy
SIGNIFICANT
• Bleeding disorder
• Duodenal ulcer
• Gastric ulcer
• Peptic ulcer
• Stomatitis
• Systemic lupus
• Erythematosus
• Ulcerative colitis
• Upper gastro-intestinal disease
POSSIBLY SIGNIFICANT
• Bronchial asthma
• Cardiac disease
• Congestive heart failure
• Hepatic function impairment
• Hypertension

Table 1.2 Drug disease contraindications

The risk of potentially serious adverse gastro-intestinal effects should be considered in patients receiving ibuprofen, particularly in patients receiving chronic therapy with the drug (4,9,16,24). Ibuprofen should be used with caution in patients with peptic ulcer disease, gastro-intestinal perforation or bleeding, bleeding abnormalities (especially in patients who may be adversely affected by prolongation of bleeding time), impaired renal function, hypertension or compromised cardiac function (4,5).

Renal prostaglandins may play a supportive role in maintaining renal perfusion in patients with pre-renal conditions, therefore administration of ibuprofen to such patients may cause a dose-dependent reduction in prostaglandin formation and thereby precipitate overt renal decompensation. Patients at greatest risk of this reaction include those with impaired renal function, heart failure or hepatic dysfunction; those with extracellular fluid depletion, those receiving a nephrotoxic drug concomitantly, those with elevated levels of angiotensin II or catecholamines and geriatric patients (5).

Ibuprofen is contraindicated in patients with known hypersensitivity to the drug and in patients in whom bronchospasm, angioedema or nasal polyps are precipitated by aspirin or other NSAIDs. Ibuprofen is contraindicated in patients in whom urticaria, angioedema, bronchospasm, severe rhinitis or shock is precipitated by aspirin or other NSAIDs since anaphylactoid reactions have occurred in such patients (4,5,9).

The safe use of ibuprofen during pregnancy has not been established. Ibuprofen inhibits prostaglandin synthesis and release which may cause dystocia, interfere with labour, delay parturition and may also have adverse effects on the foetal cardiovascular system. Use of ibuprofen is not recommended during pregnancy (especially during the last trimester) or during labour and delivery (5).

1.2.6 Drug Interactions

In several short-term, controlled studies, ibuprofen did not have a substantial effect on the prothrombin time of patients receiving oral anticoagulants. However, because ibuprofen may cause gastro-intestinal bleeding, inhibit platelet aggregation and prolong bleeding time, the drug should be used with caution and the patient carefully observed if the drug is used concomitantly with any anticoagulant or thrombolytic agent (4,5).

Aspirin and ibuprofen should not be given together because of the displacement of ibuprofen from serum binding sites and thus a reduction in plasma ibuprofen levels. In addition, concomitant administration of ibuprofen and salicylates, phenylbutazone, indomethacin or other NSAIDs could potentiate the adverse gastro-intestinal effects of these drugs and therefore ibuprofen probably should not be administered with these agents (4,5).

Ibuprofen has been reported to increase plasma or serum lithium concentrations by 12 - 67% and to reduce renal lithium clearance. The mechanism involved in the reduction of lithium clearance by NSAIDs (including ibuprofen) is not known but has been attributed to inhibition of prostaglandin synthesis, which may interfere with the renal elimination of lithium. Some clinicians recommend that patients receiving lithium should not receive ibuprofen. However, if ibuprofen and lithium are used concurrently, the patient should be closely observed for signs of lithium toxicity, and plasma or serum lithium concentrations should be monitored carefully during the initial stages of combined therapy or subsequent dosage adjustment (25-28).

1.2.7 Toxicity

Adverse effects associated with an overdose of ibuprofen usually depend on the amount of drug ingested and time elapsed, however, because individual response may vary, each occurrence should be evaluated individually. Occasionally overdosage of ibuprofen has been associated with severe toxicity, including death (6,29).

The most frequent manifestations of an ibuprofen overdose are abdominal pain, nausea, vomiting, lethargy, and drowsiness. In addition, other adverse effects, including headache, tinnitus, central nervous system depression, seizures, hypotension, bradycardia, tachycardia and atrial fibrillation may occur (30-34). Metabolic acidosis, coma, acute renal failure, hyperkalemia, apnoea (mainly in young children), respiratory depression and respiratory failure have rarely been reported (6,29). There appears to be little correlation between severity of manifestations associated with ibuprofen overdosage and plasma ibuprofen concentrations (30-34).

1.3 PHARMACOKINETICS OF TOPICAL IBUPROFEN

The pharmacokinetics of a topically applied ibuprofen cream have been investigated and compared to the results obtained after oral administration of ibuprofen (38). In this study, a maximum serum concentration (C_{max}) of 0.64 μg/ml of ibuprofen was found after two hours for a topical application of 300 mg ibuprofen. The maximum serum concentration is understandably significantly lower than that found after oral administration of 400 mg (C_{max} = 38.9 μg/ml at 2 hours). Assuming the absorption after oral administration was 100%, and adjusting for the differing dosage sizes, an absorption rate of 5% was calculated for topical administration.

Investigations into the pharmacokinetics of a topically applied ibuprofen gel have also been conducted. In one study a percutaneous absorption rate of 3% was found, with reference to an equivalent ibuprofen dose (39). This study yielded a maximum serum concentration of 0.2 μg/ml after 11 hours for a topical application of 400 mg of ibuprofen. A second study of a topically applied ibuprofen gel (200 mg ibuprofen) found the maximum serum concentration to be 1.40 ± 0.40 μg/ml which occurred at 4.83 ± 1.83 hours (40). In this study the use of an 8 hour occlusion period led to higher plasma profiles. A third study of a topically applied ibuprofen gel (500 mg ibuprofen) found the maximum serum concentration to be 7.1 ± 4.4 μg/ml which occurred at 2.4 ± 0.8 hours (41). The relative bioavailability of the ibuprofen gel formulation was reported as 22 ± 12%, determined as the dose corrected value with the oral dose set at 100%.

Several factors contribute to the differences between the results reviewed in the literature for topical ibuprofen. In general, the maximum serum concentration is dependent on the application site, the application surface, the duration of occlusion, the dosage and type of formulation. In three of the studies reviewed the application site and surface were identical [20 x 20 cm area on the upper back (38,39,41)]. In the fourth study, the application site was 20 x 18 cm on the upper back (40). The dose varies from 200 to 500 mg ibuprofen, which may also account for the differences in the results.

Two of the cited literature studies were performed without occlusion (38,39). Under non-occluded conditions, the rate of transepithelial water loss is about 6 $g/m^2/h$ (45). The use of occlusion prevents transepithelial water loss and thus causes swelling of the stratum

corneum. Swelling facilitates the penetration of ibuprofen through the stratum corneum into the epidermis, resulting in higher concentrations in the epidermis and eventually in the plasma.

The much higher maximum serum concentration observed in the study conducted by Kleinbloesem (41) can be related to the type of formulation. The product contains, among other ingredients, isopropanol and propylene glycol. Both of these substances are classified as humectants and are thus capable of bringing about hydration and hydrophilisation of the stratum corneum, facilitating the absorption of ibuprofen. Propylene glycol is also classified as a penetration enhancer, which causes increased penetration of ibuprofen through the stratum corneum.

Promotion of penetration is also brought about by the pH 5.0 of the gel used in the study conducted by Kleinbloesem (41). The pKa of ibuprofen is 4.41 (35), thus at pH 5.0, about 40% of the ibuprofen is present in its neutral, undissociated form. This neutral form of ibuprofen is better able to pass through the lipophilic stratum corneum into the epidermis (45), where the pH of 7.4 causes a shift to about 99.9% dissociated ibuprofen. In this dissociated form, ibuprofen is better able to travel through the hydrophilic epidermis and reach the target tissues.

Concentrations of ibuprofen in the plasma are therapeutic at levels greater than 10 μg/ml (42). After topical administration, ibuprofen levels in the plasma reach detectable levels surprisingly quickly (within the first half hour), however maximum serum concentration falls short of reaching therapeutic plasma levels (41). This is a good indication that the topical administration of ibuprofen does not lead to systemic adverse effects, as therapeutic concentrations in the circulatory system are not achieved. In contrast, it is known from the literature that therapeutic concentrations are achieved in the target tissues after topical application of ibuprofen (39,43,44).

The principle products of metabolism, as seen in figure 1.4, are formed by 2-hydroxylation to 2-[4-(2-hydroxy-2-methylpropyl) phenyl]-propionic acid (~9% of the dose in urine) followed by conjugation (~17%) and oxidation to the 2-carboxy product, 2-[4-(carboxypropyl)- phenyl]propionic acid (~16%) followed by conjugation (19%). None of the metabolites are pharmacologically active (4,37).

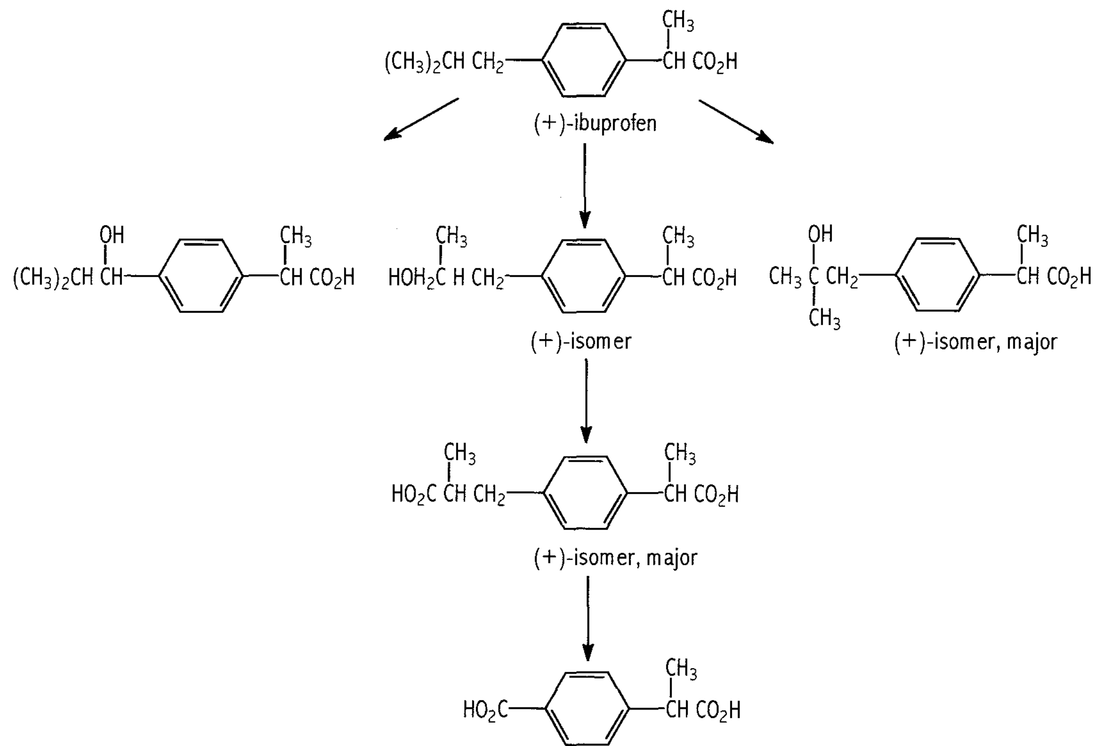


Figure 1.4 Metabolism of ibuprofen

CHAPTER TWO
THEORY OF TRANSMEMBRANE DIFFUSION

2.1 TRANSMEMBRANE DIFFUSION

2.1.1 Introduction

The skin is the primary area of body contact with the environment. Introduction of chemicals into the body via the skin occurs both through passive contact with the environment and through direct application of chemicals on the body for purposes of medical therapy and as cosmetics. Delivery systems may be placed against the skin to deliver drugs to the local tissues immediately beneath the application site; deep regions in the vicinity of, but still somewhat remote from, the application site; and the systemic circulation, the latter to mediate pharmacological changes somewhere totally removed from the application site (144).

Free diffusion or passive transport of substances through liquids, solids, and membranes is a process of considerable importance in the pharmaceutical sciences.

2.1.2 Kinetics of the Diffusion Process

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 permeation of a molecule through a membrane, either biological or synthetic, *in vivo* or *in vitro*, would normally occur as follows (152):

1. The molecule must diffuse through the vehicle in which it is contained to the membrane interface and must partition from the vehicle into the upper lamina of the medium.

2. The molecule must diffuse within the membrane, equilibrating laterally and must emerge, eventually under steady state conditions, from the distal surface of the medium. Adsorptive interaction may be extensive in this layer, forming a reservoir of the molecule.

3. The molecule then partitions either into the neighbouring membrane strata or into the receptor fluid under the influence of the concentration gradient, and adsorption may occur once again. Diffusion through any one of the layers or any of the partitioning events may control the overall rate of permeation.

The experimental handling of animal membrane data resembles that for diffusion through homogenous synthetic media. The steady state diffusion through a homogenous medium may be described in terms of Fick's first law (46) which states that the rate of transfer of diffusant through unit area of membrane (the flux) is proportional to the concentration gradient measured normal to the section:

$$J = -D\, dC/dx \qquad \text{Equation 1}$$

Where J is the flux, D is the diffusion coefficient of the diffusant in the membrane, C is the concentration of the diffusing substance (usually taken as the concentration of the diffusant in the vehicle in contact with the donor surface of the membrane) and x is the distance of movement perpendicular to the surface of the barrier. The negative sign signifies that diffusion occurs in a direction opposite to that of increasing concentration, thus flux is always a positive quantity (152).

Fick's first law is only applicable to diffusion in an isotropic medium and where the diffusant concentration at the distal surface of the membrane is zero. While synthetic media may comply with the isotropic constraint of the equation, the heterogeneous environment of snake skin may cause significant deviations from Fickian behaviour. In the case of diffusion through anisotropic membranes, such as snake skin, an equation for mass transport that emphasises the change in concentration with time at a definite location rather than the mass diffusing across a unit area of barrier in unit time is more applicable. For most experimental situations effective diffusion occurs only in one direction through the membrane, that of the concentration gradient (152). Fick's second law (46) states that the change in concentration with time in a particular region is proportional to the change in the concentration gradient at that point in the system:

$$dC/dt = D\, d^2C/dx^2 \qquad \text{Equation 2}$$

where t is the time, and x is the diffusion distance in the direction of the concentration gradient.

In the *in vitro* diffusion systems used, the Franz and European diffusion cells, a membrane separates two chambers, one containing a constant donor concentration, and the other containing sink receptor conditions. Initially the concentration gradient across the membrane will not be linear as the diffusant equilibrates within the medium, however after sufficient permeation time has elapsed steady state will be achieved and the effective diffusant concentration at all points in the membrane will remain constant (152). For an infinite dose of drug applied to the donor surface of a membrane in an *in vitro* diffusion cell under sink conditions, (the permeation situation that has prevailed in this experimental work) Fick's second law has been expanded to the following expression as time approaches infinity (46):

$$M = DC_0/h\ (t - h^2/6D) \qquad \text{Equation 3}$$

where M is the cumulative mass of diffusant that has passed through unit area of membrane, C_0 is the concentration of diffusant in the membrane lamina juxtaposed to the donor vehicle, and h is the thickness of the membrane. This equation is applicable to the typical cumulative mass versus time diffusion plots that have an initial non-linear lag time followed by a linear steady state plot.

By differentiating Equation 3 with respect to time an expression is obtained for the steady state flux (dM/dt) which is the gradient of the straight line (46):

$$DM/dt = DC_0/h \qquad \text{Equation 4}$$

Normally the concentration of the diffusant in the membrane lamina at the donor surface is not known, however the concentration of the diffusant in the donor vehicle in contact with the membrane can be measured:

$$DM/dt = DCK/h \qquad \text{Equation 5}$$

where K is the partition coefficient of the diffusant between the vehicle and the membrane. The product of the partition coefficient and the donor vehicle concentration will yield the diffusant concentration in the membrane lamina. The main variables influencing the rate of diffusion are thus the partition coefficient and the effective diffusion coefficient, the donor concentration and the membrane thickness (152).

The dimensions of these variables may be unknown and the equation is simplified to (46):

$$dM/dt = PC \qquad \text{Equation 6}$$

where P is the composite permeability coefficient. The permeability coefficient is often quoted where the diffusion and partition coefficients cannot individually be calculated.

The diffusion coefficient of the solute in the membrane may be estimated if the lag time (L) is obtained from the diffusion profile by extrapolating the linear steady state portion of the plot to the abscissa where M = 0. If the membrane thickness is known, the diffusion coefficient is then given by (46):

$$D = h^2/6L \qquad \text{Equation 7}$$

Knowing the concentration of the diffusant in the donor vehicle, the partition coefficient may be estimated from the mass of the diffusant that has passed through a unit area of membrane. Using Equation 6, the partition coefficient may then be calculated using:

$$P = KD/h \qquad \text{Equation 8}$$

Therefore, all the parameters of the steady state diffusion equation may be estimated from the measured permeation data, if the medium thickness and donor concentration are known.

In certain instances the partitioning characteristics of the drug between the vehicle and membrane may be highly favourable, generating high drug concentrations within the medium. Alternatively, the barrier potential of the membrane may be minimal or violated by disease, chemical or, as is the case with shedding of the snake skin, physical trauma. In these cases the rate of drug diffusion through the medium will be high and the replenishment of the partitioned diffusant at the membrane interface from the bulk vehicle may become the rate controlling step in the diffusion process, rather than the sensitivity of the membrane itself (152). Higuchi (47) proposed the mathematical relationships that are applicable to this situation in which the membrane is regarded as having negligible resistivity and sink clearance conditions are maintained throughout the diffusion time.

The rate of permeation would then be governed by the rate of diffusion through the vehicle and is expressed by the simplified equation:

$$M = 2C_0 (D_v t/\pi)^{1/2} \qquad \text{Equation 9}$$

Where D_v is the diffusion coefficient of the drug in the vehicle and C represents the initial concentration of diffusant in the vehicle. It is apparent from this equation that the mass of drug permeating to the sink is proportional to the square root of the contact time between vehicle and membrane. Permeation of this nature has a characteristic curved profile, exhibiting relatively high flux at early contact times which decreases as the diffusant front regresses into the bulk vehicle, away from the membrane. The path is progressively more tortuous, and it takes longer for drug molecules to diffuse from the region of high concentration in the vehicle to replenish the drug molecules at the membrane interface that have partitioned into the membrane, therefore the flux rate decreases with time (152).

Equations 1-9 describe the process of diffusion through an isotropic medium and these mathematical functions have been applied to the analysis of the data from this experimental study. Certain assumptions are made in their application: that sink diffusion conditions are maintained throughout the experiment, that extensive adsorption of the diffusant does not occur, and that the barrier medium is homogeneous.

2.1.3 Modification of the Diffusion Process

Many physicochemical factors may influence the rate of drug diffusion through a membrane. Although several of these factors may be controlled in laboratory diffusion experiments, some influences may be unavoidable and may thus affect the results obtained.

2.1.3.1 Hydration

An important factor which influences drug partitioning from a topical vehicle into a membrane, especially biological tissues, is the degree of hydration. Maintaining adequate membrane hydration is essential to the dissolution of the drug into, and subsequent diffusion through, the membrane.

During the diffusion experiments performed in this study, the receptor fluid was in continuous contact with the membrane resulting in complete hydration of the membrane. Further hydration was provided through the application of external occlusion which prevented the evaporation of water and volatile constituents from the formulation. Moreover, the composition of the vehicles in the occluded mode remained relatively constant throughout the delivery period.

2.1.3.2 Diffusant Concentration

The rate of diffusion is directly proportional to the concentration of drug in solution at the donor surface of the membrane. The greatest thermodynamic potential of an experimental system would be generated by a saturated solution of the diffusant in the vehicle (128). The viscosity of the formulation may hinder intra-vehicle drug diffusion and result in a steadily decreasing concentration at the membrane interface. In general, any physicochemical factor that may change the concentration of the drug at the membrane surface will alter the permeation behaviour.

2.1.3.3 Skin Damage

Any pathological or physical condition that alters the character of shed snake skin will probably alter its resistance to diffusion. Although *in vitro* permeation studies would normally use undamaged shed snake skin, there is a possibility that these membranes may be damaged by preparative techniques or by experimental methodology. This problem is not as critical when synthetic media are used as these are relatively resistant to physical damage.

2.1.3.4 Partition Coefficient

The influence of the vehicle composition on the partition coefficient is important in that any change in the partition coefficient will change the drug concentration established in the superficial lamina of the membrane. The magnitude of the partition coefficient will be influenced by any factors that affect the potential of the molecule to escape from the donor vehicle, including the degree of ionisation, complexation and adsorption.

The solubility of the drug in the membrane would influence its affinity for that medium. The drug structure-activity relationship suggests that the more lipophilic molecules would have greater affinity for the skin and would partition to a greater extent than polar moieties, however some bipolar character is essential.

Surfactant molecules in the donor vehicle may enhance partitioning by reducing the surface tension between the vehicle and the membrane surface, but may also influence the barrier potential of the membrane (48).

2.1.3.5 Vehicle/Dosage Form

Many researchers have evaluated a large number of vehicles and various dosage forms for their effects on drug permeation through the skin (129-141). These evaluations were conducted in relation to both transdermal and dermatological drug delivery and based on both *in vitro* and *in vivo* methods. From the published literature, it is not always clear whether the vehicle concentration was optimal (128). This is especially true for extemporaneous dosage forms such as creams and ointments as these products usually contain a large number of excipients including vehicles, surfactants, stabilisers, preservatives, and thickening agents (128).

Vehicles alone can cause dramatic effects on the permeation of drugs, and even for dermatological products, vehicles can dramatically influence drug uptake into the skin (142). It is now becoming apparent that the manner in which vehicles influence transdermal drug delivery is different from the manner in which they affect local drug delivery to the skin (128). This is believed to be due to the simultaneous uptake of the vehicles and other formulation components into the skin, which can cause alterations in the skin integrity and changes in the microenvironment of the skin (142).

2.1.3.6 Penetration Enhancers

Chemical and physical approaches have been used to improve or enhance the permeation characteristics of drugs of choice (128). The chemical approach involves using certain chemicals in the formulation that improve drug permeation by modifying the

thermodynamic properties of the drug or by altering the skin integrity to make it more permeable.

A confusing aspect of the chemical approach is that it is difficult to differentiate typical vehicle effects from typical enhancer effects, as the point at which a chemical becomes an enhancer has not been well defined. Even if a certain chemical is labelled as an enhancer in a given formulation, its effectiveness is highly influenced by the vehicle(s) used (128).

It is the complex interaction between the biochemical constituents of the skin, the drug and the constituents of the donor vehicle that may enhance the permeation of the drug through the membrane, or retain it within the vehicle environment.

2.1.4 Diffusion Boundary Layer Formation

The drug concentration gradient is the driving force for diffusion and any increase in the drug concentration on the receptor side of the barrier would decrease the magnitude of the gradient and retard further permeation. In many cases of *in vitro* diffusion cell use, absolute sink conditions do not prevail for the entire experimental period because of accumulation of the permeant in the receptor phase. However, if this increase in concentration is not substantial then it may be assumed that sink conditions have not been violated.

The characteristics of the mass transfer within the receptor chamber of the diffusion cell are determined by the nature of the liquid motion: either laminar or turbulent flow may exist dependent on the degree of fluid agitation. Laminar flow is uniform in character with little variation in the velocity of the fluid eddies throughout the chamber. Turbulent flow is irregular in direction and velocity, and mass transfer in these cases is by irregular fluid pulsations. In both cases, the properties of fluid flow at the membrane surface are important as the diffusant molecules that partition into the receptor fluid from the distal surface of the membrane must be rapidly swept away by the bulk fluid motion so that the concentration differential may be maintained across the barrier.

In both flow conditions the velocity of the fluid will decrease in the layers adjacent to the membrane, and will be zero at the barrier surface. This layer of fluid near the membrane,

that is not agitated at the same rate as the bulk fluid, is termed the boundary diffusion layer and is generated by the viscous, frictional drag forces between one theoretical fluid layer passing over another. The rate of agitation is one of the primary determinants of boundary layer thickness. The greater the degree of agitation of the bulk fluid the greater will be the shearing stress on the fluid layers adjacent to the membrane, and a thinner boundary layer will result. Conversely, slow stirring of the chamber fluid will generate much thicker stationary diffusion layers with a more gradual decrease in the fluid velocity. Turbulent flow is generally regarded as more efficient than laminar flow in the dispersion of these relatively stationary fluid layers. The viscosity of the receptor fluid is equally important in determining the thickness of the diffusion layer. Only under conditions of optimal fluid agitation would the diffusion boundary layers in the receptor chamber of the diffusion cell be minimised and therefore, negligibly influence the overall resistance to mass transfer (46).

2.2 MEMBRANES FOR USE IN *IN VITRO* DIFFUSION EXPERIMENTS

2.2.1 Biological Membranes

2.2.1.1 Human Skin

Human tissue would be the best medium to use in a diffusion cell so that appropriate extrapolation of conclusions to the *in vivo* situation could occur. Excision of the skin does not alter its permeability properties significantly, provided the stratum corneum remains intact, and several studies have shown that the stratum corneum performs similarly *in vitro* and *in vivo* even after several postmortal days (70,71). It should therefore be possible to design representative *in vitro* systems using excised human or animal tissue as a similarity between laboratory animal and human skin has been observed (72,73).

The use of human skin for experimentation is not without problems. Its availability is limited and permeability varies greatly between specimens taken from the same or different anatomical sites of the same donor (27% variance *in vivo* and 43% *in vitro*), and greater variations (45% variance *in vivo* and 66% *in vitro*) are noted between specimens from different subjects of different age groups (74-76). Complicating these difficulties, metabolism and biotransformation of chemicals applied to the skin may continue for long periods after excision of the tissue from the donor and may be extensive in some cases (77).

2.2.1.2 Animal Models

Given the limited availability of human tissue and the fact that a number of percutaneous investigations may be too toxic to be carried out on living subjects, a number of animal models have been investigated for their usefulness in predicting percutaneous absorption kinetics. These animal models include mouse (53,80,81,83), hairless mouse (53,80,83-92), rat (53,73,80,93-96), guinea pig (80,97), rabbit (53,64,78,83,98,99), monkey (52,53,80,81,100), pig (53,79-81,94,178), snake skin and egg-shell membranes (80,101).

The skin of experimental animals differs markedly from that of humans in features such as thickness and biochemical composition of the stratum corneum, and especially the

presence or density of hair follicles and glands (49). Furthermore it has been suggested that the lipid content of the skin is a major determinant in its barrier potential and that differences between species or between sites are due to varying lipid composition (40,51).

Recently there has been considerable interest in the use of shed snake skin as a membrane for *in vitro* diffusion studies monitoring the release of a number of drugs from semisolid formulations (102-104,108-111,177,244). Shed snake skin has also been suggested as a membrane for the study of the effects of a number of penetration enhancers (112-120,244). Although shed snake skin is not a mammalian integument, many compounds penetrate snake skin and human stratum corneum at similar rates (102,103,172,179,180). Both shed snake skin (149) and human stratum corneum (150) are composed of keratinized proteins and lipids. Water permeation characteristics of snake skin are similar to those of human skin (136,151).

In some countries it is particularly difficult to obtain human skin for *in vitro* experimentation and it is therefore important to have alternate biological or synthetic membranes which mimic human skin for diffusion experiments (111). In South Africa, shed snake skin is easily obtainable from the many snake parks present in the country. Since snakes moult periodically, a single animal can provide repeated sheds, thus eliminating inter-individual variability. Skins can be obtained without injury to the animal and do not have to be subjected to chemical or heat stress before use (80). The epidermis is shed as a large, intact sheet, thus a single snake skin can provide multiple samples (104). Shed snake skin is not a living tissue, can be stored at room temperature for relatively long periods and is easily transported. Stored or fresh skins appear to show no differences in permeability. Since snake skin lacks hair follicles, the problems associated with transfollicular routes of penetration, which may be significant in mammalian skins, can be avoided (80).

Snakes shed their skin every two to four months, depending upon the species, age of the animal and quantity of food ingested. Skins are shed in different ways. This shedding has been observed for three species of snakes in a South African snake park. It was reported that the *Naja melanoleuca* (forest cobra) and the *Bitis nasicornis* (rhinoceros viper) rub their noses against a hard surface, normally a rock, to break the old skin. They then, with very gentle muscular contractions, loosen the whole skin and ease their bodies out of the

hole in front, leaving behind a complete shed skin. The *Python sebae natalensis* (African rock python), on the other hand, tends to rub its body against the rocks, thus the skin comes away in fragments (111).

Shed snake skin is composed of two very different regions as depicted in figure 2.1. These comprise the scales which are separated by hinges. The scales are fairly rigid, whereas the hinge region is relatively elastic. The scales on the dorsal surface are much smaller than the scales on the ventral surface and, in addition, the size of the scales varies considerably between species.

Unlike human stratum corneum, which consists of 10-20 layers of an alpha-keratin-rich intracellular layer and a lipid-rich intercellular layer (172), shed snake skin consists of three distinctive layers (113). These are the beta-keratin-rich outermost beta layer, alpha-keratin- and lipid-rich intermediate mesos layer, and alpha-keratin-rich innermost alpha layer (149). Further, the mesos layer shows three to five layers of multilayer structure with cornified cells surrounded by intercellular lipids, which is similar to human stratum corneum. This mesos layer is also a major depot of lipids, and the mesos layer and alpha layer are considered to be the main barrier to water penetration through the skin (172,180-186).

Lipid compositions of shed snake skin and human stratum corneum are also similar, *i.e.*, neutral lipids are a major lipid component in both skins and fatty acids, with carbon chain lengths of C16 and C18 predominant (172). Lipids are important components of the skin in controlling the permeability of compounds, and delipidization has been shown to increase water permeability through both shed snake skin and human skin (151).

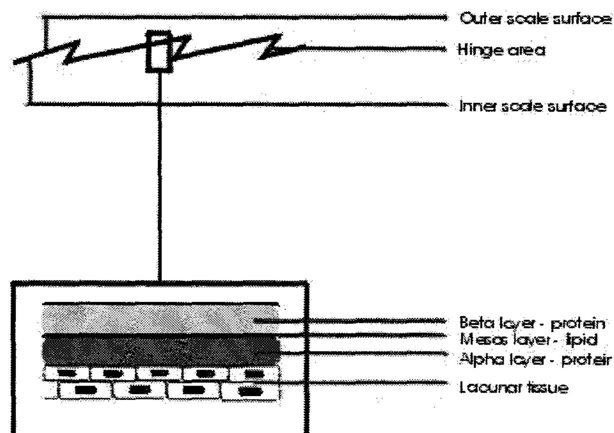


Figure 2.1 Diagrammatic representation of the transverse section of shed snake skin

Previous studies indicate that different snake species have been used as well as both dorsal and ventral skin. Some studies report which species of snake was utilised and whether dorsal or ventral skin was used in the experiments. Other studies did not report whether dorsal or ventral skin was used (103,108,112-114,118,121,244) and two studies did not indicate which species was used (116,117). This study addressed the diffusion characteristics of four different species of snake and investigated the differences between the dorsal and ventral skin sites as well as the orientation of the skin with respect to the semisolid formulation surface.

It has been shown in an investigation involving the *Naja melanoleuca* (forest cobra), the *Bitis nasicornis* (rhinoceros viper) and the *Python sebae natalensis* (African rock python) that the most permeable is the python skin with dorsal skin being significantly more permeable than ventral skin. Cobra skin displayed intermediate permeability with dorsal and ventral skin producing identical results. Viper skin was found to be the least permeable with dorsal skin being more permeable than ventral skin. The apparent release constants obtained from regression analysis of the diffusion curves, displayed the same trend. The lag time was found to be very similar for python and cobra skins but virtually zero for viper skin (111).

The same investigation found that the ventral skin of all three species is always thicker than the dorsal skin both in the scale and hinge regions. This was attributed to the fact that when the snake moves, the ventral surface is in contact with the ground. It would therefore have to be more robust than the dorsal surface. In addition, the hinge region was found to be considerably thinner than the scale region, especially in the forest cobra and rhinoceros viper. Generally, the ventral scales are much larger than the dorsal scales resulting in a much larger hinge:scale ratio for dorsal skin than for ventral skin. Since permeation appears to be more facile through dorsal skin, it may imply that diffusion occurs mainly through the hinge region of the skin (111).

The study concluded that shed snake skin may be a useful membrane for comparing the diffusion of specific drugs from different formulations or the effects of different enhancers but care must be exercised when extrapolating to the *in vivo* situation since each species displays different permeation characteristics (111).

2.2.2 Synthetic Membranes

The use of artificial membranes is common in laboratory percutaneous absorption studies. Synthetic membranes are not intended to mimic the barrier properties or the heterogeneous nature of the skin but, instead, to serve as predictive models for topical drug release (175). They are useful in preformulation studies designed to determine the leaving potential of an active drug from its vehicle and in ensuring batch-to-batch uniformity of the formulated topical vehicles (176). Furthermore, the stability and uniformity of synthetic membranes makes them desirable for use in diffusion experiments.

The barrier potential of porous membranes is dictated by the probability of a diffusant molecule entering and diffusing through the pores. The only factor governing selectivity to diffusion would be the relative molecular size and shape. Conversely, aporous media appear to offer some rate-limiting factor to permeation and therefore may more closely simulate the diffusion through biological tissue. The barrier properties here generally relate to the solubility of the diffusant in the polymer matrix and partition coefficient between donor and membrane (54).

2.2.2.1 Filter Membranes

Porous filter membranes have seen relatively little usage in diffusion systems in comparison to the synthetic polymers. Generally, porous filter media appear to be most useful as a dividing medium or as supporting screens where the release rate of drug from the delivery system is under investigation, and not the transdermal kinetics of the permeant. In these cases the filter medium does not simulate the skin and provides no significant barrier to diffusant passage (55).

2.2.2.2 Cellulose Media

Cellulose is a relatively rigid structure consisting of glucopyranose rings combined by β-1,4-inkages. This conformation allows only two types of movement in the chains: inversion of the pyranose ring or rotation around the glycosidic linkage (3). In addition, the cellulose chains exist in a partially crystallised form due to inter-chain hydrogen bonding (54). Commercial cellulose membranes have a cut off of 8000-15000 daltons for molecular dialysis and on purchase normally contain a number of softener, preservative

and plasticizer additives which may affect drug permeation depending on the membrane pre-treatment prior to experimentation.

Cellulose acetate (dialysis) media have been extensively used in diffusion cell systems (56-61). Generally, cellulose membranes are reported to be more permeable than biological membranes or aporous synthetic media (62) and are non-discriminatory to the characteristics of the diffusant molecule.

2.2.2.3 Synthetic Polymers

Diffusion of a molecule through continuous synthetic polymer is dependent on the frequency of void formation of sufficient size to accommodate the diffusant. Voids are formed by the random oscillation of polymer chains and the larger the diffusant species the greater the number of neighbouring polymer units which would have to move in a specific manner in order to generate a void of sufficient volume to accommodate the diffusant (46,66).

Molecular flux through continuous polymer is analogous to flux through liquids, but in this case molecular voids are formed by the random oscillation of polymeric units. Hydrogen and other small molecules may require only monomeric unit displacements whereas larger diffusional species need cooperative oscillations, possibly involving polymer segments containing tens of units (66). The degree of bonding interaction between the polymer chains will determine the rigidity of the matrix and thus, the propensity for hole formation and resultant permeability. Furthermore, crystallinity within the matrix generates regions of low diffusivity and the presence of solvents facilitates the oscillation of polymeric segments, both situations altering the overall permeability of the membrane (63,64). Crystallinity introduces regions of very low diffusivity relative to the diffusivity in the surrounding amorphous mass. At a minimum, solvent tends to facilitate the oscillation of polymeric segments by reducing chain interactions (plasticization) and changing permeability. In the extreme, the solvent may become the diffusional medium (66). Silicone elastomer is above its glass transition point (point of appearance of crystallinity) at ambient temperature and, in addition, solvent is denied access by the physicochemical nature of the polymer, therefore these complexities are absent in this study.

Silicone polymers such as polydimethylsiloxane are lipophilic in nature and highly permeable to many non-ionic drugs which dissolve in the barrier matrix and diffuse across it (62,65,66). An important variable affecting the permeation process is the partition coefficient between donor or receptor vehicle and membrane, as is the polymer solubility of the drug. These two factors are highly dependent on structure and even small molecular modifications may greatly alter partitioning and solubility (63).

Commercial products may contain additive fillers, such as 20-30% silica or graphite, to improve the membrane strength and their presence may increase the barrier potential to permeation. This filler is randomly orientated, non-uniform in size and is impervious to permeants but readily enters into adsorption interactions. Thus, permeation rates are generally lower through filled media (63,67-69) and lag times are increased (66). Generally, it is concluded that these fillers simply reduce the volume of polymer which is available for the steady state passage of permeating molecules and makes their diffusive path longer and more tortuous.

Therefore, the most important parameters with regard to permeation appear to be polymer chain mobility (rigidity) and the solubility of the diffusant in the matrix, which influences the partition coefficient. Silicone membranes are considered the most useful of the synthetic media for use in diffusion cell systems. Its relatively inert, lipophilic nature makes it an ideal environment for partitioning and permeation of drugs while its aporosity provides some rate-limiting function to this process.

2.2.3 Conclusions

It is evident that no single animal or synthetic model can mimic percutaneous absorption in man for the diverse range of chemicals that are investigated for clinical and toxicological effects. A specific test system comprising membrane, diffusion cell type (105), cell receptor fluid, analytical procedure (106), agitation and temperature (107), must be established for each drug and delivery vehicle under investigation.

Presently, biological membranes are used more extensively than synthetic membranes in laboratory diffusion systems. In addition, the comparison of results from different laboratories is difficult because of the plethora of different diffusion cells and experimental

protocols currently used worldwide. As the technology of polymer membranes and cell culture techniques improves, it may be expected that these media would become convenient standard membranes for use in topical drug delivery systems (80).

2.3 DIFFUSION CELL APPARATUS

2.3.1 Introduction

A number of experimental methods have been developed to investigate the percutaneous delivery of topically applied drugs in an attempt to isolate physical and chemical factors that govern drug absorption. In many instances the diverse experimental techniques tend to obscure absorption controlling factors and complicate inter-study comparisons.

The benefits of utilising an *in vitro* cell system for the preliminary testing of drug diffusion in the laboratory are numerous. The environment, specific permeation parameters and variables may be controlled in an attempt to elucidate specific factors affecting the kinetic processes, prior to undertaking studies in human volunteers. The permutations of these variables that are selected for study will dictate the design and protocol of the experiment. Experimental design that allows the greatest number of variables and different variable permutations to be investigated by the same *in vitro* diffusion apparatus is highly desirable.

Drug absorption factors which may require consideration when selecting an *in vitro* system include (152):

1. The intrinsic diffusivity of the permeating molecule, its structure-activity relationship and its apparent diffusion coefficient.

2. The route of penetration that will predominate during the experiment and the relative extents of drug binding and metabolism that will occur.

3. The rate-limiting factor in the permeation process: drug solubilization and/or diffusion in the formulation, partitioning from the vehicle, diffusion through the test membrane or partitioning and removal by the receptor phase.

4. The intrinsic barrier potential of the test membrane and the effects that vehicle components may have on its retardive properties. Hydration of the membrane may be important in this regard as may be the presence of penetration enhancers.

Inter-specimen variability between membranes of the same type may greatly influence experimental results.

Several diffusion cell designs incorporating rate-limiting membranes have been reported in the literature, each design having specific features which it was hoped would overcome certain experimental limitations. Most designs share common features which include two chambers, one containing the donor vehicle and the other containing agitated receptor phase fluid, the two chambers being separated by the membrane under test. The appearance of the drug in the receptor phase (or diminution from donor phase) may then be analytically monitored as a function of time. The donor vehicle is assumed to expose the membrane to a constant concentration of the drug and the receptor phase is assumed to maintain sink clearance conditions for the permeant. It has been reported that a depletion of donor phase or an increase in receptor phase concentration not exceeding 10% does not significantly violate zero-order flux conditions or deviate from the initial thermodynamic driving force for diffusion (156).

The influence of adequate receptor solvent agitation on the results obtained from *in vitro* diffusion experiments have shown that as stirring rate increased, the thickness of the unstirred boundary diffusion layer decreased and drug permeation rate increased. Moreover, if stirring increased sufficiently the rate of detected permeation would no longer be partially governed by the diffusion layer thickness (55,157). Many experimental observations are made when boundary diffusion layers represent a significant portion of the total barrier presented to the diffusing solute. The greater the portion of this representation the less accurate are the results obtained with respect to the membrane itself and the greater will be the deviation between *in vitro* and *in vivo* results. In some cases the existence of the diffusion boundary layer is acknowledged but is ignored in comparative work because of its uniformity throughout the experiments (54), or it is assumed to be negligible (158). This is acceptable as long as it is realised that calculation of the diffusion coefficient will include the contribution of the boundary layer barrier and thereby only yield an apparent diffusion coefficient value.

Although several solvents have been used for the donor and receptor vehicles, the vast majority of the reported experiments use aqueous media because the assay of drugs in aqueous solution is facile by a number of techniques. However, drug solubility must be

considered when aqueous solvents are used. If the diffusant is only slightly soluble in the receptor fluid then it will not partition from the distal surface of the membrane and further diffusion will be retarded (159-161). The question of hydration should also be addressed when membranes are immersed for prolonged periods in aqueous vehicles. The effect that hydration may have on permeation appears to vary depending on the drug under investigation and membrane characteristics (75,162,163). On the other hand, the use of alcoholic or nonpolar organic solvents must in no way affect the biochemical composition of the barrier membrane (161).

Although diffusion cells are manufactured with many different designs, there are two basic models that have been identified: one-chambered and two-chambered cells (153). Each type has its own use for skin absorption experiments.

2.3.2 Two-Chambered Cell

Researchers have used variations of the two-chambered cell for measuring the diffusion of a compound in solution from one side of a membrane to the other (154). A dose that is large enough to maintain constant concentration during the course of an experiment (an infinite dose) is added to one side of the diffusion cell, and the rate of diffusion across a concentration gradient into a solution on the opposite side of the membrane is determined. Usually, the solution on each side of the membrane is stirred to ensure that the concentration of test compound remains uniform throughout the experiment. Studies that compare absorption through membranes according to principles of Fick's membrane diffusion theory are usually conducted with these cells. The two-chambered cell is also useful for studying mechanisms of diffusion through membranes and for measuring membrane permeation (153).

2.3.3 One-Chambered Cell

The actual exposure of the membrane to substances that are absorbed usually occurs under conditions different from those simulated by the two-chambered cell. The amount of material applied to the surface of the membrane is frequently small (finite dose), and as permeation proceeds, a steady-state rate of absorption is not achieved. The surface of the membrane in this type of cell is frequently open to the environment. The membrane is not

excessively hydrated by continued exposure to an aqueous solution, as in the two-chambered cell. The chamber beneath the membrane holds the receptor fluid, which is continually mixed with a magnetic stirring bar. Aliquots of the receptor fluid are removed through a side arm for analysis, and the rates of penetration through the membrane are determined (153).

Infinite doses can also be applied to the membrane in the one-chambered cell for determination of steady-state absorption kinetics and lag times when semisolid formulations are applied to the membranes. In these investigations it is assumed that the drug concentration in the donor phase does not diminish significantly during the time course of the experiment. After the initial transient period of membrane saturation, and provided sink conditions are maintained, the concentration gradient across the membrane and the drug permeation rate should remain constant (153).

In this experimentation the characteristics of the donor vehicle may vary greatly from the solutions used in the two-chambered cell design. A film of drug deposited by solvent evaporation, one of the numerous types of topical formulations, or even a transdermal delivery device, may be used as the drug source. Other benefits include the ability to control parameters of the donor compartment such as humidity, thereby simulating occluded or unoccluded clinical conditions. Additionally, the membrane may sequentially be treated with chemicals before or during exposure to the permeant to ascertain what effect they have on the retardative properties of the barrier medium (164-167).

There are two basic designs of one-chambered cell: flow-through and static.

2.3.3.1 Flow-Through Diffusion Cell

A flow-through cell system was introduced to automate sample collection from a one-chambered cell (82). The flow-through design also facilitates the maintenance of membrane viability because the physiological receptor fluid is continually replaced. Receptor fluid eluting from the diffusion cells is automatically collected in a fraction collector. Possibly the most important feature of flow-through cell design is the volume of the diffusion cell receptor, which must be small (less than 0.5ml) so that it can be properly flushed out during sample collection intervals with a manageable volume of receptor fluid.

Cells should be made of a material that does not bind or retain test compounds. Glass and teflon are preferred for construction of cells because they are inert. A glass window fitted in the bottom of the cell allows viewing of the receptor contents and thereby verifying the absence of air bubbles (153,156).

2.3.3.2 Static Diffusion Cell

Most of the static diffusion cells described in the literature are of similar design: a lower, agitated, solvent-filled receptor chamber with an inclined, stoppered, sampling side-arm. The receptor volume of the static one-chambered cell can vary with the diameter of the opening for the membrane specimen. Heated water is usually used to maintain a physiological temperature in the chamber leaving the flange at ambient temperature so that the donor vehicle is not heated, although there is some debate as to the appropriate temperature that will give the desired skin temperature (153). Frequently, glass cells require assembly where the membrane is mounted horizontally between the flanged edges of the lower chamber and the upper donor container, which are sealed with a metal clamp making the joint water-tight. Fluid is added to the cell to the level of the membrane, so that no hydrostatic strain is imposed on the medium, and any air bubbles which form may be expelled through the sampling port by tipping the cell. Magnetic stirring bars are usually used for mixing thereby avoiding unmixed regions directly underneath the skin.

Finite-dose techniques and the design of a static one-chambered diffusion cell were described by Franz (155). The Franz cell is probably the most widely used diffusion cell and has been commercially available for many years (153). The cell has the normal bichamber arrangement but has a dumbbell-shaped receptor chamber (168). An unstoppered sampling port is connected to the upper segment of this compartment and only the central, cylindrical portion of the receptor chamber is surrounded by a thermostatically controlled water jacket. A stirrer bar in the lower, ellipsoid bulb generates agitation.

Keshary and Chien (88,169) have summarised the shortcomings of the Franz cell after comparative evaluation, and Chien and Valia (170) have compared the hydrodynamics of this cell to their own design. They report that the architecture of the Franz cell does not provide adequate solution hydrodynamics, mixing efficiency and temperature control required for quantitative permeation evaluations. On the basis of these shortcomings

Keshary and Chien proposed a number of modifications to the Franz cell design which were adopted in this study (see figure 2.2). Their stoppered receptor compartment is a simple cylinder, shorter in height than the Franz design, completely enclosed by a water jacket and a star-head magnet is used to agitate the fluid. Equilibrium temperature maintenance, boundary diffusion layer thickness and solution mixing efficiency were substantially improved by these modifications.

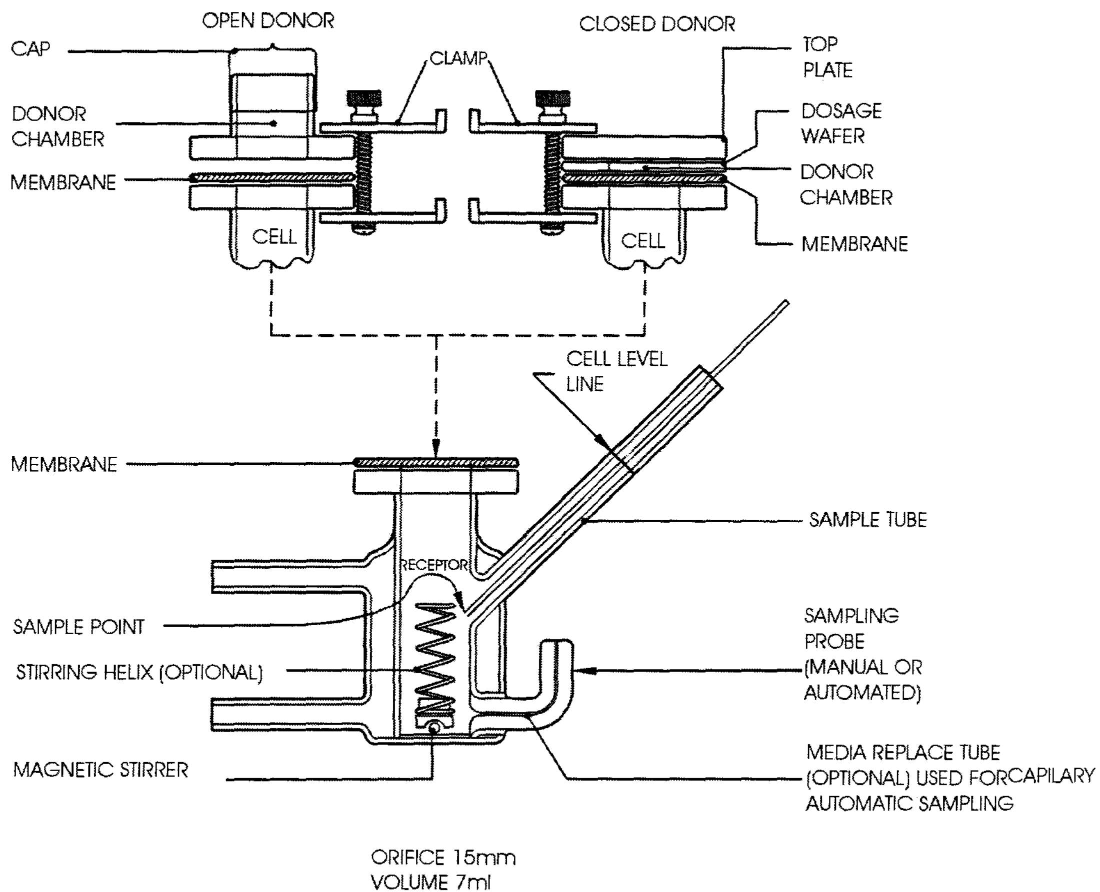


Figure 2.2 Schematic of a modified Franz diffusion cell assembly

An alternative to the vertical Franz-type cells is the USP tablet dissolution apparatus modified to accommodate a newly introduced accessory, the Enhancer cell. This dissolution cell is easy to load, and most laboratories have a USP dissolution apparatus. Furthermore, the apparatus allows variable paddle speeds, good temperature control and avoidance of air bubbles.

An *in vitro* release study of triamcinolone acetonide from hydroalcoholic gel was conducted using a Franz vertical diffusion cell and a modified USP dissolution apparatus

(171). It was found that the release rate for triamcinolone acetonide from hydroalcoholic gel was slightly higher and diffusion coefficients more consistent based on release data obtained from the dissolution cells as compared with the vertical diffusion cells. Notwithstanding these relatively minor differences, both types of apparatus gave similar results.

The European cell dissolution apparatus (187) is similar to the modified USP tablet dissolution apparatus. The diffusion cell consists of a support, a cover and a membrane. All parts of the apparatus that may come into contact with the topical formulation being examined or with the receptor medium are chemically inert and do not adsorb, react with or interfere with the topical formulation being examined. All metal parts of the apparatus that may come into contact with the receptor medium must be made from a suitable stainless steel or coated with a suitable material to ensure that such parts do not react or interfere with the receptor medium. No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation or vibration beyond that due to the smoothly rotating element.

The support contains a central depression which acts as the reservoir for the transdermal dosage form. The depth of the central reservoir is 2.6 mm, while the diameter may be 27 mm, 38 mm, 45 mm or 52 mm; which corresponds to volumes of 1.48 ml, 2.94 ml, 4.13 ml and 5.52 ml respectively. The diameter chosen for this study was 38 mm. The cover has a central aperture, the diameter of which determines the surface area of the reservoir from which the topical formulation diffuses. The diameter of the aperture may be 20 mm, 32 mm, 40 mm or 50 mm; which corresponds to surface areas of 3.14 cm^2, 8.03 cm^2, 12.56 cm^2 and 19.63 cm^2 respectively. The diameter corresponding to the chosen support for this study was 32 mm with an aperture area of 8.03 cm^2. The cover is held firmly in place above the support by four screws extending into the support base.

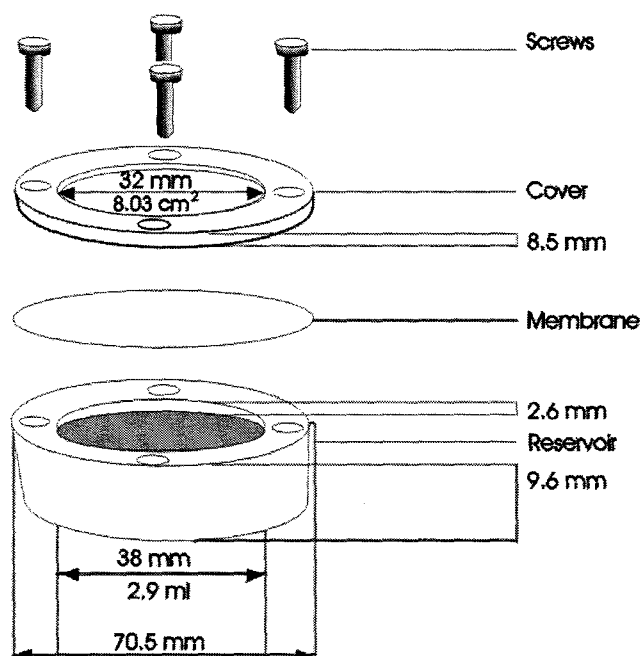


Figure 2.3 European Pharmacopoeia diffusion cell (187)

The diffusion cell is assembled and placed into a cylindrical vessel with the diffusion surface facing upwards and parallel to the horizontal plane. The cylindrical vessel is made from borosilicate glass or other suitable transparent material, with a hemispherical bottom and with a nominal capacity of 1000 ml. The vessel has a flanged upper rim and is fitted with a lid that has a number of openings, one of which is central. The apparatus has a motor with a speed regulator capable of maintaining the speed of rotation of the paddle within ± 4% of 100 rpm. The motor is fitted with a stirring element which consists of a drive shaft and a paddle. The paddle is lowered to a depth corresponding to 10 ± 2 mm above the diffusion cell surface. The temperature of the receptor fluid is maintained at 32 ± 0.5 °C. Evaporation is prevented by the fitted lid. Samples are taken from the cylindrical vessel at suitable time intervals.

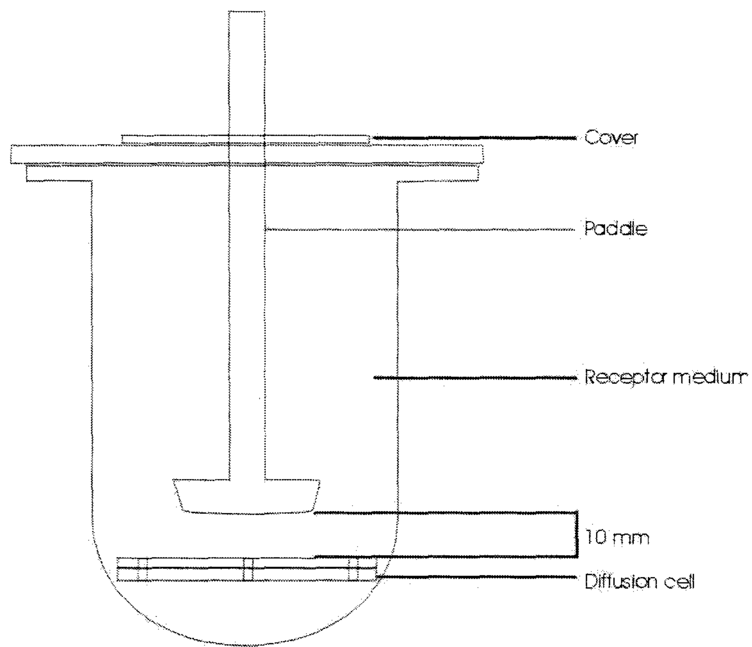


Figure 2.4 Schematic of a modified European Pharmacopoeia diffusion cell assembly (187)

2.4 FORMULATION ASPECTS

2.4.1 Introduction

Topical delivery can be defined as the application of a drug-containing formulation to the skin to directly treat cutaneous disorders or the cutaneous manifestations of general disease, with the intent of confining the pharmacological or other effect of the drug to the surface of the skin or within the skin. Topical activities may or may not require intra-cutaneous penetration and deposition, and while systemic absorption may be unavoidable, it is always unwelcome (144). Ointments and creams dominate the systems for topical delivery, but gels, foams, sprays, medicated powders, solutions, lotions (fluid emulsions and suspensions), and medicated adhesive systems are also in use.

Regional delivery, by contrast, involves the application of a drug to the skin for the purpose of treating diseases or alleviating disease symptoms in deep tissues beneath the application, where the intent is to effectuate or accentuate the pharmacological actions of the drug beneath and around the site of application (144). A selectivity of action over that which can be achieved by the systemic administration is sought. Regional delivery is accomplished with traditional ointments and creams and also large adhesive patches, plasters, and poultices.

Transdermal delivery involves the application of a drug to the skin to treat systemic disease and is aimed at achieving systemically active levels of the drug. While traditional dosage forms can be employed in this kind of therapy, adhesive systems of precisely defined size are the rule. Here, percutaneous absorption with appreciable systemic drug accumulation is absolutely essential. Local build-up is unavoidable as the drug is forced through the relatively small diffusional window defined by the contact area of the patch. Consequently, high and potentially irritating or sensitising concentrations of a drug in the viable tissues underlying the patch are preordained by the nature of the delivery process (144).

The functional features of dermatological systems such as ointment, creams, and gels stand in stark contrast with those of transdermal delivery systems. Most topical applications are left open to the atmosphere with an estimated thickness of an application ranging between

10μm and 30μm in ordinary circumstances. Their area to thickness ratios are large, thus any volatile materials contained within the topical film will invariably evaporate rapidly following its application. Since ointments are made mostly of high molecular weight hydrocarbons of low volatility, these are presumed to be relatively compositionally stable upon application, whereas creams, gels, topical solutions, and lotions are completely different in this regard with as much as 90% of the system's composition being appreciably volatile. Evaporation of these ingredients begins immediately upon application of the dosage form, and it continues until all of the volatile substances are exhausted. Extraordinary physicochemical changes accompany the evaporative concentration of the formula, and, as a consequence, the thermodynamic activity of every ingredient in a topically applied film is continually varying. Since it is the thermodynamic activity of a drug that exists at the surface of the skin which determines the partitioning of the drug into the skin, and since this is time-dependent in ways related to compositional changes, topical delivery is seldom a steady-state process for any appreciable duration (144).

The most common pharmaceutical vehicles for topical ibuprofen used in clinical practice are gels, creams and aerosol quick-break foams. The extent to which ibuprofen is released from these vehicles is closely related to the solubility of the drug in the vehicle, the particle size of the drug, the occlusivity of the vehicle and the presence of penetration enhancers.

2.4.2 Vehicles

The percutaneous absorption of a drug is frequently an extremely inefficient process and the importance of the vehicle into which the drug is incorporated is recognised. The vehicle should, to the largest possible extent, facilitate the migration of the drug to the target site so as to allow the drug to exert its pharmacological effect. The ideal vehicle should be cosmetically acceptable, physically stable, physiologically inert and provide an environment in which the drug is stable and from which the drug in low concentrations is readily released. Maximisation of the release and penetrability of the drug, without unfavourably altering other relevant vehicle properties, should therefore be a general goal in topical vehicle design (122-126).

The *in vitro* release of drugs from topical vehicles is a function of the solubility of that drug in both the vehicle and its surrounding medium. The drug must be sufficiently

soluble in a non-aqueous vehicle to allow for its release into an aqueous medium but not so soluble as to remain in that vehicle preferentially. If the drug is insoluble in its vehicle, only the drug particles available at the surface of the vehicle apparently dissolve into an aqueous medium. If the drug is partly soluble in the vehicle, it seems to dissolve and diffuse throughout the medium as it dissolves from the surface and then returns to the surface for release into the surrounding medium (124).

The design of a vehicle for topically administered drugs should take into consideration general formulation aspects, the physical characteristics of the drug and the skin barrier, as well as the biophysics of skin transport. It is important to bear in mind that a vehicle that provides good release of one drug may not be suitable for another drug (122).

2.4.2.1 Liquid Preparations

Such systems for external application to the skin include simple soaks or baths, liniments, lotions, paints, varnishes, tinctures, and eardrops. Usually, these formulations are physicochemically simple and pose few difficulties to the prediction of their abilities to deliver drug to the skin once account has been taken of evaporation. Among the most complex are the micellar systems consisting of solutions of surface active agents in which the surfactants equilibrate between the monomer and micellar pseudophases (143).

2.4.2.2 Simple Suspensions

Simple suspensions may be fluid, gel-like, or semisolid and hydrophilic or hydrophobic. A gel-like system may consist of entangling polymer chains, adjuvant crystals, and solid drug particles in equilibrium with dissolved molecules at saturation. The dissolved medicament may be in free solution between the polymer chains, bound (usually reversibly) to the polymer chain, or adsorbed at the surface of non-drug solid. The excess drug provides the ultimate source, which, as molecules leave the vehicle to penetrate the skin, dissolves to replenish free and bound molecules and keeps the chemical potential at the maximum for a saturated system (143).

2.4.2.3 Gels

A gel may be defined as a solid or semisolid system of at least two constituents, consisting of a condensed mass enclosing and interpenetrated by a liquid. Gels may be classified as either two-phase or as single-phase systems. The gel structure in a two-phase system may consist of floccules of small particles which are not always stable and may be thixotropic, forming semisolids on standing and becoming liquids on agitation. On the other hand, a gel may consist of macromolecules existing as twisted matted strands where the units are often bound together by stronger types of van der Waals forces so as to form crystalline and amorphous regions throughout the entire system. These gels are considered to be one-phase systems since no definite boundaries exist between the dispersed macromolecules and the liquid (46).

2.4.2.4 Emulsions

Emulsions may be mobile lotions or semisolid creams. Essentially an emulsion is a heterogeneous system containing two immiscible phases: an aqueous or hydrophilic phase and a lipid or oil phase. If the mixture is one of hydrophilic droplets dispersed in oil, it is referred to as a water-in-oil (w/o) emulsion; the reverse is an oil-in-water (o/w) system.

2.4.2.4.1 Oil-in-Water Emulsions

Topical o/w creams ("vanishing" creams) rub into the skin and disappear with little or no trace of their former presence. In the preparation of dermatological o/w creams, mixed emulsifiers of the surfactant/fatty amphiphile type, in which the amphiphile is usually a long-chain acid or alcohol, are often employed. The water-soluble surfactant may be anionic, cationic, or nonionic. Combining a surfactant with a fatty amphiphile in the correct ratio produces a powerful emulsifying blend of the o/w type, with superior stabilising and thickening properties (143).

2.4.2.4.2 Water-in-Oil Emulsions

Water-in-oil emulsions provide a wide range of consistencies depending on the components in the oil and aqueous phase and their relative proportions, the emulsifier

blend, and various auxiliary agents. Oily creams contain w/o emulsifying agents, typically wool fat, wool alcohols, fatty acid esters of sorbitan, or the salts of fatty acids with a divalent metal such as calcium (143).

It has been found that the vehicle has a substantial influence on the hydration of the skin barrier, with greases and oils being the most occlusive types of vehicle and consequently inducing the greatest degree of hydration. The inclusion of a surfactant into these vehicles results in water-in-oil emulsions which induce less hydration than greases and oils. Oil-in-water emulsions, formed by an increase in the water/oil ratio, induce the lowest degree of hydration (124). It should be noted that greases and oils cause hydration by preventing evaporation of moisture from the skin whereas the emulsions both wet the skin and have varying degrees of occlusivity. The onset of hydration due to the former will therefore not be as rapid.

2.4.2.5 Aerosol Quick-Break Foams

The quick-break aerosol foam has the advantages of high activity (ibuprofen is in solution), ease of application, controlled dosage from a metering valve, economy in use and suitability for smooth or hairy skin.

2.4.3 Penetration Enhancers

Penetration enhancers utilised in ibuprofen formulations are propylene glycol, alcohol and possibly lower molecular weight polyethylene glycol (the molecular weight was not reported in the formulation composition). Evidence for the effects in promoting skin penetration of such solvents as propylene glycol and ethanol is conflicting as, in many instances, the liquids simply function as cosolvents to produce saturated or nearly saturated solutions of the active ingredient, thereby maximising the thermodynamic activity of the penetrant (145). In addition to affecting drug solubility in the vehicle, cosolvents may alter the structure of the skin and modify the penetration rate. Moreover, the use of a cosolvent in combination with a potential penetration enhancer may offer synergistic enhancement (146,147).

Ethanol and other volatile solvents initially build up a high concentration gradient of the drug on the skin surface. However, as a result of progressive evaporation of the solvent, the residual drug will precipitate and the absorption process will be interrupted. In general, higher molecular weight polyethylene glycols do not penetrate the skin and may form hydrogen bonds with penetrants, thus reducing the therapeutic activity of the compound and penetration rate. Propylene glycol appears to aid penetration best when combined with surfactants (145).

2.4.4 Surfactants

Surfactants are often included in pharmaceutical formulations as emulsifying agents. While penetration is poor, there is evidence that anionic and cationic agents do penetrate the skin, with anionics penetrating best followed by cationics and nonionics (124).

The factor determining whether a nonionic surfactant will increase the absorption rate appears to be the configuration of the surfactant molecule rather than the hydrophilic-lipophilic balance or surface activity. Where the surfactant has several long hydrophilic chains (more than five ethylene oxide units for example) rather than a single or several short ethylene oxide chains, drug absorption is not increased. This indicates that the effectiveness of the surfactant is due to the ease with which the surfactant molecules penetrate lipid membranes (124).

The only surfactant encountered in the ibuprofen formulations was that of a polyoxyethylene fatty acid ester which is classified as a hydrophilic nonionic surfactant (3). This surfactant was present in both cream formulations. It has been shown that the addition of a polyoxyethylene fatty acid ester to a 4% w/w sodium carboxymethylcellulose gel improved the permeation parameters of ibuprofen through a standard cellophane membrane as compared to a control gel (174). Gels containing a polyoxyethylene fatty acid ester exhibited a higher release rate and significantly decreased lag time as compared to the control gel.

CHAPTER THREE
THE *IN VITRO* ANALYSIS OF IBUPROFEN

3.1 ANALYTICAL METHOD DEVELOPMENT

3.1.1 Introduction

A suitable analytical technique for ibuprofen should be able to quantify the drug and be stability indicating. The ideal assay should be precise, accurate, sensitive, rapid and specific. It should be capable of processing small volumes, be inexpensive and simple to perform.

Many analytical procedures for the determination of ibuprofen have been reported including methods employing paper chromatography (189), gas chromatography (190-194,201) gas chromatography-mass spectroscopy (195), and hplc techniques (196-213). Other quantitative procedures include electron and nuclear magnetic spectroscopy and infrared and ultraviolet spectrophotometry (214,215).

Proprietary topical preparations may not contain the exact concentration of drug as specified on the label. This could be due to factors such as overage addition by the manufacturer, degradation or adsorption onto the inner surface of the container. If these formulations are accepted as label strength, misleading conclusions regarding the drug release characteristics of the vehicle may be drawn from the results of *in vitro* or *in vivo* assays. Discrepancies in performance may be due to drug concentration differences. It is therefore imperative that a reliable, analytical technique be available so that the exact drug content of all formulations used in diffusion studies may be measured. Appendix I lists the exact drug content of the formulations used in this study. No significant difference, evaluated at a 95% confidence interval, was found between the different drug concentrations as specified on the labels of the formulations. Only in this manner may conclusions be made from flux data with some degree of validity and confidence (152).

Of all the possible analytical techniques, hplc has the required sensitivity, selectivity and versatility to provide fast and efficient drug analysis for formulation assessment.

3.1.2 Principles of High Performance Liquid Chromatography

The feature that distinguishes chromatography from most other physical and chemical methods of separation is that two mutually immiscible phases are brought into contact; one phase is stationary and the other mobile. A sample introduced into a mobile phase is carried through a column containing a distributed stationary phase. Molecules in the sample undergo repeated interactions between the mobile phase and the stationary phase. At the end of the process, separated components emerge in order of decreasing interaction with the stationary phase. Interaction between the phases exploits differences in the physical and/or chemical properties of the components in the sample (188).

Hplc is able to separate macromolecules and ionic species, labile natural products, polymeric materials, and a wide variety of other high molecular weight polyfunctional compounds. With an interactive liquid mobile phase, a parameter is available for selectivity in addition to an active stationary phase. Chromatographic separation in hplc is the result of specific interactions of molecules with both the stationary and mobile phases. Hplc offers a variety of stationary phases, which therefore allow a variety of these selective interactions and greater possibilities for separation. Sample recovery is usually quantitative (barring irreversible adsorption on a column), and separated sample components pass through unchanged (188).

The stationary phase of the hplc system may be either a totally porous particle (or macroporous polymer) or a superficially porous support (porous layer beads or pellicular supports). Either of these types may have a polymer bonded to its surface. Molecules separated by these adsorbent packings are retained almost exclusively on the internal surface of the pores of the particles because only a small fraction of the active area is found on the outside of the particles (188).

Knowledge of the molecular structure of the sample components can be very helpful in selection of a liquid chromatographic method. A general guide for the choice of a method is shown in table 3.1.

METHOD	PREDOMINANT MECHANISM
Liquid-solid or adsorption	Adsorption on surface
Liquid-liquid	Partition between liquid phases, one mobile and the other stationary
Bonded phase	Partition and/or adsorption between mobile and bonded phases
Ion pairing	Separation of ion pairs between mobile and bonded phases
Ion exchange	Exploitation of charge by adsorption on fixed ionic site via cation or anion exchange
Steric exclusion	Exploitation of size via diffusion into pores by molecules able to enter
Affinity	Use of structure of immobilised ligand to bioselectively bind the desired protein

Table 3.1 Liquid Chromatographic Methods

The partition technique in its various forms is the most widely used mode in hplc and comprises nearly half of all liquid chromatographic methods described in the literature. This technique is the most likely to provide optimum retention and selectivity when compounds have a predominant aliphatic or aromatic character (188).

Partition chromatography comprises a hydrophobic bonded packing, usually with an octadecyl (C-18) or octyl (C-8) functional group and a polar mobile phase and often a partially or fully aqueous mobile phase. Polar substances prefer the mobile phase and elute first. As the hydrophobic character of the solutes increases, retention increases. Generally, the lower the polarity of the mobile phase, the higher is its eluent strength. Hydrocarbons are retained more strongly than alcohols, with water as the weakest eluent. Methanol and acetonitrile are popular solvents because they have low viscosity and are readily available with a high degree of purity. Eluents intermediate in strength between these solvents and water are usually obtained by preparing mixtures (188).

3.1.3 Experimental

3.1.3.1 Reagents

All chemicals used were at least of analytical reagent grade. Acetonitrile and methanol (Burdick and Jackson Laboratories, Muskegon, Michigan, USA) were of hplc grade. Ibuprofen (batch IBU9905390) was obtained from Shasun Chemicals and Drugs, Ltd. (Pondicherry, India). The sodium hydroxide pellets were obtained from Riedel-deHaen (Germany) and the orthophosphoric acid (85%) was obtained from Saarchem-Holpro Analytic (Pty.) Ltd., (Krugersdorp, South Africa).

Water used for buffer preparation, extraction and chromatography was initially purified by a reverse-osmosis Milli-RO 15 Water Purification System (Millipore, Bedford, MA, USA) that consisted of a Super-C carbon cartridge, two Ion-X ion exchange cartridges and an Organex-Q cartridge. The water was filtered through a 0.22 μm Millipak stack filter prior to use.

3.1.3.2 High Performance Liquid Chromatographic System

The hplc system used consisted of an IsoChrom Isocratic pump, a Spectra 100 UV-Vis detector and a Spectra-Physics DataJet integrator all from Spectra-Physics Inc. (California, USA). Samples were introduced into a 20 μl fixed loop injector using a 100 μl syringe (Scientific Glass Engineering, Australia). The analytical column was a 4.0 mm x 250 mm stainless-steel column, packed with Waters Sherisorb 5 μm octadecylsilane (C-18) material (Waters Corporation, Massachusetts, USA).

3.1.3.3 Ultraviolet Detection

Optical detectors based on ultraviolet-visible absorption are the most commonly used hplc detection systems. Three types of absorption detectors are available: a fixed-wavelength detector, a variable-wavelength detector, and a scanning (in real time) wavelength detector. A variable-wavelength detector was used in the hplc method developed for the analysis of ibuprofen.

A variable-wavelength detector offers a range of wavelengths from 190 to 600 nm, which permits a wavelength to be chosen where the solute absorbance is maximal and little interference is observed from additional solutes or the mobile phase. The absorption maximum (λ_{max}) of ibuprofen was measured at 222 nm. Published methods for the analysis of ibuprofen vary considerably, utilising wavelengths from 214 to 263 nm, but many of these methods are optimised for the detection of more than one compound.

3.1.3.4 Column Selection

The choice of an analytical column is based on physical properties of the molecule, such as molecular mass, solubility and ionic character (188). Table 3.2 shows silica-based column packing material for partition chromatography.

SAMPLE	COLUMN PACKING
Low/moderate polarity (soluble in aliphatic hydrocarbons)	Bonded C-18
Moderate polarity (soluble in methyl ethyl ketone)	Bonded C-8
High polarity (soluble in lower alcohols)	Bonded C-2

Table 3.2 Silica-based column packing material for partition chromatography

Column packings can be described in terms of their chemical composition, whether silica, alumina, or carbon. The most commonly used adsorbant column packing material is silica particles, although alumina columns find applications in special situations.

Many hplc separations are performed on columns with an internal diameter of 4-5 mm. Such columns provide a good compromise between efficiency, sample capacity, and the amount of packing and solvent required. Column packings feature particles that are uniformly sized and mechanically stable. Particle diameters commonly lie in the range 5-10 μm. The smaller the microparticle, the greater the surface area resulting in a greater number of sites available for interactions, therefore, the better the resolution of the sample and the greater the sensitivity of the analytical method. Smaller particle sizes give more theoretical plates and better sensitivity, but higher backpressure than the larger particles.

The number of theoretical plates is a quantitative measure of the efficacy of a chromatographic system (188).

The most commonly used column previously reported for the analysis of ibuprofen was octadecasilyl, C-18 (144,197,199,205-207,212,217-218). All columns used in this study were partition hplc columns.

3.1.3.5 Mobile Phase Selection and Preparation

The relative extent of the three-way interaction between the molecule, the stationary phase and the mobile phase will determine the efficiency and efficacy of the method used. If the molecule is highly soluble in the mobile phase, it will undergo limited partitioning onto the stationary phase, and retention times will be short. Conversely, if the molecule displays limited solubility in the mobile phase, it will partition into the stationary phase and thus result in a longer retention time. The pH of the aqueous component of partition systems is capable of modifying the retention time of weak acids and bases, as the un-ionised species is retained for a longer period of time because it is less polar. The use of organic modifiers in predominantly aqueous mobile phases will also alter the retention characteristics of the molecule. The choice and amount of modifier required will depend on the properties of the molecule and the stationary phase (219). Mobile phases with pH values outside the range of 3 to 9 may interact with the selected stationary phase, thus altering its properties or integrity. This interaction occurs via hydrolysis of the bonded phase or dissolution of the silica (219). The mobile phase must not interfere with the detection of the molecule, particularly when using uv detection. Many solvents exhibit some degree of uv absorbance at lower wavelengths, but wavelengths above 210 nm have been used with limited interference. It is important that the uv cut-off values for the solvents used are known and evaluated (219). Water and acetonitrile, the two solvents used in the study, have cut-off values at 195 nm.

The effect of the mobile phase on the hplc system must also be considered. All mobile phases should be filtered through at least a 2 µm filter and degassed prior to use. This prevents the accumulation of particulate matter in the system with the resulting increase in backpressure. The presence of air may interfere with detection as well as cause pressure fluctuations if caught in the pump. All solvents used should be chemically pure to ensure

RHODES UNIVERSITY LIBRARY

that no build up of impurities on the column or in the system occurs, and that no impurities interfere with the analysis. The viscosity of the solvent can influence backpressure. More viscous solvents and solvent combinations generally give rise to higher backpressures. High concentrations of buffer salts are also undesirable as there is a potential for precipitation with subsequent damage to pump seals and pistons and blockages with consequent increases in backpressure (219).

The initial mobile phases used were based on those published or were modifications of these. The mobile phases used during development and the corresponding retention times are reported in table 3.3.

MOBILE PHASE COMPOSITION	RETENTION TIME	REFERENCE
Acetonitrile:0.01 M Phosphate buffer (pH 3.0) 50:50	No peak eluted by 35 minutes	144
Acetonitrile:0.02 M Phosphate buffer (pH 4.0) 40:60	No peak eluted by 35 minutes	197
Methanol:Water (pH 3.3 adjusted with concentrated phosphoric acid) 65:35	22.9 minutes	207
Methanol:Water plus 1 ml concentrated phosphoric acid per litre 65:35	26.3 minutes	212
Acetonitrile:Water (pH 2.0 adjusted with concentrated phosphoric acid) 55:45	No peak eluted by 35 minutes	217,218
Acetonitrile:0.04 M Phosphate buffer (pH 7.0) 30:70	6.3 minute, sharp peak, well resolved from solvent front	211

Table 3.3 Effect of mobile phase on ibuprofen retention time

As can be seen from table 3.3 a mobile phase comprising acetonitrile and 0.04 M phosphate buffer (pH 7.0) in a ratio of 30:70 gave sharp peaks at a retention time of 6.3 minutes, which ensured good resolution from the solvent front. Two other mobile phases

yielded ibuprofen peaks at 22.9 and 26.3 minutes that were resolved from the solvent front. These retention times are too long for multiple sample analysis.

3.1.3.6 Guard Columns

To prolong the life of analytical columns, guard columns are often inserted ahead of the analytical column where they act as both physical and chemical filters. A Spheri-5 RP-18, 5 µ 30 x 4.6 mm guard column (Perkin Elmer, Norwalk, USA) was necessary in order to filter contamination arising from formulation components as well as the biological membranes used. Guard columns are relatively short (usually 5 cm) and contain a stationary phase similar to that in the analytical column. They protect the analytical column from particulate contamination that arises from a contaminated mobile phase or from degrading sample-injection valves (188).

Samples were loaded onto the analytical column, both with and without the guard column in line, and it was confirmed that the guard column made no significant contribution to dead volume or extra column effects. Guard columns are by design expendable and were periodically replaced when they were seen to have lost efficiency or caused backpressure in the system to rise.

3.1.3.7 Preparation of Stock Solutions

Approximately 100 mg of ibuprofen was accurately weighed into an A-grade 100 ml volumetric flask and made up to volume with hplc grade water or 0.2 M phosphate buffer (pH 7.2) prepared with hplc grade water. Standards ranging in concentration from 10 to 150 µg/ml were prepared by serial dilution of this stock solution.

3.1.4 Optimisation of the Chromatographic Conditions

3.1.4.1 Detector Wavelength

The wavelength at which maximum absorption of ibuprofen occured (λ_{max}) was found experimentally to be 222 nm. Some published methods have used alternative wavelengths (199,216), but detection in this region has been employed by the majority of investigators (144,197,205-207,212,217,218), and it was shown to be suitably sensitive for the purposes of this *in vitro* study. The effect of different wavelengths on the peak area representing maximum absorption of ibuprofen was investigated. As expected, peak area was affected by the wavelength used. The data are listed in table 3.4.

CONCENTRATION (µg/ml)	**214 nm**	**220 nm**	**222 nm**	**254 nm**
26.6	85.5	96.2	100	18.0
53.2	86.7	92.1	100	21.3
79.8	88.6	98.1	100	18.8
133	86.9	94.5	100	16.4
MEAN	**86.9**	**95.2**	**100**	**18.6**
STANDARD DEVIATION	2.3	2.6	0	2.0
%RSD	2.6	2.7	0	10.7

Table 3.4 Effect of wavelength on the peak area of ibuprofen

Results are shown as a percentage of peak area obtained with the 222 nm wavelength. The results indicate that absorption was maximal at 222 nm, with decreasing absorbance through 220 nm, 214 nm and 254 nm. Based on the results of this experiment 222 nm was chosen as the wavelength of detection.

3.1.4.2 Choice of Column

A C-18 column was selected for the analysis of ibuprofen. Ibuprofen is a weak acid and is soluble in the aqueous-organic mobile phase combination, thus retention times are expected to be short with a non polar stationary phase, as ibuprofen will partition preferentially into the mobile phase.

3.1.4.3 Mobile Phase Composition

Methods to improve the mobile phase are numerous and include slight variations of pH, an increase or decrease of ionic strength and changes in the amount of modifier in the mobile phase. In order to determine an optimum mobile phase system, the organic phases, various buffer compositions, and different ratios of organic phase to aqueous phase were varied. Ionisation control by means of a buffer was considered when the drug appeared to be poorly retained with weakly eluting mobile phases. Different pH values and concentrations of salts (table 3.3) were tested until a suitable retention time and symmetrical peaks were obtained. A mobile phase comprising acetonitrile and 0.04 M phosphate buffer (pH 7.0) in a ratio of 30:70 gave sharp peaks at a retention time of 6.3 minutes, which ensured good resolution from the solvent front, and therefore was selected for method validation (211).

The buffer solution was prepared by accurately pipetting 2.5 ml of 85% orthophosphoric acid into a 1 l A-grade volumetric flask and made up to volume with hplc grade water. The pH was then adjusted to 7.0 using sodium hydroxide pellets. A Crison pH meter (Crison, LASEC, South Africa) was used for pH measurements. The buffer solution was then combined with acetonitrile and filtered through a 0.45 µm filter (Millipore, Maryland, USA) before use.

3.1.5 Chromatographic Conditions

The optimal chromatographic conditions established during the method development were:

Mobile Phase	Acetonitrile:0.04 M Phosphate Buffer (pH 7.0) 30:70
Flow Rate	1.0 ml/min
Detection Wavelength	222 nm
AUFS (Attenuation)	0.05
Injection Volume	20 µl
Retention Time	6.3 minutes
Temperature	Ambient

3.1.6 Conclusion

The effects of altering system variables on the elution of ibuprofen were established in these preliminary investigations. By optimising the choice of mobile phase, detection wavelength and analytical column, an hplc method which provided well resolved symmetrical peaks, suitable retention times and minimal baseline noise was developed.

3.2 METHOD VALIDATION

3.2.1 Introduction

One of the most important aspects of method development is the validation of the method. Validation of an analytical method is the process by which it is established that the performance characteristics of the optimised method meet the requirements for the intended analytical applications. Performance characteristics are expressed in terms of analytical parameters (3).

Laboratories must perform method validation in order to be in compliance with Food and Drug Administration (FDA) regulations. The FDA designated the specifications in the USP as those legally recognised when determining compliance with the Federal Food, Drug and Cosmetic Act. For method validation the analytical parameters are precision, accuracy, limit of detection, limit of quantitation, specificity, linearity and range, ruggedness and robustness. The International Conference on Harmonisation (ICH) stated the validation characteristics somewhat differently as precision, accuracy, limit of detection, limit of quantitation, specificity, linearity, range, robustness and system suitability. Analytical parameters considered in the validation of the method used in this study are specificity, linearity, precision, limit of quantitation, limit of detection, accuracy and bias, and robustness.

Once the chromatographic conditions have been optimised with respect to detector wavelength, choice of column and mobile phase composition, an attempt should be made to provide analytical figures of merit which are needed to meet the assay requirements, *i.e.*, the required detection limits, limits of quantitation, accuracy and precision of quantitation and specificity must be defined. Results obtained during the validation procedure must be evaluated against the goals of the analysis set forth by the analytical figures of merit. This evaluation may reveal that additional development and optimisation are needed to meet some of the initial method requirements.

3.2.2 Accuracy and Bias

Accuracy is the measure of exactness of an analytical method, or the closeness of agreement between the measured value and the value that is accepted either as a conventional, true value or an accepted reference value. Accuracy is expressed as percent error. Bias assesses the influence of the analyst on the performance of the method (220).

To document the accuracy and bias, the ICH guideline on methodology recommends collecting data from nine determinations over three blinded concentration levels covering the specified range. Three replicate determinations of each of three concentration levels were used. The measurements were performed in consecutive and non-consecutive sequences (220). The percent error obtained during the determination of blinded unknowns is shown in table 3.5.

CONCENTRATION (μg/ml)	MEAN DETERMINED CONCENTRATION (μg/ml) (n=3)	STANDARD DEVIATION	PRECISION (% RSD)	PERCENT ERROR
103.3	107.4	1.2	1.1	-3.9
51.7	50.4	0.8	1.6	+2.4
25.8	26.1	1.2	4.6	+0.9

Table 3.5 Percent error obtained during the determination of blinded unknowns

The figures of merit for acceptable accuracy were taken as less than or equal to 10% relative standard deviation and less than or equal to 10% percent error (222). The analytical method was therefore deemed to be acceptable in terms of accuracy and bias.

3.2.3 Precision

Precision is the measure of the degree of repeatability of an analytical method and is expressed as the percent relative standard deviation for a statistically significant number of samples. According to the ICH, precision should be performed at three different levels: repeatability, intermediate precision, and reproducibility.

3.2.3.1 Repeatability

Repeatability refers to the results of the method operating over a short time interval under the same conditions (inter-assay precision). It is an expression of the degree of variation arising during replicate assays performed consecutively and non-consecutively, but on the same day. Repeatability should be determined from a minimum of nine determinations covering the specified concentration range of the procedure (220). The percent relative standard deviation obtained from three replicate determinations of each of four concentration levels is shown in table 3.6.

CONCENTRATION (µg/ml)	**MEAN PEAK AREA (n=3)**	**STANDARD DEVIATION**	**PRECISION (%RSD)**
125.3	1750108	20861	1.2
12.5	211779	14067	6.6
6.3	98711	2533	2.6
1.3	23304	671	2.3

Table 3.6 Repeatability with respect to four concentrations

The figures of merit for acceptable precision were taken as less than or equal to 10% relative standard deviation and less than or equal to 10% percent relative standard deviation (222). The analytical method was therefore deemed to be acceptable in terms of repeatability.

3.2.3.2 Intermediate Precision (Ruggedness)

Intermediate precision refers to the results from intra-laboratory variations due to random events such as differences in experimental periods, analysts and equipment (220). In determining intermediate precision, data from three replicate determinations of each of four concentration levels covering the specified range were collected. Inter-day precision of the calibration standards were assessed over a one week period.

The percent relative standard deviation obtained from three determinations over four different concentration levels is shown in table 3.7.

CONCENTRATION (µg/ml)	MEAN PEAK AREA (n=3)	STANDARD DEVIATION	PRECISION (%RSD)
125.3	1784999	30824	1.7
12.5	205329	12354	6.0
6.3	99182	3831	3.9
1.3	22370	1477	6.6

Table 3.7 Intermediate precision with respect to four concentration levels

The figures of merit for acceptable precision were taken as less than or equal to 10% relative standard deviation and less than or equal to 10% percent relative standard deviation (222). The analytical method was therefore deemed to be acceptable in terms of intermediate precision.

3.2.3.3 Reproducibility

Reproducibility refers to the results of collaborative studies between laboratories, and was not assessed.

3.2.4 Specificity

Specificity is the ability to measure accurately and specifically the molecule of interest in the presence of other components that may be expected to be present in the sample matrix. It is the measure of the degree of interference from such things as other active ingredients, excipients, impurities, and degradation products, ensuring that a peak response is due only to a single component; *i.e.*, that no co-elutions exist (220). Specificity was assessed by analysing samples made up from serial dilutions of a 5% m/m proprietary ibuprofen-containing gel and a 0.2 M phosphate buffer (pH 7.2) solution. The peaks obtained were well resolved from the solvent front and no interference was observed, indicating that the method was specific for ibuprofen.

3.2.5 Limit of Quantitation and Limit of Detection

The limit of detection (LOD) is defined as the lowest concentration of a molecule in a sample that can be detected, though not necessarily quantitated. The limit of quantitation (LOQ) is defined as the lowest concentration of a molecule in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. Both are expressed as a concentration, with the precision and accuracy of the measurements also reported (220).

There are four commonly used methods for determining LOD and LOQ reported in the literature (220,222,224). The method most applicable to this validation states that the LOQ is the lowest concentration with a percent RSD of less than 10% on multiple determinations (n=6 for the purposes of the study), and that the LOD is 30% of the LOQ. The LOQ was found to be 0.5 μg/ml, which had a percent RSD of less than 10%. The LOD was found to be 0.3 μg/ml, which showed a percent RSD of less than 20%.

3.2.6 Linearity

Linearity is the ability of the method to elicit test results that are directly proportional to molecule concentration within a given range. The ICH guidelines recommend that five concentrations spanning the concentration range to be studied be used (220).

A calibration curve was constructed over the concentration range 0.6 – 125.3 μg/ml by linear regression of the peak areas obtained versus the concentration. Three replicate determinations of each of seven concentrations were used. The calibration curve was linear over the concentration range studied, with $r^2 = 0.9998$. The equation of the regression line is $y = 14197x + 9271$.

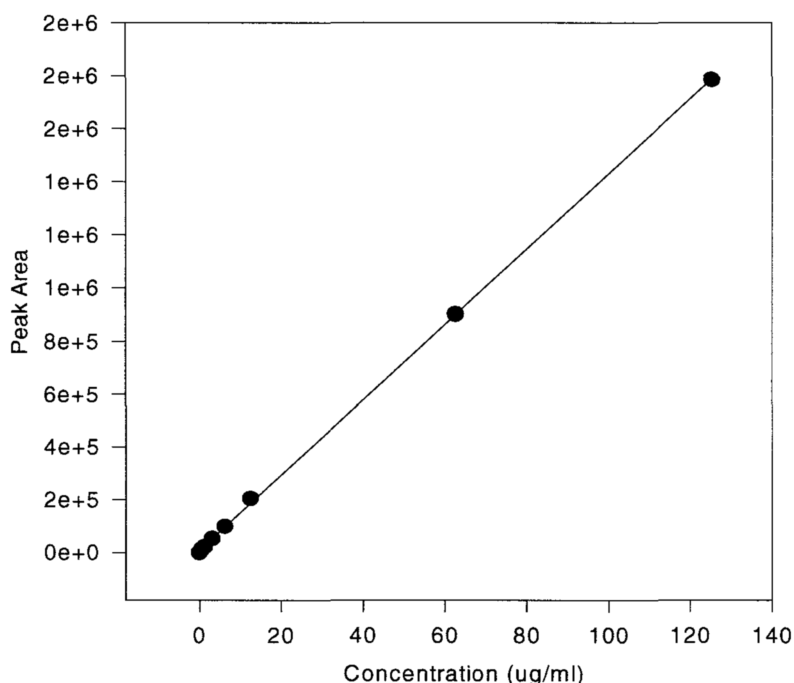


Figure 3.1 Calibration curve

3.2.7 Stability of the Molecule

The molecule must remain stable in solution for the duration of the analysis and under the conditions of an analytical run. Ibuprofen remained stable throughout the analytical procedure, as selected samples from an *in vitro* diffusion experiment were re-analysed after a storage period of two months at ambient conditions and showed no statistically significant differences in detector response when compared to the initial analysis.

3.2.8 Conclusion

Validation of the analytical method established that the performance characteristics of the optimised method met the requirements for the intended analytical applications. The results obtained with this analytical method show that it is precise, accurate, sensitive, rapid and specific with regard to ibuprofen. It is capable of processing small volumes, is inexpensive and simple to perform.

CHAPTER FOUR
THE *IN VITRO* RELEASE OF IBUPROFEN

4.1 EXPERIMENTAL METHOD DEVELOPMENT

4.1.1 Introduction

In vitro approaches, such as *in vitro* dissolution, are standard methods used to assess the performance characteristics of solid oral dosage formulations. *In vitro* dissolution may be used specifically to guide formulation development, monitor formulation quality from batch to batch, monitor control of the formulation manufacturing process, and possibly predict *in vivo* performance. When used as a quality control procedure, *in vitro* dissolution testing can demonstrate changes in drug and/or excipient characteristics or in the manufacturing process. Extension of *in vitro* dissolution methodology to semisolid dosage forms would be useful for the same reasons that such methodology has proved valuable in the development, manufacture, and batch-to-batch quality control of solid oral dosage forms. Present quality control tests to assure the identity, strength, quality, purity and potency of the active ingredient in semisolid dosage forms may provide little or no information about the drug release properties of the product, stability of the product or effects of manufacturing and processing variables on the performance of the dosage form. A drug release test for topical products, analogous to a dissolution test for a solid oral dose form, is therefore of interest (223).

4.1.2 *In Vitro* Release Testing

In vitro release is one of several methods used to monitor performance characteristics of a topical dosage form. Important changes in the characteristics of a drug product or in the thermodynamic properties of the drug substance in the dosage form may be manifested as a difference in drug release. The shape and design of the *in vitro* diffusion cell is standardised by the guidelines describing the Franz and European Pharmacopoeia cell systems, however, other parameters such as membranes and receptor solutions have to be investigated individually. The characteristics of the membrane and receptor phase composition may influence the drug release from topical formulations. The membrane should be chemically inert and permeable to the drug and should not be rate limiting in the

release process. The receptor fluid must be able to solubilise the drug and should not alter the dosage form by back diffusion through the membrane. The choice of the medium depends on the physicochemical characteristics of the drug and the formulation (225).

An FDA guidance document (224) recommends the following methodology for *in vitro* release studies:

1. A static diffusion cell system as described in figure 2.2.

2. Appropriate inert, porous, and commercially available synthetic membrane of appropriate size to fit the diffusion cell diameter.

3. Appropriate receptor medium with proper justification.

4. A minimum of six samples is recommended to determine the release rate of the topical formulation.

5. About 300 mg of the semisolid preparation should be placed uniformly on the membrane and kept occluded to prevent solvent evaporation and compositional changes. This corresponds to an infinite dose condition.

6. Multiple sampling times over an appropriate time period to generate an adequate release profile and to determine the drug release rate is suggested.

7. Appropriate validated, specific, and sensitive analytical procedure, generally hplc, should be used to analyse the samples and to determine the drug concentration and the amount of drug released.

8. A plot of the amount of drug released per unit membrane area ($\mu g/cm^2$) versus square root of time should yield a straight line. The slope of the line (regression) represents the release rate of the product.

9. The *in vitro* release test can be completely automated.

4.1.3 Choice of Apparatus

The FDA guidelines were considered when choosing the apparatus to be used in the comparative diffusion study on proprietary ibuprofen-containing topical preparations from three countries and the verification of the usefulness, or otherwise, of shed snake skin as a biological membrane for the assessment of the permeation of ibuprofen.

Two diffusion techniques were considered appropriate for the measurement of the amount of ibuprofen released from a topical formulation during *in vitro* testing. One was the Franz diffusion cell, as modified by Keshary and Chien (88,169) and the other was the European Pharmacopoeia diffusion cell (187).

4.1.4 Choice of Diffusion Test Conditions

4.1.4.1 Membrane Selection

Three different synthetic membranes were assessed: filter, cellulose acetate and silastic media. The *in vitro* diffusion was performed in modified Franz cells (88,169) using three different synthetic membranes with 100 mg of 5% ibuprofen gel (Ibuleve, South Africa) in the donor compartment. The cells were maintained at 37°C throughout the 72 hour period. The receptor fluid (20% methanol/water) was analysed for ibuprofen at 1, 2, 4, 6, 8, 12 and 24 hours by the hplc method described. The Franz cells were completely emptied at each sampling time and refilled with fresh receptor fluid.

The choice of synthetic membrane for the *in vitro* comparative diffusion study on proprietary ibuprofen-containing topical preparations was dictated by the diffusion profiles (cumulative amount released *vs.* time) obtained as well as membrane characteristics. The diffusion profiles are shown in Figure 4.1.

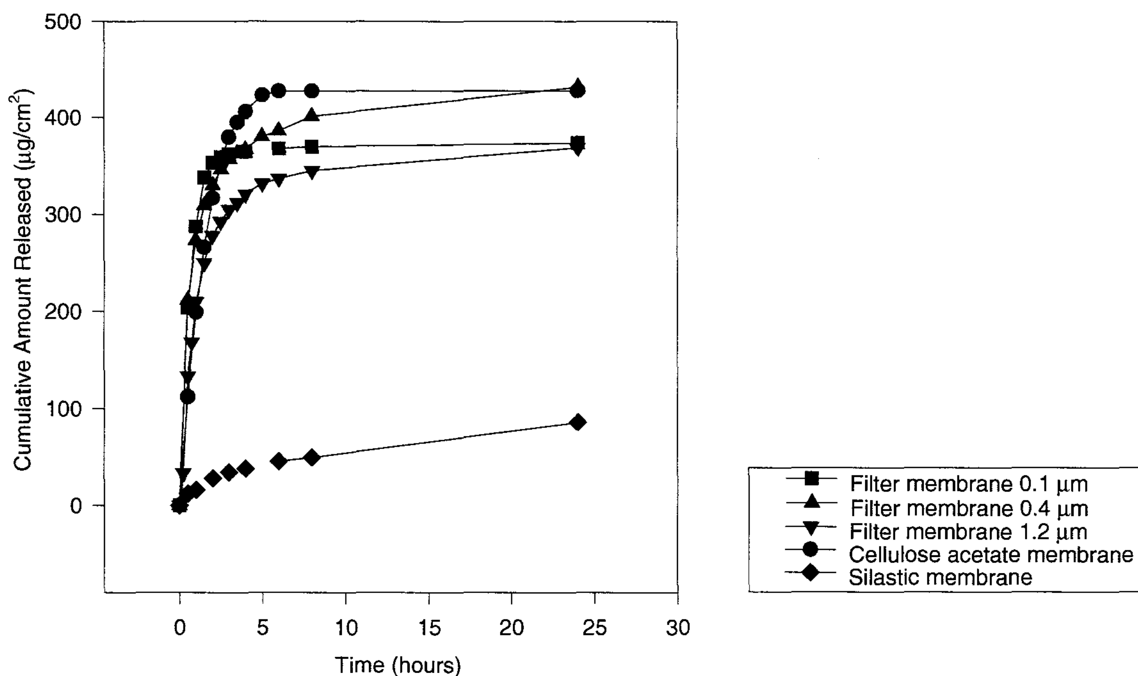


Figure 4.1 Effect of synthetic membranes on the diffusion of ibuprofen

The filter membranes investigated were Isopore Membrane Filters (Millipore, Ireland), with pore sizes of 0.1 µm, 0.4 µm and 1.2 µm. The porous filter media appear to be most useful as a dividing medium or as supporting screens where the release rate of drug from the topical formulation is under investigation, and not the transdermal kinetics of the permeant. The filter medium provides no significant barrier to diffusant passage. This can be seen as a dose-dumping effect which occurred between 0 and 8 hours after which time steady state was reached.

Generally, cellulose membranes are reported to be more permeable than biological membranes or aporous synthetic media (62) and are non-discriminatory to the characteristics of the diffusant molecule. This was found to be true as the diffusion profile of ibuprofen through the cellulose acetate membrane (Bel-Art Products, New Jersey, USA) mimicked that of the filter membranes and provided no significant barrier to the passage of ibuprofen through the membrane.

Silicone membrane is considered the most useful of the synthetic media for use in diffusion cell systems. Its relatively inert, lipophilic nature makes it an ideal environment for partitioning and permeation of drugs while its aporosity provides some rate-limiting

function to this process. The diffusion profile of ibuprofen through the Silatos Silicone Sheeting (Atos Medical, Horby, Sweden) indicated that the membrane exhibited a rate-limiting function. The Silatos Silicone Sheeting was deemed to be an appropriate inert, porous, and commercially available synthetic silastic membrane of appropriate size to fit the diffusion cell diameter and was therefore used in the comparative diffusion study on proprietary ibuprofen-containing topical preparations from three countries.

Shed snake skin from four species of snake was used for the verification of the usefulness, or otherwise, of shed snake skin as a biological membrane for the assessment of the *in vitro* permeation of ibuprofen. These were *Naja nivea* (Cape cobra), *Naja haje haje* (Egyptian cobra), *Naja melanoleuca* (forest cobra), and *Python sebae natalensis* (African rock python). The shed snake skins were cut to an appropriate size to fit the diffusion cell diameter.

4.1.4.2 Receptor Medium Selection and Preparation

Ibuprofen is practically insoluble in water, but is readily soluble in most organic solvents and is soluble in aqueous solutions of alkali hydroxides and carbonates (1-3). Based on the solubility of ibuprofen, five solutions were chosen as possible receptor media for investigation. The five solutions are listed in table 4.1.

20 %	Methanol / Water
0.2 M	Phosphate Buffer Solution at pH 7.2
0.5 %	Sodium Lauryl Sulphate / Water
6 %	Polyethyleneglycol / Water
0.1 %	Sodium Carbonate / Water

Table 4.1 Receptor media investigated

The *in vitro* diffusion was performed in modified Franz cells (88,169) using Silatos Silicone Sheeting membrane with 100 mg of 5% ibuprofen gel (Ibuleve, South Africa) in the donor compartment. The cells were maintained at 37°C throughout the 72 hour period. The receptor fluid was analysed for ibuprofen at 2, 4, 8, 24, 48 and 72 hours by the hplc method described. The Franz cells were completely emptied at each sampling time and

refilled with fresh receptor fluid. The effect of the receptor medium on the diffusion profile of ibuprofen is shown in figure 4.2.

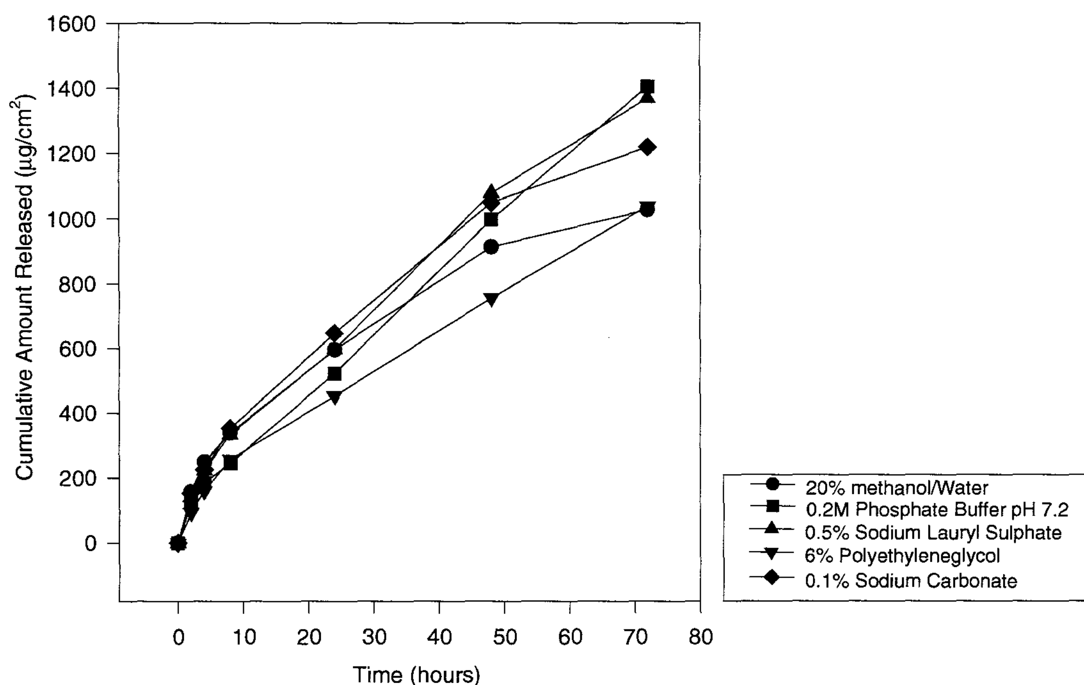


Figure 4.2 Effect of receptor medium on the diffusion profile of ibuprofen

The alcoholic solution produced excess bubble formation on the undersurface of the membrane which reduced the surface area available for diffusion and was subsequently rejected. The 0.2M phosphate buffer solution at pH 7.2 proved to be the most efficient of the aqueous receptor solutions over 0.5% sodium lauryl sulphate/water, 0.1% sodium carbonate/water and 6% polyethyleneglycol. The phosphate buffer solution was therefore chosen for subsequent determinations since there was little bubble formation beneath the membrane in the Franz cell and it showed the lowest tendency for evaporation. This is in accordance with the dissolution medium as specified by the USP for ibuprofen (3). With regard to the European Pharmacopoeia dissolution cells, bubble formation and evaporation are not problematic as neither of these factors are present when the apparatus is assembled.

The receptor medium was prepared as follows: 61.5 ml of 85% orthophosphoric acid (Saarchem-Holpro Analytic, Pty., Ltd., Krugersdorp, South Africa) was pipetted into a 5 l volumetric flask and made up to volume with hplc grade water. The pH was then adjusted to 7.2 using sodium hydroxide pellets (Riedel-deHaen, Germany). A Crison pH meter (Crison, LASEC, South Africa) was used for pH measurements. The solution was filtered

through a 0.44 µm filter (Millipore, Maryland, USA) and degassed under vacuum before use.

4.1.4.3 Temperature of the Receptor Medium

The *in vitro* diffusion experiment to determine the optimum temperature of the receptor medium was performed in modified Franz cells (88,169) using Silatos Silicone Sheeting membrane with 100 mg of 5% ibuprofen gel (Ibuleve, South Africa) in the donor compartment. The cells were maintained at 37°C (n=5) and at 32°C (n=5) throughout the 72 hour period. The receptor fluid (0.2M phosphate buffer at pH 7.2) was analysed for ibuprofen at 2, 4, 8, 24, 48 and 72 hours by the hplc method described. The Franz cells were completely emptied at each sampling time and refilled with fresh receptor fluid.

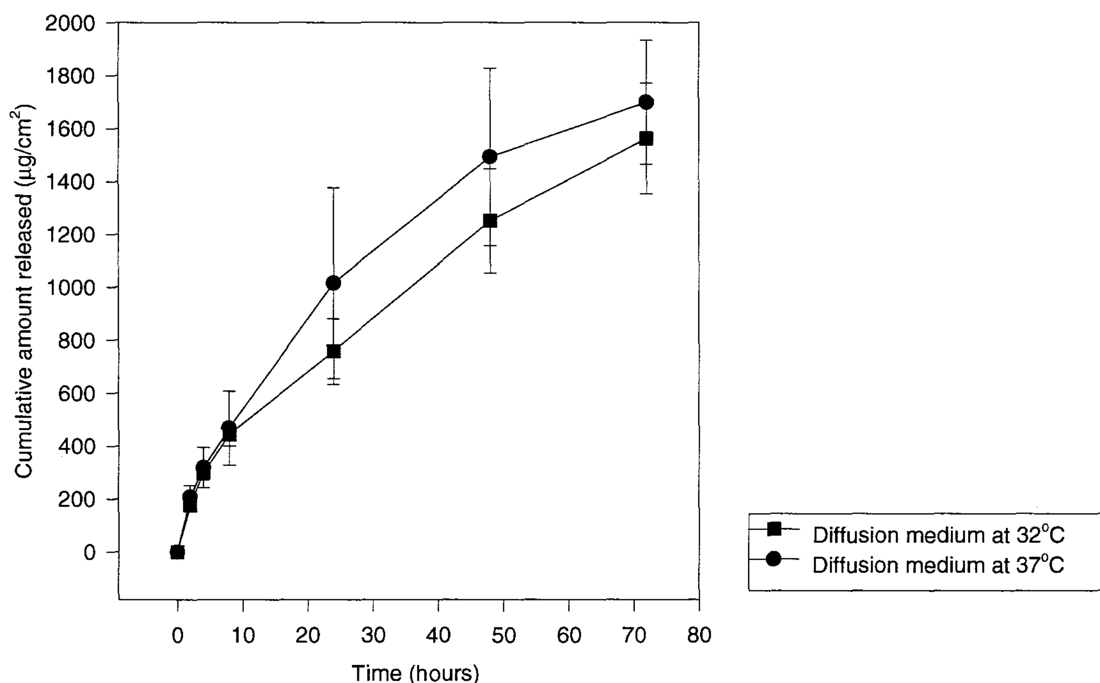


Figure 4.3 The effect of temperature on the diffusion profile of ibuprofen

As can be seen in figure 4.3, no significant difference in the diffusion profile of ibuprofen is apparent due to temperature differences of the diffusion medium, therefore 32°C was chosen as the temperature at which the diffusion experiments were to be performed as less evaporation of the receptor medium occurred at the lower temperature, and possibly less degradation of the active.

4.1.4.4 Number of Samples and Sampling Time

A minimum of six samples is recommended to determine the release rate (diffusion profile) of a topical formulation (224). The recommended number of six samples is based on the design of the diffusion cell apparatus which commonly has six sample stations. The design of the diffusion cell follows the imperial numerical system which has been replaced by the metric system. In accordance with the metric system five samples taken at each sampling time were deemed to be sufficient to give a diffusion profile from which the kinetics of the diffusion process could be calculated.

Samples of receptor fluid were taken at 2, 4, 8, 24, 48 and 72 hours. These multiple sampling times were chosen over an appropriate time period to generate an adequate diffusion profile and to determine the drug release rate. The Franz cells were completely emptied at each sampling time and refilled with fresh receptor fluid. Aliquots of 2 ml were taken from the European Pharmacopoeia cells at each sampling time before analysis using the hplc method described in chapter 3. The total percentage of receptor fluid removed for sample analysis was 1.2% which was deemed to be too small a volume to disrupt sink conditions.

4.1.4.5 Sample Applications

The effect of the mass of the sample application was investigated in modified Franz cells (88,169) using Silatos Silicone Sheeting membrane with 50 mg (n=3), 100 mg (n=5) and 200 mg (n=2) of 5% ibuprofen gel (Ibuleve, South Africa) in the donor compartment. The cells were maintained at 32°C throughout the 72 hour period. The receptor fluid (0.2M phosphate buffer at pH 7.2) was analysed for ibuprofen at 2, 4, 8, 24, 48 and 72 hours by the hplc method described. The Franz cells were completely emptied at each sampling time and refilled with fresh receptor fluid. Figure 4.4 shows the effect of mass on the diffusion profile of ibuprofen.

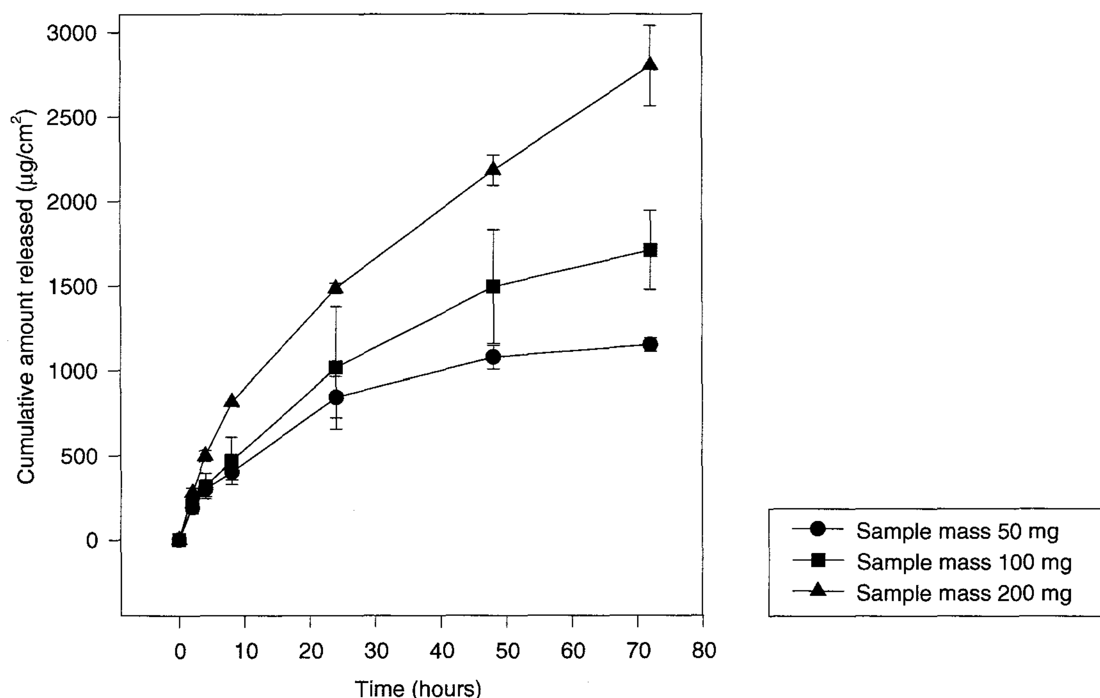


Figure 4.4 Effect of mass on the diffusion profile of ibuprofen

All three diffusion profiles are significantly different. 100 mg was chosen as the sample application over 50 mg as the cumulative amount of ibuprofen released is greater, thus giving a better diffusion profile. 200 mg was deemed to be excessive both in economic terms as well as the possibility that a large sample application may result in saturation kinetics.

Approximately 100 mg of the proprietary ibuprofen-containing topical formulation (5% m/m) was uniformly placed on the membrane and kept occluded, in the case of the Franz cell, to prevent solvent evaporation and compositional changes. The amount of topical formulation per unit membrane area available to the diffusant in the Franz cell donor chamber was calculated to be 60.63 mg/cm^2. From this figure the corresponding sample application was calculated for the European Pharmacopoeia cell. Approximately 500 mg of the proprietary ibuprofen-containing topical formulation (5% m/m) was uniformly placed on the membrane, ensuring no air bubbles were present between the surface of the formulation and the membrane. As the mass of topical formulation to the unit of surface area available to the diffusant was identical for both the Franz and the European Pharmacopoeia cells, direct comparisons of the diffusion profiles was possible.

4.1.5 Dissolution Profile Comparison

Dissolution profile similarity depends on the similarity between the profiles at each time point and the similarity between the overall profiles. The similarity between overall profiles is particularly important for topical dosage forms. When comparing dissolution profiles it is important to ensure that the test conditions are identical (219). The diffusion profiles of ibuprofen using four different species of shed snake skin as a biological membrane and the diffusion profiles of proprietary ibuprofen-containing topical preparations from three countries were compared.

4.2 ELECTRON MICROSCOPY

4.2.1 Introduction

An electron microscope is an instrument in which an electron beam is used to create an enlarged image of a subject under investigation. Electron microscopes allow the observation of much finer detail than any other form of microscope which uses light in the creation of enlarged images. The two main types of electron microscopes are the transmission electron microscope (TEM) and the scanning electron microscope (SEM). A scanning electron microscope (JEOL JSM 840) was used to determine the integrity, surface morphology and thickness of the snake skins investigated in this study.

4.2.2 Scanning Electron Microscopy

An electron beam is focussed by electro magnetic lenses on to the surface of a specimen. Scanning coils are used to make the beam scan in a raster fashion across the specimen (up to 3000 scans per rectangular field of view). The interaction between the incident electron beam and the specimen causes the liberation of secondary electrons, and other electromagnetic radiation such as X-rays, back-scattered electrons and light. The secondary electrons are attracted by a small positively-charged grid to a detector where the secondary electrons emitted from any given point on the raster create a signal which is amplified and used to modulate a beam on a cathode ray tube which is scanning in synchrony with the beam in the SEM column. This creates a pattern of light and dark areas corresponding to the emission of secondary electrons from the specimen from the same area, as registered by the detector. As the number of electrons produced at any given point can be related directly to the topography of the specimen with respect to the detector, the pattern created on the viewing screen represents the surface topography of the specimen. Magnification is created by setting the scanning coils to scan progressively smaller rectangular areas of the specimen. The images are recorded on conventional film by photography of the scan on a second cathode ray tube.

The elemental composition of the surface of the specimen will also affect the electron emission. While this is used as an analytical tool in some cases, for surface morphology

studies such as those conducted on the snake skins the specimen surface is coated with a thin layer of gold and thus has a uniform elemental composition (243).

4.2.3 Specimen Preparation for Scanning Electron Microscopy

The environment in the specimen chamber of the scanning electron microscope (vacuum, heat generated by the electron beam) demands stabilisation of the biological specimens to ensure that images obtained by the SEM are reproductions of the structure of the specimen in its natural state. There are both physical and chemical ways of achieving this objective.

4.2.3.1 Chemical Methods

Chemical-based methods for preparing biological specimens for SEM involve fixation, dehydration, drying and metal coating.

Fixation is a procedure involving the use of chemicals to counteract normal post-mortem enzymatic action and cross-link the chemical constituents of the living material into a form which will withstand the effects of the alien environment within the SEM specimen chamber.

Dehydration involves the use of a series of ascending concentrations of a dehydrating agent (ethanol, acetone) to remove water from the specimen.

Drying of the specimen is a crucial step in the process in that it should not result in any distortion of the shape, size and form but leave it in a state which reproduces its shape, size and form in its natural state. This step is necessary, however, as free water cannot be present in the specimen because of the vacuum environment in the SEM specimen chamber. Some degree of shrinkage is almost inevitable but procedures such as critical point drying (CPD) and air-drying from low vapour pressure solvents (HMDS) provide good preservation of structure. The choice of procedure depends upon available technology and the specimen under investigation.

Metal coating is applied before the dried biological specimens can be examined successfully in the SEM. This coating, which is usually a thin (<100nm) layer of gold or

gold alloy deposited on the specimen surface, either by sputter-coating or vacuum evaporation, has four functions:

1. To increase the level of secondary electron emission from the surface of the specimen and thus optimise the brightness and contrast of the image and the achievable resolution of fine detail.

2. To eliminate the effects of atomic number contrast in the surface layers of the specimen.

3. To prevent the build-up of electrical charges in the specimen by allowing these charges to discharge to earth.

4. To ensure that secondary electron emission is confined to the surface layers of the specimen and thus avoid confusion caused by emission from deeper layers within the specimen.

Fixation and dehydration were unnecessary steps in the preparation of the shed snake skins. The specimens are essentially non-living structures and do not require chemicals to counteract post-mortem enzymatic action and cross-link the chemical constituents of the living material nor do they require dehydration as the shed snake skins were examined in their naturally dehydrated state. The specimens were therefore cut with scissors, dried and sputter-coated with a thin layer of gold.

4.2.3.2 Physical Methods

These are based on stabilising the specimen by freezing the free water within it. It is most important when using such methods to ensure that the freezing process and the formation of ice-crystals dies not cause distortion of the specimen.

Once frozen the specimens can be further prepared for SEM observation by direct observation in frozen state by cryoSEM or after freeze drying. Metal coating is still required for optimal results both for cryoSEM and for observation of freeze-dried specimens by conventional SEM. Freeze-drying involves the rapid freezing of a specimen,

with or without cryo-protection, transfer of the frozen specimen to a vacuum followed by drying through sublimation of ice from the specimen (243).

4.2.4 Scanning Electron Microscopy Instrumentation and Conditions

A scanning JEOL JSM 840 microscope was used at the following settings:

Acceleration voltage	12.0 kV
Optical aperture	3
Probe current	10 amp
Working distance	39 mm

The images were recorded using a Mamiya roll film adaptor camera on conventional Agfa Agfapan (APX 100/120) film by photography of the scan on a second cathode ray tube.

4.3 THE DIFFUSION PROFILES OF PROPRIETARY IBUPROFEN-CONTAINING TOPICAL PREPARATIONS FROM THREE COUNTRIES

4.3.1 Introduction

One of the factors that requires consideration during the development of topical formulations is the climatic conditions under which the medicament is to be utilised. Preparations formulated for use in temperate climates may not be suitable for use in tropical or sub-tropical climates and *vice versa* (226). The vehicle may greatly influence the release of the drug. This study investigated the diffusion profiles obtained from proprietary ibuprofen formulations purchased in three countries, namely South Africa (SA), France (FR) and the United Kingdom (UK). South Africa has a sub-tropical climate, while France and the United Kingdom have a temperate climate. The formulations used in this study are listed in table 4.2. The composition in terms of the ingredients in each formulation, made available by the manufacturer, is listed in appendix II.

PRERARATION (5% m/m)	**PURCHASED**	**MANUFACTURED**
Deep Relief Gel	South Africa	United Kingdom
Ibuleve Gel	South Africa	South Africa
Deep Relief Gel	United Kingdom	United Kingdom
Ibuleve Gel	United Kingdom	United Kingdom
Ibuleve Mousse	United Kingdom	United Kingdom
Nurofen Gel	United Kingdom	United Kingdom
Proflex Cream	United Kingdom	United Kingdom
Radian B Gel	United Kingdom	United Kingdom
Ralgex Gel	United Kingdom	United Kingdom
Cliptol Gel	France	France
Dolgit Cream	France	France
Ibutop Gel	France	France
Intralgis Gel	France	France
Syntofene Gel	France	France
Tiburon Gel	France	France

Table 4.2 Proprietary ibuprofen-containing topical formulations

The French and UK preparations were transported to South Africa on a commercial flight in the hand luggage of the purchaser thereby avoiding possible adverse effects of temperature extremes during transportation. Each of the different products from the three countries was assayed using the experimental and analytical methodology described in chapter 3 and section 4.1.

Numerous authors have stated that commercially available products were used in studies involving topical ibuprofen, but have not reported the tradenames. In several cases where proprietary products were used, the name or names of only the principal products are presented. Inter-laboratory comparisons are unreliable unless tradenames or formulae of vehicles are reported. Authors should therefore, where possible, report the tradenames or formulae of the formulations used (226). Since many tradenames are the same in different countries, the country of manufacture should also be reported.

4.3.2 Comparisons of Ibuprofen Creams, Gels and Mousses

The *in vitro* permeation of ibuprofen from commercially available formulations through a silastic silicone membrane was monitored over a period of 72 hours. The experimental methodology as outlined in section 4.1 was adopted.

Individual and mean apparent release constants were obtained by linear regression analysis of plots of cumulative amount of released drug ($\mu g/cm^2$) versus square root of time ($h^{1/2}$). This relationship between drug release and square root of time was previously shown to be valid for topical formulations with either fully dissolved or suspended active ingredients (227-233). The "square root of time law" can be applied to ibuprofen release from the various topical formulations because firstly, only a single drug diffuses out of the dosage forms with a percentage of drug release less than 30% after a 20 hour period. Secondly, sink conditions are ensured by the large receptor volume of the European Pharmacopoeia diffusion cell and the replacement of receptor fluid at each sampling time when using the Franz diffusion cell.

By linear regression of the curves representing cumulative ibuprofen release per centimetre squared versus square root of time, an apparent release constant corresponding to the measured slope was obtained. The lag time, defined by the intercept on the square root of

time axis was also used to compare drug release from different formulations. This lag time corresponds to the time it takes for drug molecules to be released from the dosage form and to diffuse across the synthetic membrane, the latter not being rate limiting.

The diffusion profiles obtained for the ibuprofen formulations studied using both the Franz and European Pharmacopoeia diffusion cells are shown in figure 4.5 and figure 4.6.

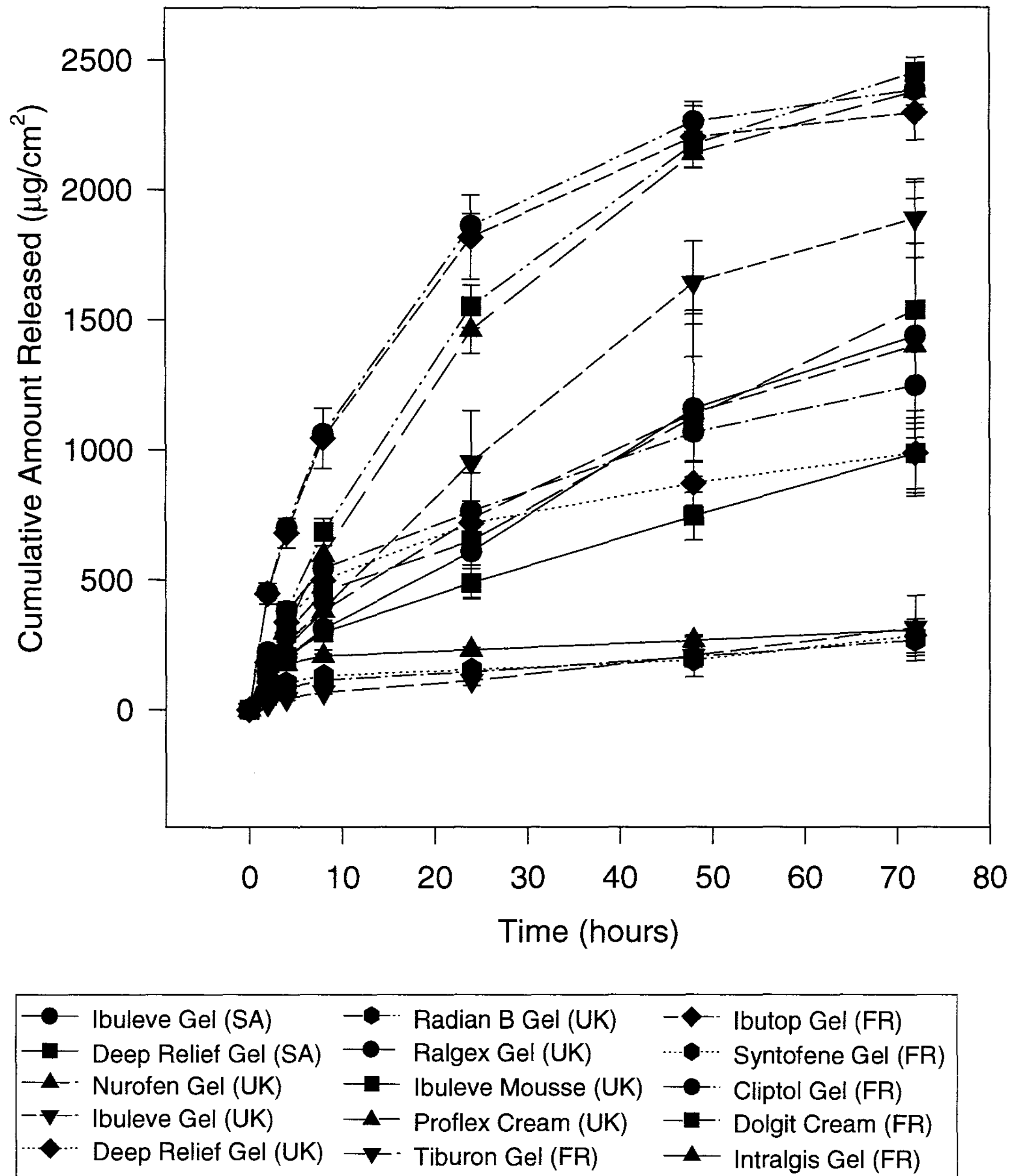
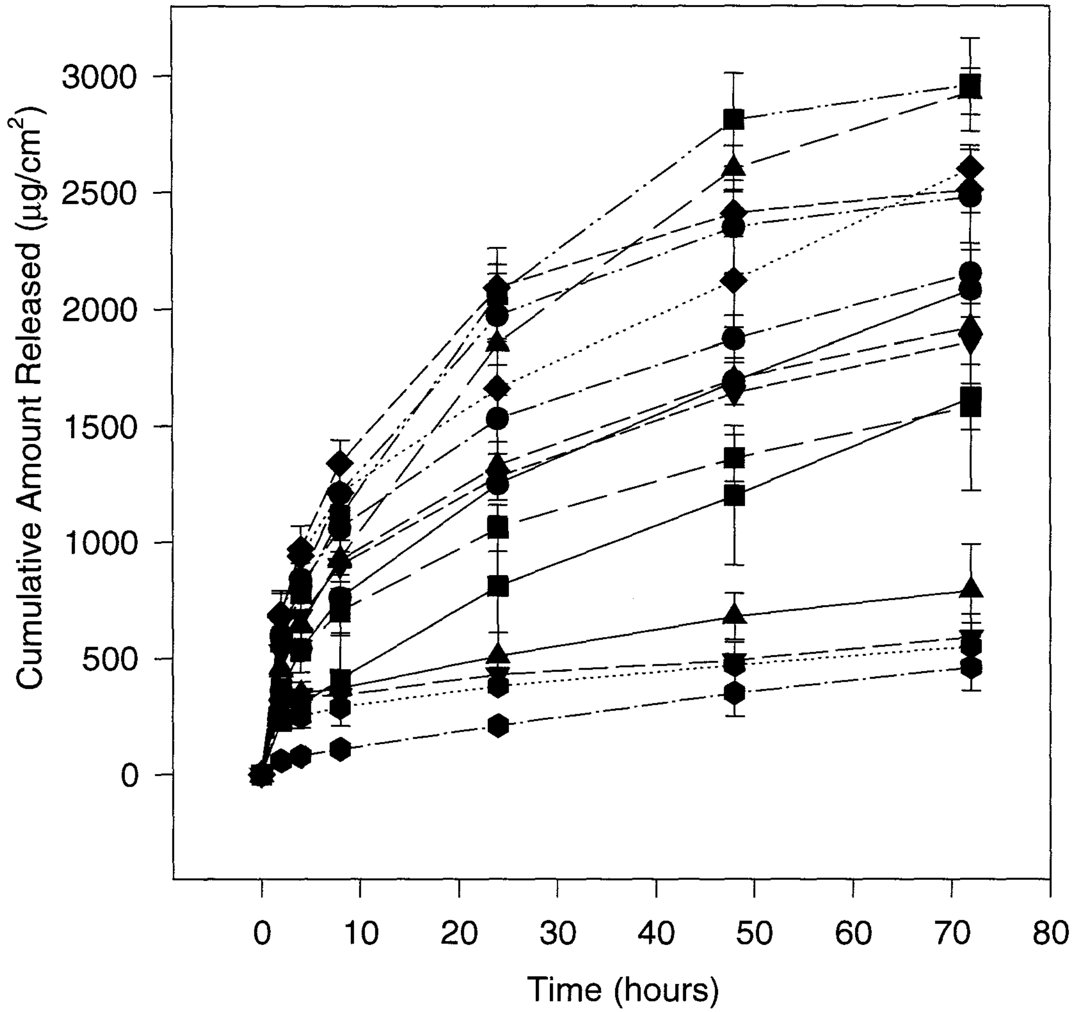


Figure 4.5 The diffusion profiles obtained for ibuprofen formulations using the Franz diffusion cell



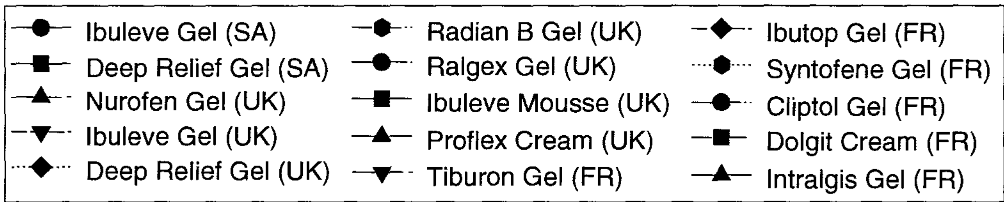


Figure 4.6 The diffusion profiles obtained for ibuprofen formulations using the European Pharmacopoeia diffusion cells

The diffusion profiles obtained for the ibuprofen formulations using both the Franz and European Pharmacopoeia diffusion cells have been divided into three groups based on the range of the respective apparent release constants (μg/cm^2 /h$^{1/2}$). This enabled the data to be presented in clear graphic images, thus facilitating interpretation.

4.3.2.1 Results Obtained Using the Franz Diffusion Apparatus

The diffusion profiles using the Franz diffusion cell apparatus are depicted as follows:

RANGE (μg/cm^2 /h$^{1/2}$)	FIGURE
286 – 318	4.7
115 – 241	4.8
28 – 36	4.9

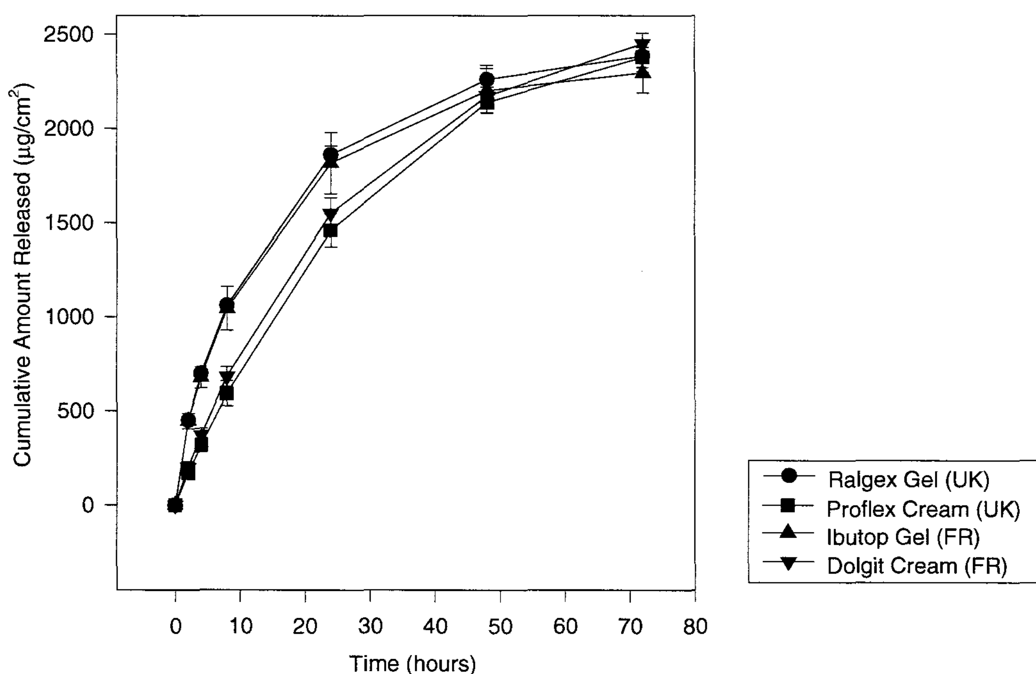


Figure 4.7 Ibuprofen diffusion profiles (range 286-318 μg/cm^2 /h$^{1/2}$)

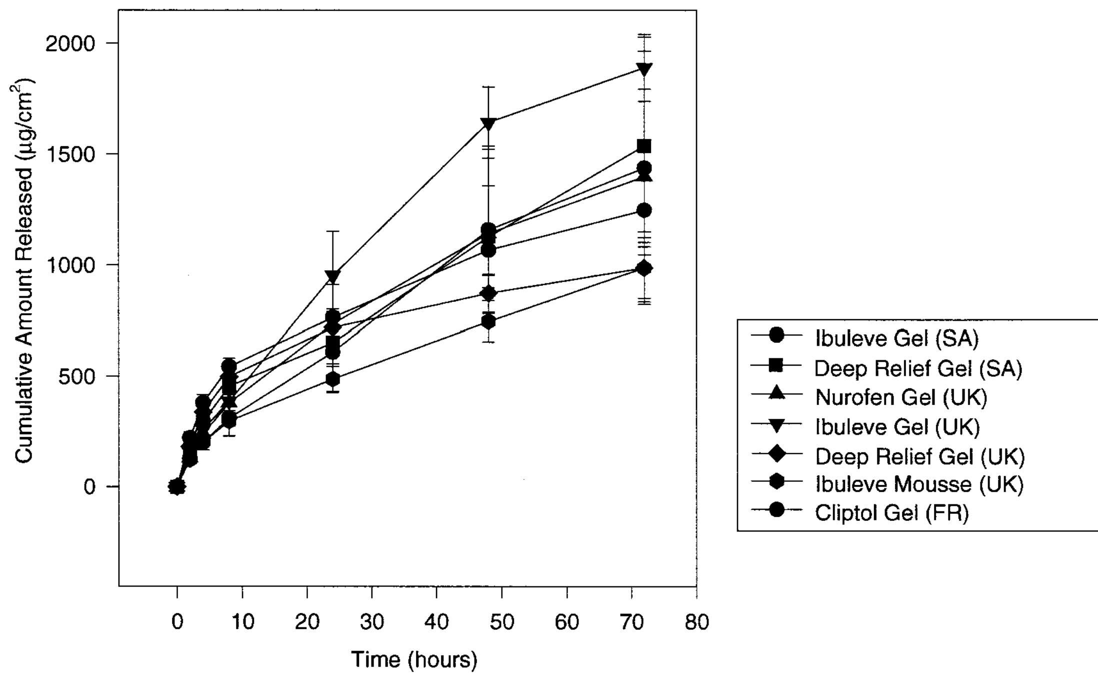


Figure 4.8 Ibuprofen diffusion profiles (range 115-241 $\mu g/cm^2/h^{1/2}$)

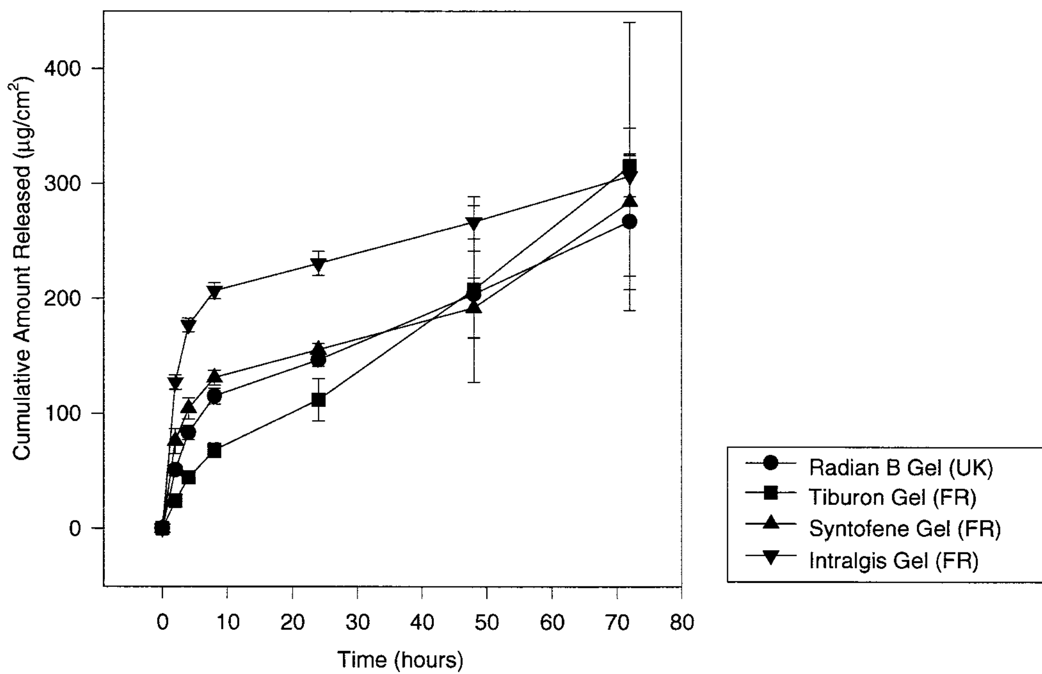


Figure 4.9 Ibuprofen diffusion profiles (range 28-36 $\mu g/cm^2/h^{1/2}$)

The release constants, lag times and correlation coefficients (r^2) for ibuprofen-containing topical formulations using the Franz diffusion cell apparatus are shown in table 4.3.

FORMULATION	**APPARENT RELEASE CONSTANT (μg/cm^2/h$^{1/2}$)**	**LAG TIME (h$^{1/2}$)**	r^2
Dolgit Cream (FR)	318.03	0.46	0.9891
Proflex Cream (UK)	312.91	0.57	0.9803
Ralgex Gel (UK)	296.37	0.41	0.9641
Ibutop Gel (FR)	286.09	0.44	0.9603
Ibuleve Gel (UK)	241.87	0.67	0.9794
Deep Relief Gel (SA)	176.25	0.37	0.9783
Ibuleve Gel (SA)	176.0	0.67	0.9752
Nurofen Gel (UK)	171.93	0.45	0.9937
Deep Relief Gel (UK)	155.81	0.64	0.9627
Cliptol Gel (FR)	145.02	0.37	0.9842
Ibuleve Mousse (UK)	114.46	0.25	0.9931
Tiburon Gel (FR)	35.86	0.73	0.9515
Intralgis Gel (FR)	29.86	2.5	0.8295
Radian B Gel (UK)	29.21	0.44	0.9808
Syntofene Gel (FR)	28.15	0.99	0.9348

Table 4.3 The release constants, lag times and r^2 values for ibuprofen-containing topical formulations, using the Franz diffusion cell apparatus

The rank order of drug permeation from the formulations (μg/cm^2) at 72 hours is Dolgit Cream (FR), Ralgex Gel (UK), Proflex Cream (UK), Ibutop Gel (FR), Ibuleve Gel (UK), Deep Relief Gel (SA), Ibuleve Gel (SA), Nurofen Gel (UK), Cliptol Gel (FR), Deep Relief Gel (UK), Ibuleve Mousse (UK), Tiburon Gel (FR), Intralgis Gel (FR), Syntofene Gel (FR) and Radian B Gel (UK). There is a significant statistical difference at a 95% confidence interval between the formulations of groups one, two and three.

The gel formulations in the first group have the highest initial release rates (Ralgex 218 ± 9 μg/cm^2/h and Ibutop 217 ± 19 μg/cm^2/h) which steadily decrease over the sampling time period to a release rate that is less than that of the two cream formulations. Steady state is gradually approached at 72 hours. The cream formulations exhibit the same steady decrease in release rates (initial release rates: Dolgit 97 ± 14 μg/cm^2/h and

Proflex 82 ± 11 μg/cm^2/h). The gel and mousse formulations in the second group show a similar decrease from an average initial release rate of 77 μg/cm^2/h. The individual release rates are shown in appendix III. The third group of gel formulations have much lower initial release rates and reach a relatively low constant rate of delivery of approximately 2 μg/cm^2/h after 12 hours.

When the cumulative amount of drug released per square centimetre was plotted against the square root of time, a linear relationship was obtained for the cream, mousse and gel formulations from group one and two (r^2>0.96), showing that the release of ibuprofen from the formulations was well described by the Higuchi model, where the rate controlling step is the process of diffusion through the gel matrix. This relationship exists for formulations in which the drug is fully dissolved and in which the drug is present as a suspension. Hence, the membrane has no significant effects and the properties of the formulation control the release of the drug (229). The gel formulations in group three, excluding Radian B gel which has a correlation coefficient greater than 0.98, do not conform to the same linear relationship. The exception of Radian B gel may be due to the fact that although all four of the formulations in group three contain the same ingredients, the percentages of the ingredients in Radian B gel may differ from those of the other three gels.

The two cream formulations have the greatest apparent release constants. Dolgit cream (318 μg/cm^2/h$^{1/2}$) has a slightly greater apparent release constant than Proflex cream (313 μg/cm^2/h$^{1/2}$). The apparent release constants of the gel formulations ranged from 28 – 242 μg/cm^2/h$^{1/2}$. The mousse formulation has an apparent release constant of 114 μg/cm^2/h$^{1/2}$, which falls roughly mid-way in the gel range. The formulations are listed in table 4.4 in decreasing order of the apparent release constants.

The observed lag times were short, ranging from 0.25 – 0.67 h$^{1/2}$ for the cream mousse and gel formulations in groups one and two. Longer lag times over a greater range (0.44 – 2.5 h$^{1/2}$) were observed for group three. The longer lag time values indicate that ibuprofen is preferentially retained by the formulation base.

4.3.2.2 Results Obtained Using the European Pharmacopoeia Diffusion Apparatus

The diffusion profiles using the European Pharmacopoeia diffusion cell apparatus are depicted as follows:

RANGE ($\mu g/cm^2/h^{1/2}$)	FIGURE
283 – 369	4.10
181 – 242	4.11
55 – 80	4.12

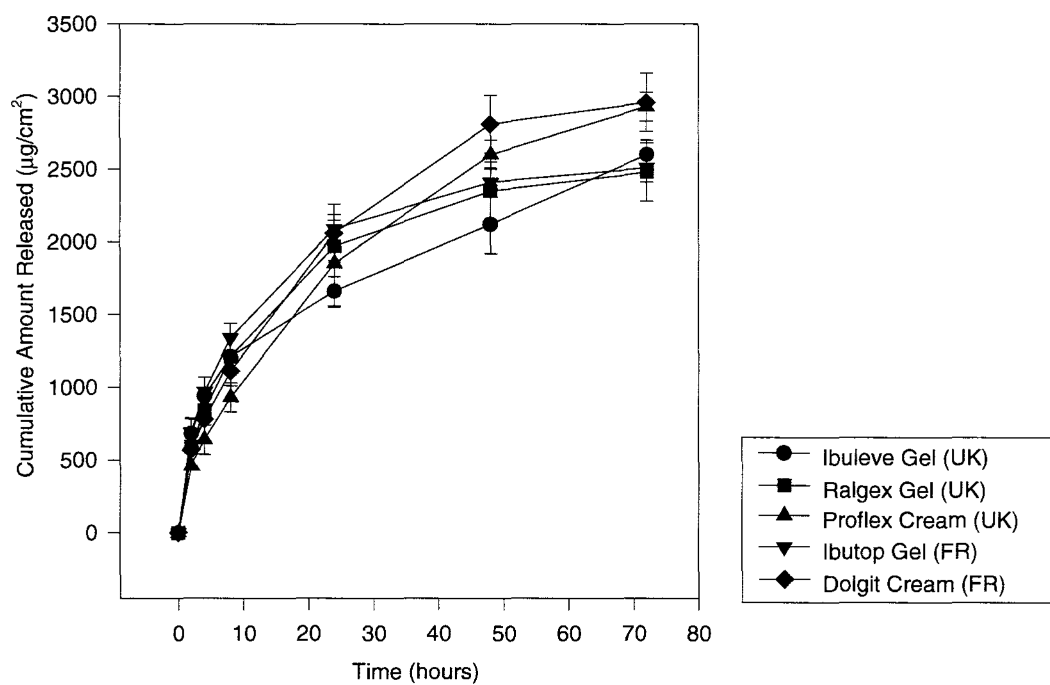


Figure 4.10 Ibuprofen diffusion profiles (range 283-369 $\mu g/cm^2/h^{1/2}$)

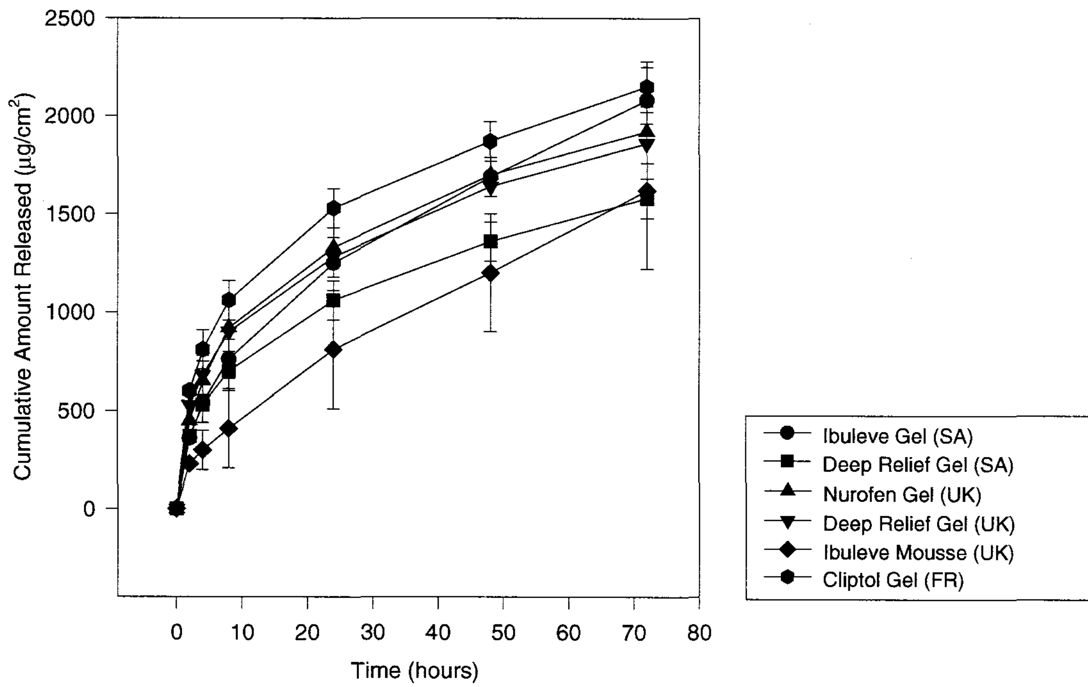


Figure 4.11 Ibuprofen diffusion profiles (range 181-242 $\mu g/cm^2/h^{1/2}$)

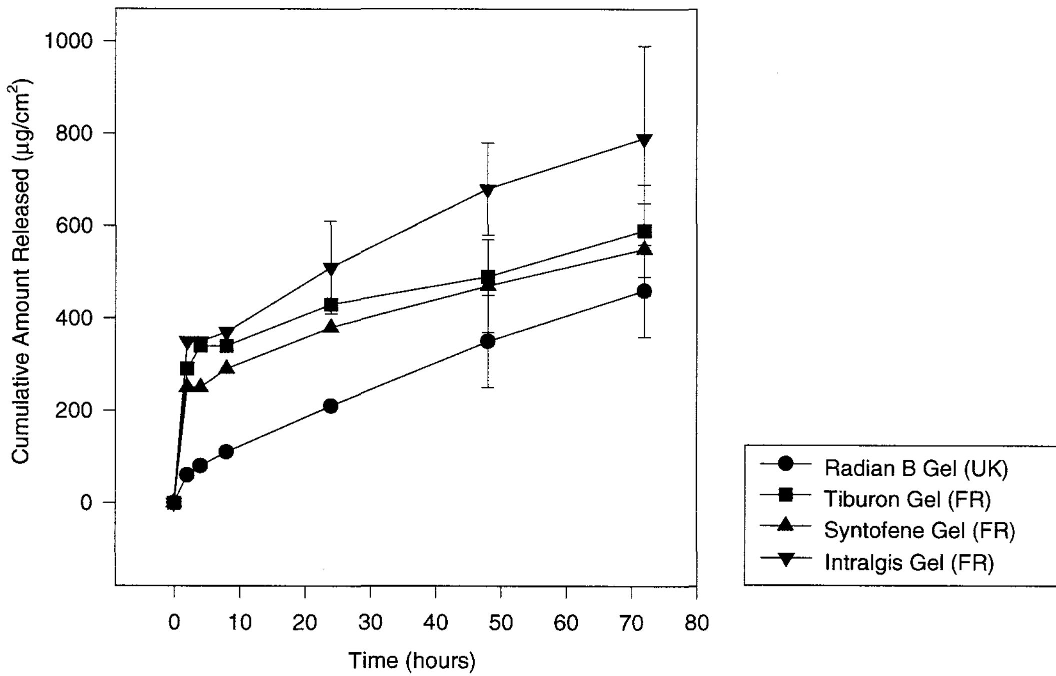


Figure 4.12 Ibuprofen diffusion profiles (range 55-80 $\mu g/cm^2/h^{1/2}$)

The release constants, lag times and r^2 values for ibuprofen-containing topical formulations, using the European Pharmacopoeia diffusion cell apparatus, are shown in table 4.4.

FORMULATION	**APPARENT RELEASE CONSTANT ($\mu g/cm^2/h^{1/2}$)**	**LAG TIME ($h^{1/2}$)**	r^2
Dolgit Cream (FR)	369.30	0.19	0.9835
Proflex Cream (UK)	363.28	0.09	0.9938
Ralgex Gel (UK)	298.65	0.72	0.9562
Ibutop Gel (FR)	296.29	1.03	0.9353
Ibuleve Gel (UK)	283.04	0.86	0.9755
Ibuleve Gel (SA)	242.39	0.14	0.9985
Cliptol Gel (FR)	239.90	0.98	0.9660
Nurofen Gel (UK)	221.49	0.70	0.9765
Deep Relief Gel (UK)	208.39	0.93	0.9717
Ibuleve Mousse (UK)	188.20	0.33	0.9903
Deep Relief Gel (SA)	180.60	0.64	0.9843
Intralgis (FR)	80.06	1.65	0.9184
Radian B Gel (UK)	55.27	0.30	0.9828
Tiburon Gel (FR)	55.04	2.64	0.8268
Syntofene Gel (FR)	55.02	1.89	0.9041

Table 4.4 The release constants, lag times and r^2 values for ibuprofen-containing topical formulations, using the European Pharmacopoeia diffusion cell apparatus

The rank order of drug permeation from the formulations ($\mu g/cm^2$) at 72 hours is Dolgit Cream (FR), Proflex Cream (UK), Ibuleve Gel (UK), Ibutop Gel (FR), Ralgex Gel (UK), Cliptol Gel (FR), Ibuleve Gel (SA), Nurofen Gel (UK), Deep Relief Gel (UK), Ibuleve Mousse (UK), Deep Relief Gel (SA), Intralgis Gel (FR), Tiburon Gel (FR), Syntofene Gel (FR) and Radian B Gel (UK). There is a significant statistical difference at a 95% confidence interval between the formulations of groups one, two and three.

The gel formulations in the first group have the highest initial release rates (Ralgex 245 ± 120 $\mu g/cm^2/h$, Ibutop 347 ± 26 $\mu g/cm^2/h$ and Ibuleve UK 283 ± 141 $\mu g/cm^2/h$) which steadily decrease over the sampling time period to a release rate that is less than that of the two cream formulations. Steady state is gradually approached at 72 hours. The cream

formulations exhibited the same steady decrease in release rates (initial release rates: Dolgit 236 ± 116 μg/cm^2/h and Proflex 193 ± 95 μg/cm^2/h). The gel and mousse formulations in the second group show a similar decrease from an average initial release rate of 195 μg/cm^2/h. The individual release rates are shown in appendix III. The third group of gel formulations have very much lower initial release rates and reach a relatively low constant rate of delivery of approximately 4 μg/cm^2/h after 12 hours.

When the cumulative amount of drug released per square centimetre was plotted against the square root of time, a linear relationship was obtained for the cream, mousse and gel formulations of group one and two (r^2>0.93), showing that the release of ibuprofen from the formulations was well described by the Higuchi model, where the rate controlling step is the process of diffusion through the gel matrix. This relationship exists for formulations in which the drug is fully dissolved and in which the drug is present as a suspension. Hence, the membrane has no significant effects and the properties of the formulation control the release of the drug (229). The gel formulations in group three, excluding Radian B gel which has a correlation coefficient greater than 0.98, do not conform to the same linear relationship. The exception of Radian B gel may be due to its percentage composition.

The two cream formulations have the greatest apparent release constants. Dolgit cream (369 μg/cm^2 /h$^{1/2}$) has a slightly greater apparent release constant than Proflex cream (363 μg/cm^2 /h$^{1/2}$). The apparent release constants of the gel formulations ranged from 55 – 299 μg/cm^2 /h$^{1/2}$. The mousse formulation has an apparent release constant of 188 μg/cm^2 /h$^{1/2}$, which falls roughly mid-way in the gel range. The formulations are listed in table 4.4 in decreasing order of the apparent release constants.

The observed lag times were found to be longer than those found using the Franz diffusion cells and ranged from 0.09 – 1.03 h$^{1/2}$ for the cream, mousse and gel formulations in groups one and two. Longer lag times over a greater range (0.3 – 2.64 h$^{1/2}$) were observed for group three. The longer lag time values indicate that ibuprofen is preferentially retained by the formulation base.

4.3.3 Discussion

Drug penetration is dependent upon the influence of the vehicle on the thermodynamic activity of the active ingredient. Ibuprofen is a poorly water-soluble drug, hence its release is favoured from hydrophilic bases such as the gels and the oil-in-water formulations which are poor solvents for the drug. Consequently, there are fewer drug-vehicle interactions leading to improved partitioning of the drug into the membrane.

Each formulation within the three groups showed similar results for correlation coefficients, apparent release constants, lag times and release rates. This may be explained by examining the compositions of the formulations within the three separate groups (Appendix II). Group one consisted of two cream formulations both containing propylene glycol and a hydrophilic non-ionic surfactant and two gel formulations with identical compositions to each another. In addition to the two creams, the gel formulations of group two contained propylene glycol. Group three consisted of four gel formulations which had identical formulations and were the only formulations that contained sodium hydroxide.

The delivery rates and apparent release constants obtained using both the Franz and European Pharmacopoeia diffusion apparatus can be explained by examining Fick's law of diffusion. The flux is proportional to the partition coefficient, the diffusion coefficient and the donor concentration and inversely proportional to the thickness of the membrane. In this experiment the intrinsic diffusion coefficient, the membrane thickness and the donor concentration were constant. Hence, in the absence of physical mechanisms, the rate of diffusion will be dictated by the partition coefficient between the donor formulation and the membrane.

Ibuprofen is poorly water soluble and therefore has a low affinity for the aqueous gel and mousse formulations. It will therefore partition into the less hydrophilic environment of the silicone membrane, hence the relatively short lag times and high flux rates observed as these processes are concentration gradient dependent.

The cream formulations and the two gel formulations from the first group (Ralgex and Ibutop) appear to present an ideal combination of solubility and physical diffusivity through the vehicle, yielding high ibuprofen release rates and apparent release constants.

A trend was observed between the cream and gel formulations when both the Franz and European Pharmacopoeia diffusion cells were used. The gel formulations gave a significantly greater initial release rate than the cream formulations and an overlap of ibuprofen release occurred at 48 hours, after which the release rate from the gel formulations decreased while the release rate from the cream formulations remained constant and a significant difference was once again observed between the gel and cream formulations. The cream is a lipophilic/hydrophilic mixture from which ibuprofen appears to partition readily into the silicone matrix. Relatively facile diffusion through this vehicle of moderate viscosity appears to allow replenishment of the drug depletion layer as ibuprofen is removed from the vehicle/membrane interface to a greater extent than that which occurs in the gel formulations. Hence the observed high flux rate and only gradual decline in diffusion rate after 72 hours.

4.3.3.1 Formulation Factors

A number of formulation factors have been considered in an attempt to explain the results obtained for both the Franz and European Pharmacopoeia diffusion cells.

4.3.3.1.1 Propylene Glycol

The formulations represented by group two consist of one mousse formulation, Ibuleve Mousse, and seven gel formulations, Ibuleve Gel (UK), Deep Relief Gel (SA), Ibuleve Gel (SA), Nurofen Gel (UK), Deep Relief Gel (UK) and Cliptol Gel (FR). The diffusion profiles vary greatly within this group and may be explained by the fact that all these formulations contain propylene glycol. No gel formulation in group one or three contains propylene glycol. The percentage mass/mass was not indicated for any ingredient, other than ibuprofen, in the formulations. Propylene glycol is classified as a humectant. The use of this substance brings about hydration and hydrophilisation of the membrane, thus facilitating the absorption of ibuprofen. Propylene glycol is also a penetration enhancer, which causes increased penetration of ibuprofen through the membrane by making it more permeable to the drug (41).

An investigation into the vehicle-skin partition coefficients and solubilities of ibuprofen in a series of aqueous propylene glycol systems with different cosolvent compositions has

determined that the flux data show essentially a steady state flux which increases progressively with propylene glycol concentration. The permeability constant, however, peaks at about 20% propylene glycol where the efficiency of drug delivery into the membrane from the saturated solution appears to be maximal. The results suggest that the vehicle may be capable of modifying the partition properties of the membrane. At concentrations of 20-30% propylene glycol there appears to be a maximum partition coefficient, probably due to penetration of propylene glycol and an enhanced solubility of ibuprofen in the membrane (217,234). The various release profiles obtained for the formulations investigated may therefore be explained by assuming that the formulations differ mainly with respect to the percentage of propylene glycol in each.

The action of propylene glycol as a potential penetration enhancer has been questioned. Several authors have reported that its action is based on its cosolvent effect (235). In this case, drug penetration is thermodynamically controlled. Only limited data demonstrate an enhancer effect in which drug penetration increases with increasing propylene glycol content of the formulation. This promoting effect is related mainly to the solvent drag effect of propylene glycol (236,237).

4.3.3.1.2 Ethanol/Isopropanol

All gel formulations contained either ethanol or isopropanol in unknown concentrations. The release profiles of the three groups may be explained by examining the action of alcohol on the system being investigated. Alcohol is classified as a humectant. The use of this substance produces varying degrees of hydration and hydrophilisation of the membrane, thus facilitating the absorption of ibuprofen (41). Different percentages of alcohol will therefore result in different diffusion profiles.

4.3.3.1.3 Purified Water

All formulations contained purified water. Addition of purified water to a formulation in which ibuprofen is less soluble increases the thermodynamic activity with consequent increase of the membrane penetration rate. When the percentage of water is high, which may be the case in the formulations with the greatest apparent release constants and release rates and the shortest lag times, more of the drug precipitates causing a substantial decrease

of solute concentration. However, the further increase in escaping tendency produces more rapid penetration and the undissolved drug provides a reservoir ensuring solution saturation with the permeant throughout the experiment (238).

4.3.3.1.4 Sodium Hydroxide

The third group of gel formulations contained sodium hydroxide which is a powerful base and is used to adjust the pH of formulations. The results obtained from this group may be explained by the specific interactions which can occur between ibuprofen and sodium hydroxide which may first decrease the activity of the drug by salt formation and even precipitate the drug as sparingly soluble complexes or salts (239). Another possible explanation may be that a certain percentage of the sodium hydroxide diffuses through the silastic membrane and into the receptor fluid where it may change the pH of the solution. The higher pH of the receptor fluid may therefore present a non-ideal environment to the diffusion of ibuprofen from the formulation.

4.3.3.1.5 Surfactant

The cream formulations contained an hydrophilic non-ionic surfactant (polyoxyethylene esters). Penetration through a membrane can be modified by surfactants. Interaction with the permeant may also occur, therefore a surfactant can either increase, decrease or have no effect on membrane absorption (238). Investigations into the action of hydrophilic non-ionic surfactants have found that gels containing a polyoxyethylene fatty acid ester exhibited a higher release rate and significantly decreased lag time as compared to control gels (174).

4.3.3.1.6 Effect of pH

Promotion of penetration is also brought about by the pH of the formulation which was not assessed in this study. The pKa of ibuprofen is 4.6 and in a gel at a pH of 5.0, about 40% of the ibuprofen is present in its neutral, undissociated form. This neutral form is better able to pass through the lipophilic silicone polymer into the receptor medium, where the pH of 7.2 causes a shift to about 99.9% dissociated ibuprofen (41).

4.3.3.2 Transport Factors

The Deep Relief Gel formulation available in South Africa is imported from Britain. The apparent release constant as well as the release rate of Deep Relief Gel (SA) is significantly less at a 95% confidence level than Deep Relief Gel (UK) assessed using both the Franz and European Pharmacopoeia diffusion cell apparatus. One possible reason for this may be that the gel was formulated for use in a temperate climate and the vehicle may not have been a good releasing medium in a subtropical climate, or that the wide temperature ranges that may have been encountered during importation may have altered the release characteristics of the vehicle.

This formulation would have been transported to South Africa by sea or air. It has been found that the temperatures in the cargo hold of a ship have been found to vary from 1-32°C (240,241) during the cool season (June/July from South Africa to Britain and December/January from Britain to Hong Kong) and may be considerably hotter during the equatorial summers. Temperatures in the cargo hold of an aircraft may be below 0°C, depending on the cruising altitude of the aircraft and the duration of the flight (242). The ideal vehicle should therefore be able to withstand storage over a temperature range of 5-30°C.

4.3.4 Comparison of Diffusion cells

The diffusion profiles obtained for each formulation, utilising the Franz and European Pharmacopoeia diffusion cells, are compared here in an attempt to demonstrate an appropriate diffusion technique for the measurement of the amount of ibuprofen released from a topical formulation during *in vitro* testing. The diffusion profiles obtained for the ibuprofen formulations have once again been divided into three groups based on the range of the respective apparent release constants in order to report the results in a consistent format.

RANGE ($\mu g/cm^2/h^{1/2}$)	FIGURE
286 – 369	4.13
115 – 283	4.14
28 – 80	4.15

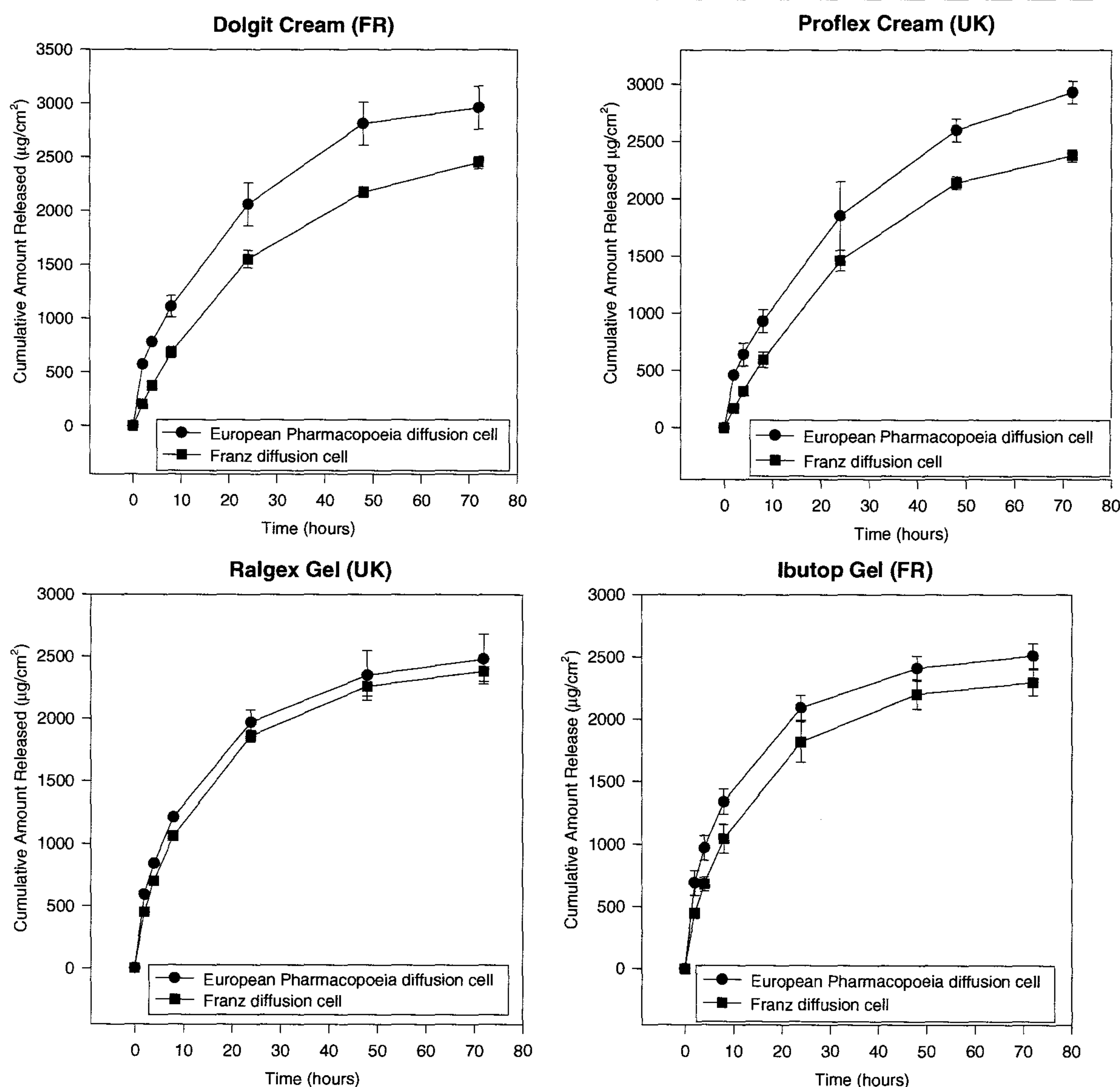
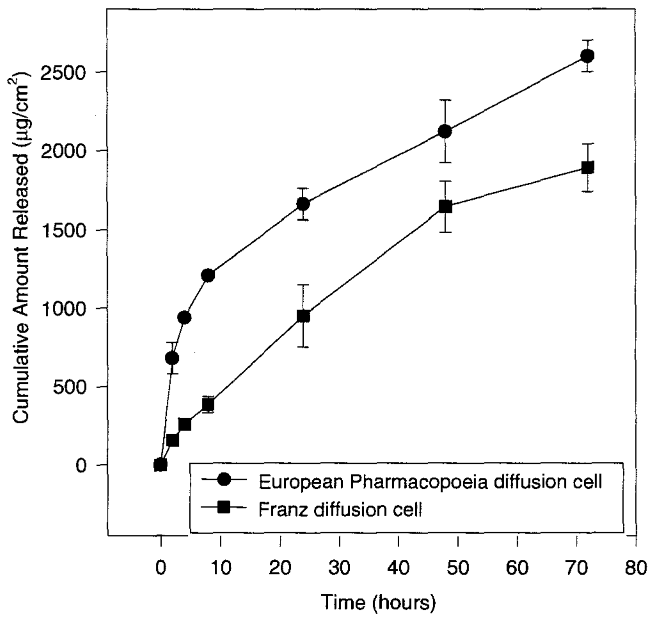
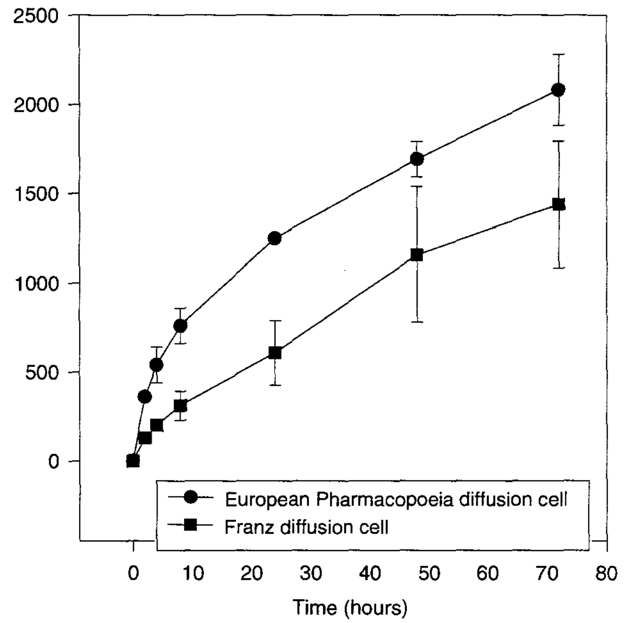
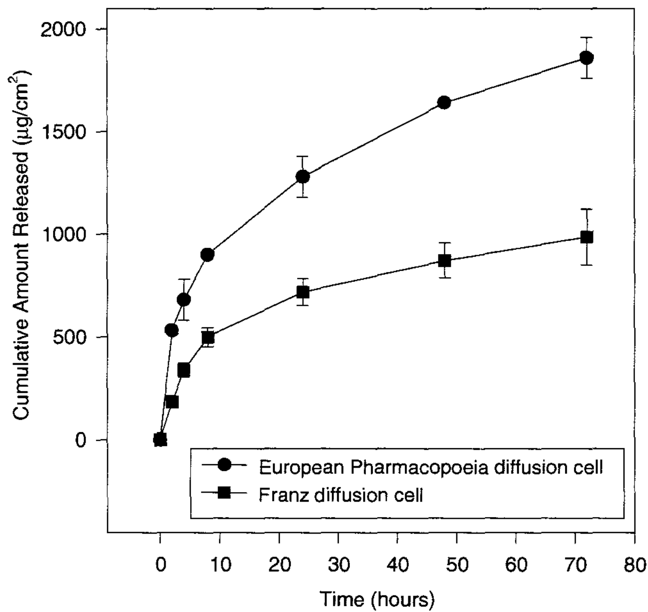
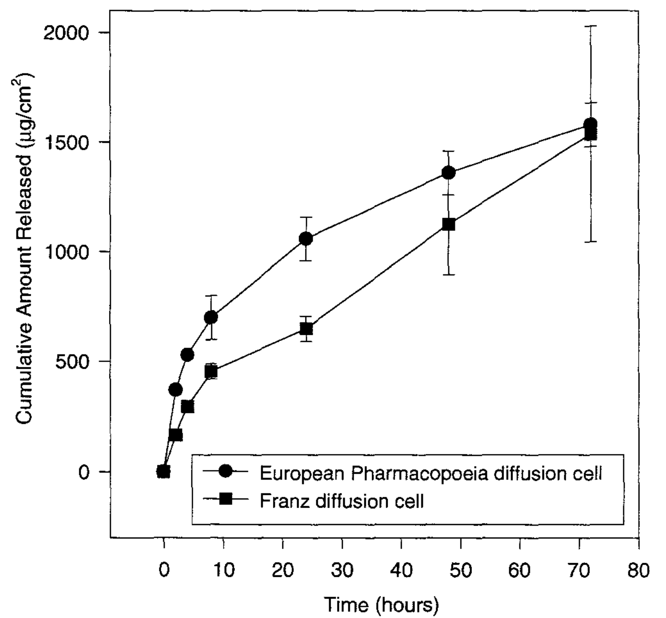


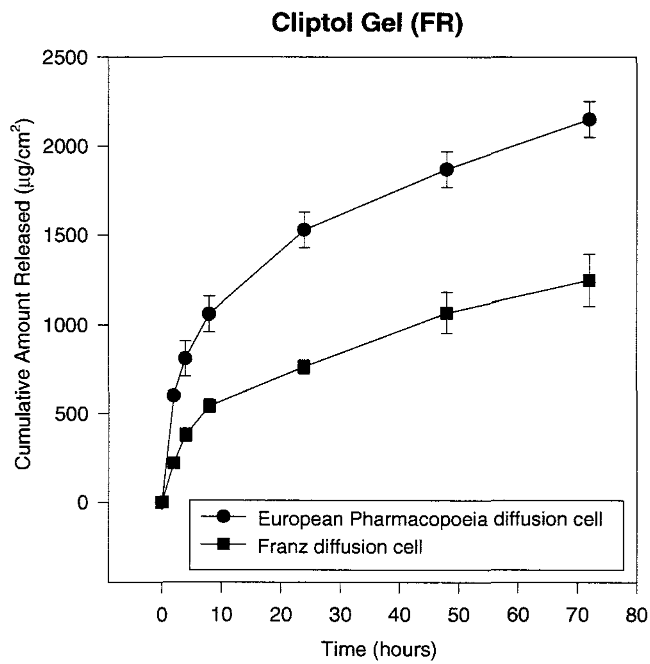
Figure 4.13 Ibuprofen diffusion profiles (range 286-369 $\mu g/cm^2/h^{1/2}$)

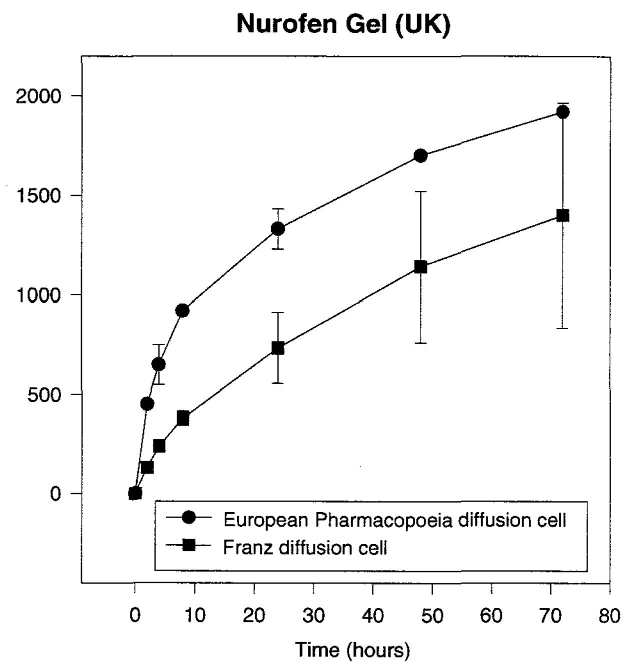

Ibuleve Gel (UK)
Cumulative Amount Released (μg/cm²)
2500
2000
1500
1000
500
0
European Pharmacopoeia diffusion cell
Franz diffusion cell
0
10
20
30
40
50
60
70
80
Time (hours)


Ibuleve Gel (SA)
2500
2000
1500
1000
500
0
European Pharmacopoeia diffusion cell
Franz diffusion cell
0
10
20
30
40
50
60
70
80
Time (hours)


Deep Relief Gel (UK)
Cumulative Amount Released (μg/cm²)
2000
1500
1000
500
0
European Pharmacopoeia diffusion cell
Franz diffusion cell
0
10
20
30
40
50
60
70
80
Time (hours)


Deep Relief Gel (SA)
Cumulative Amount Released (μg/cm²)
2000
1500
1000
500
0
European Pharmacopoeia diffusion cell
Franz diffusion cell
0
10
20
30
40
50
60
70
80
Time (hours)





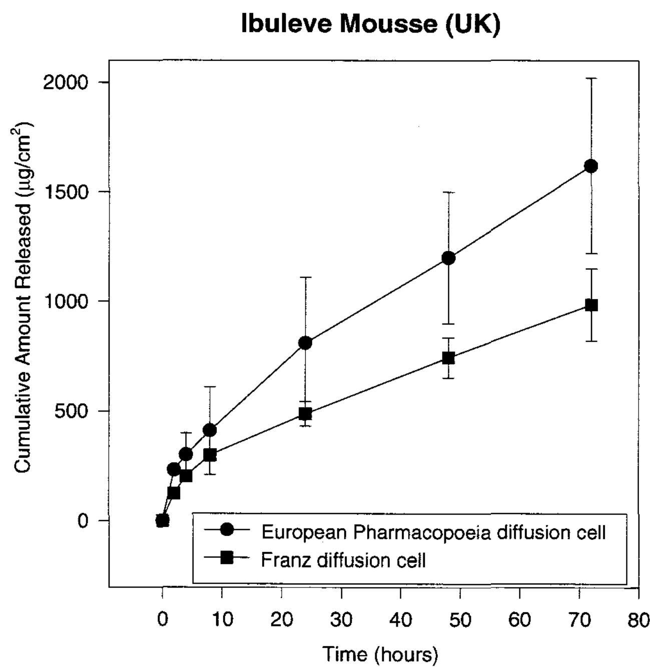
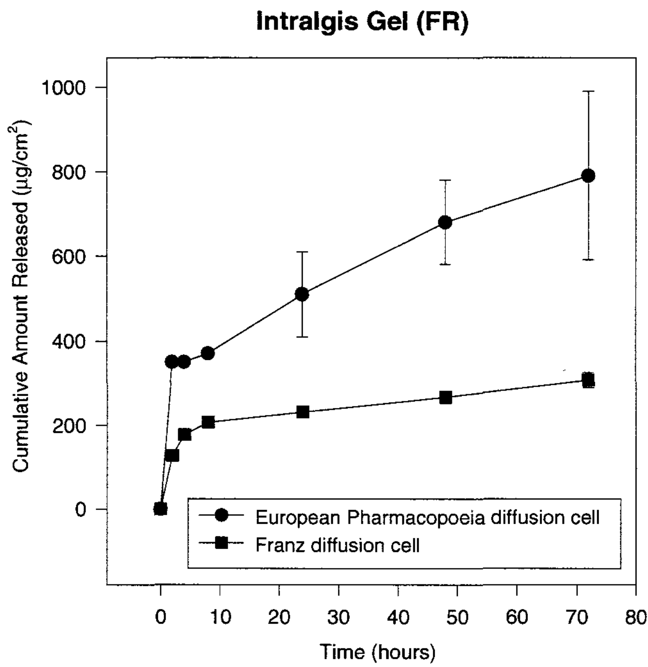
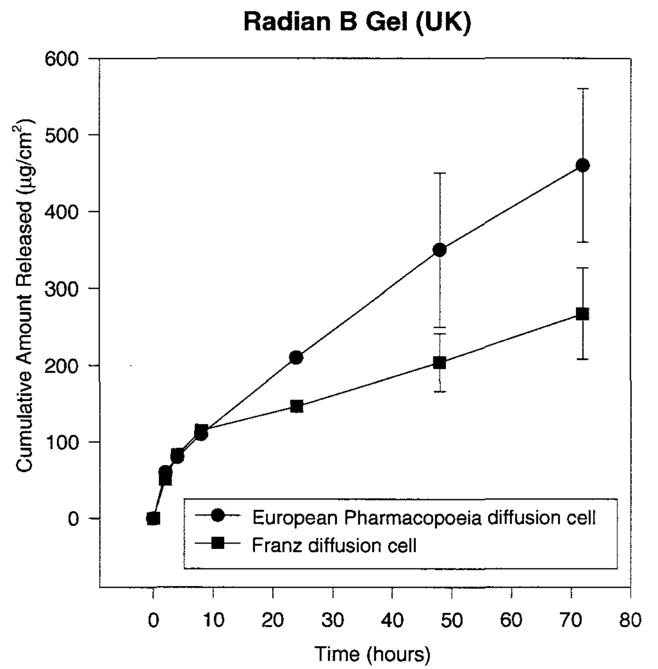
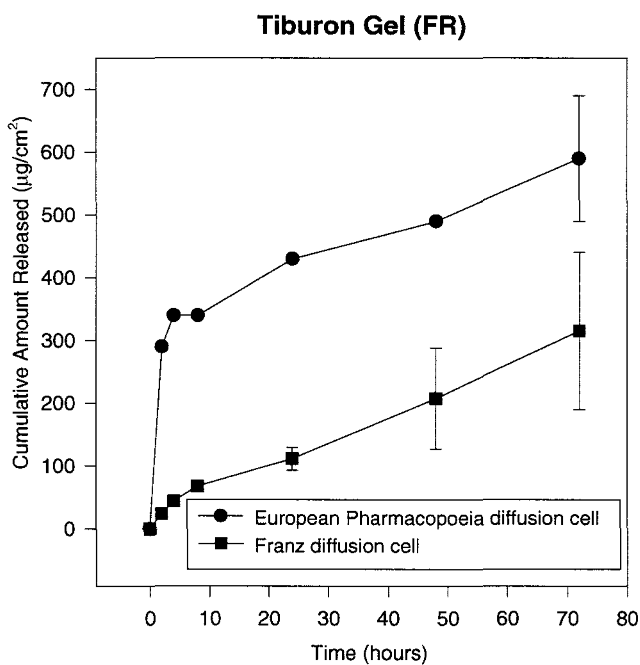


Figure 4.14 Ibuprofen diffusion profiles (range 115-283 $\mu g/cm^2/h^{1/2}$)







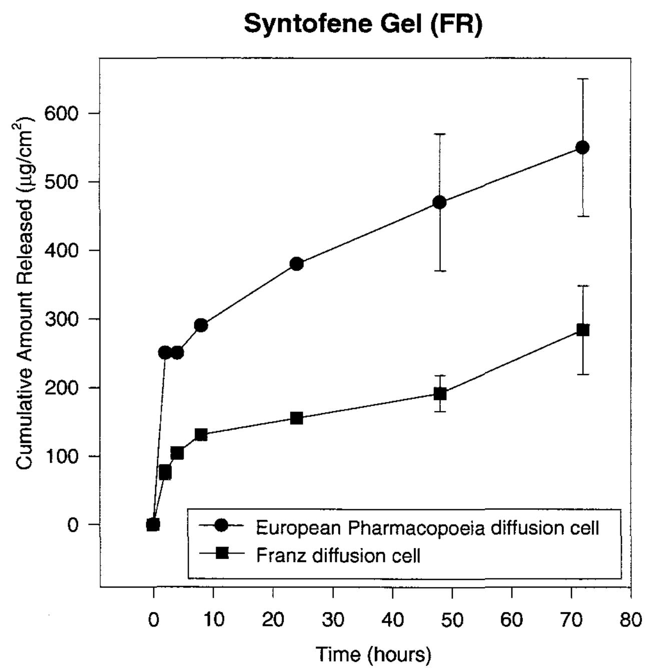


Figure 4.15 Ibuprofen diffusion profiles (range 28-80 $\mu g/cm^2/h^{1/2}$)

4.3.4.1 Discussion

A statistically significant difference, at a 95% confidence interval, was seen between the diffusion profiles obtained using the Franz and the European Pharmacopoeia diffusion cells for all formulations assessed with the exception of Ralgex Gel. The percentage standard deviation using the Franz diffusion cells was greater than the European Pharmacopoeia diffusion cells with the exception of Ibuleve Mousse. This deviation may be a result of the relatively lower viscosity of the mousse which resulted in the formation of air bubbles on the surface of the membrane in contact with the formulation when the cells were initially set up. It is therefore possible that an erratic release profile resulted as the diffusional area of membrane was not uniform in all five of the sample cells. The standard deviation of the cumulative amount of ibuprofen released per centimetre squared was generally greater using the Franz diffusion cells. In all cases the use of the European Pharmacopoeia diffusion apparatus resulted in a greater apparent release constant and release rate for the formulations.

There are a number of possible explanations for the differences observed between the two diffusion systems. Due to the design of the two systems, less evaporation occurs from the European Pharmacopoeia diffusion apparatus than the Franz diffusion cells. The formation of air bubbles under the membrane surface during the course of the experiment is possible in the Franz cell which may reduce the surface area of contact between the formulation and the receptor fluid thus resulting in a smaller diffusional area and possibly a more erratic diffusion rate.

Another possible reason for the lower values seen for the apparent release constants and release rates when using the Franz diffusion apparatus may be due to the experimental methodology adopted. The Franz cells were completely emptied at each sampling time and refilled with fresh receptor fluid in order to maintain sink conditions. A period of time therefore exists when the receptor fluid is not at 32°C but is at ambient temperature. This lower temperature may result in a smaller amount of ibuprofen diffusing across the membrane for the period of time it takes for the receptor fluid to reach 32°C.

The drug concentration gradient is the driving force for diffusion and any increase in the drug concentration on the receptor side of the barrier would decrease the magnitude of the

gradient and retard further permeation. Sample collection from the Franz diffusion cell is a non-continuous process as the cells are completely emptied at each sampling time and refilled with fresh receptor fluid. Conversely, sample collection from the European Pharmacopoeia diffusion cells is a continuous process. In the case of the Franz diffusion cell, absolute sink conditions may not prevail for the entire experimental period because of accumulation of the permeant in the receptor phase before each sampling time. This may be a result of a higher concentration of ibuprofen present in the receptor fluid due to the relatively smaller receptor volume (7 ml) as compared to 1000 ml volume of the European Pharmacopoeia cell.

The longer lag times observed over a greater range using the European Pharmacopoeia diffusion apparatus may be due to the formation of small air bubbles on the cells and over the diffusional membrane area. These air bubbles formed rapidly and may have been due to temperature equilibration between the cell which is at ambient temperature and the receptor fluid at 32°C. The receptor fluid flow created by the rotation of the paddles at 100 rpm dislodged the air bubbles after approximately four hours. The diffusional area may therefore have been decreased in a non-uniform fashion resulting in longer lag time values over a greater range.

4.3.4.2 Conclusion

The European Pharmacopoeia diffusion apparatus was judged to be the better of the two diffusion techniques assessed for the measurement of the amount of ibuprofen released from a topical formulation during *in vitro* testing. The European Pharmacopoeia diffusion apparatus shows better reproducibility, superior sink conditions and the larger values obtained for the amount of permeant diffusing across the membrane suggests that the system is more sensitive to the assessment of formulations of lower concentrations.

4.4 THE PERMEATION OF IBUPROFEN THROUGH SHED SNAKE SKIN AS THE BIOLOGICAL ANIMAL MEMBRANE

4.4.1 Introduction

Recently there has been considerable interest in the use of shed snake skin as a membrane for *in vitro* diffusion studies assessing the release of a number of drugs from semi-solid formulations. In this experiment shed snake skin from four species of snake was used for the verification of the usefulness, or otherwise, of shed snake skin as a biological membrane for the assessment of the *in vitro* permeation of ibuprofen. These were *Naja nivea* (Cape cobra), *Naja haje haje* (Egyptian cobra), *Naja melanoleuca* (forest cobra), and *Python sebae natalensis* (African rock python).

This study was designed to evaluate the barrier characteristics of four different species of shed snake skin and to investigate the differences, if any, between the dorsal and ventral skin sites, the scale and hinge regions and the orientation of the skin. Evaluation of an appropriate diffusion technique for the measurement of the amount of ibuprofen released from a topical formulation during *in vitro* testing was also assessed.

Ibuleve Gel (SA) was the formulation used in all the experiments. Of the two proprietary ibuprofen-containing topical formulations available in South Africa, Ibuleve Gel proved to be advantageous both economically and compositionally. Deep Relief Gel contains 3% levomenthol which may absorb in the same wavelength range as ibuprofen and therefore produce interference and was consequently not chosen for use.

Each of the four species of shed snake skins were assessed using the Franz and European Pharmacopoeia diffusion cells. The data obtained from the infinite dose experiments were plotted as the cumulative amount of drug per centimetre squared appearing in the receptor compartment as a function of time. Typically, these plots displayed an initial lag phase, during which no drug appeared in the receptor compartment, followed by a phase in which the cumulative amount transported was linearly related to time. This profile is consistent with steady-state Fickian diffusion through a membrane following a lag phase. Accordingly, the data was analysed using the appropriate equations for Fickian diffusion.

4.4.2 Results Obtained Using the Franz Diffusion Cell

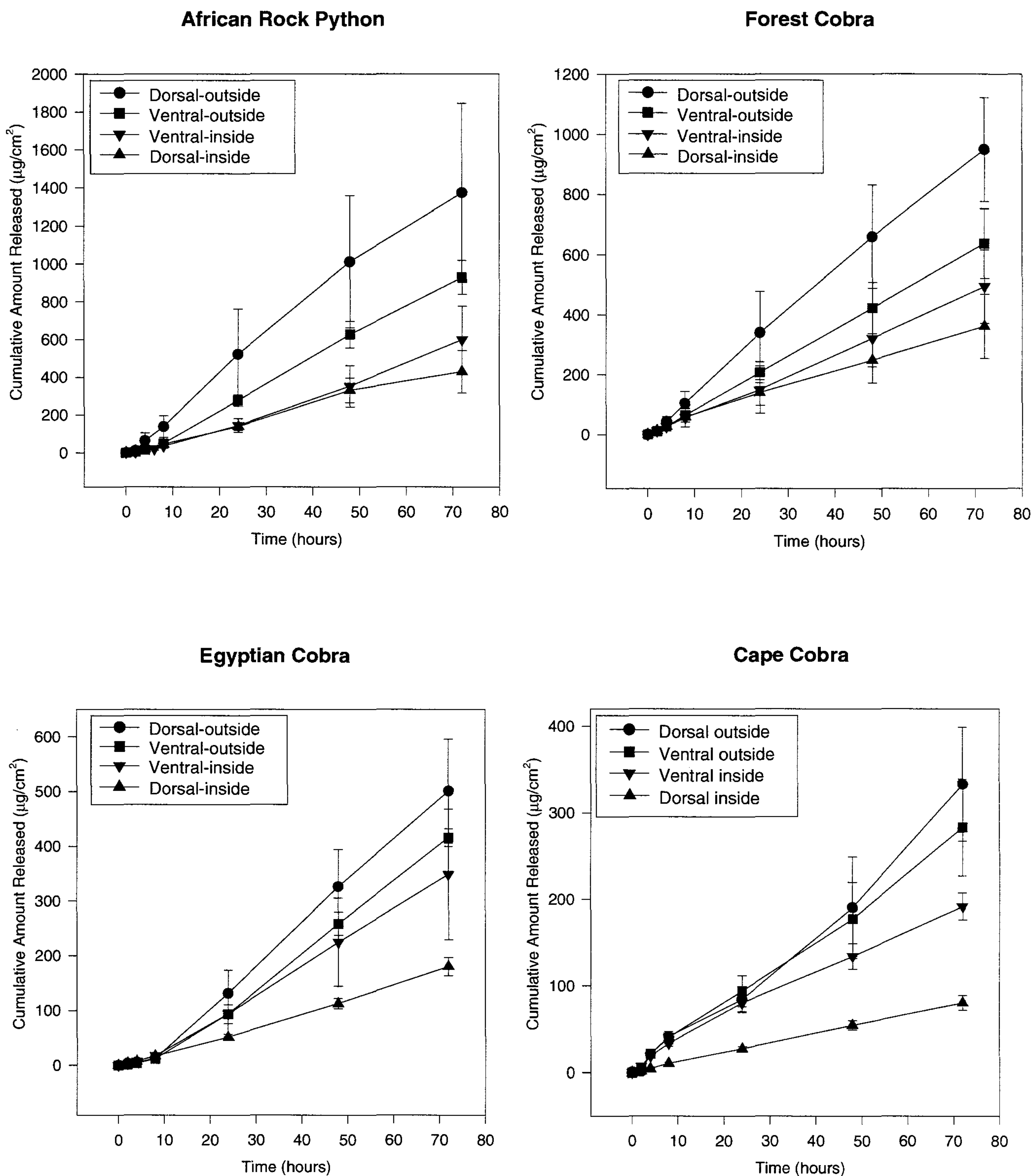


Figure 4.16 Ibuprofen diffusion profiles of four species of shed snake skin obtained using the Franz diffusion cell apparatus

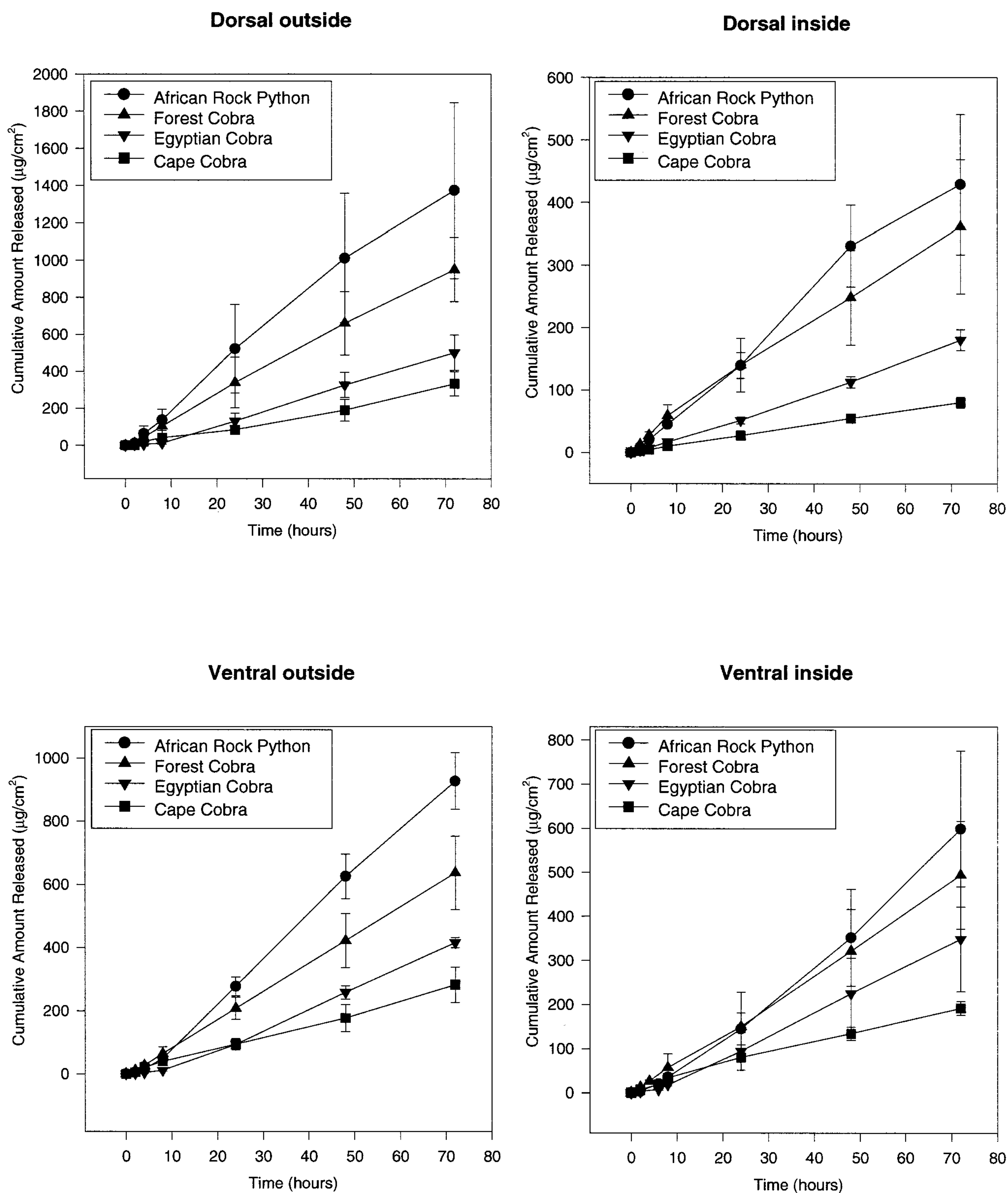


Figure 4.17 Ibuprofen diffusion profile of each shed snake skin orientation obtained using the Franz diffusion cell apparatus

It can be seen from figure 4.16 that all four species of snake produce shed skins which display different characteristics as diffusion membranes. In all four species it appears that the cumulative amount of ibuprofen released per square centimetre of membrane decreases in the following order: dorsal membrane with the outside surface in contact with the formulation, ventral with outside surface contact, ventral with inside surface contact and dorsal with inside surface contact. The apparent release constants (Table 4.5), obtained from regression analysis of the diffusion curves, display the same trend. The lag time is similar for all four species of snake regardless of the orientation of the membrane with regard to the formulation.

The dorsal membrane of the African rock python with outside surface contact is not significantly different at a 95% confidence interval to the ventral membrane with outside surface contact. However, the two outside orientations are significantly different to the two inside orientations. The ventral inside surface is not significantly different to the dorsal inside surface as a diffusion membrane. The apparent release constants range from 173.09 $\mu g/cm^2/h^{1/2}$ for the dorsal outside orientation to 54.20 $\mu g/cm^2/h^{1/2}$ for the dorsal inside orientation. The correlation coefficients are in the range of 0.8789 to 0.9466.

The dorsal membrane of the Forest cobra with outside surface contact is significantly different to the ventral membrane with outside surface contact. However, the ventral outside surface is not significantly different to the ventral inside surface which is itself not significantly different to the dorsal inside surface. The apparent release constants range from 116.83 $\mu g/cm^2/h^{1/2}$ for the dorsal outside orientation to 43.23 $\mu g/cm^2/h^{1/2}$ for the dorsal inside orientation. The correlation coefficients are in the range of 0.9248 to 0.9573.

The dorsal membrane of the Egyptian cobra with outside surface contact is not significantly different to the ventral membrane with outside surface contact which is itself not significantly different to the ventral inside surface. However, the ventral inside surface is significantly different to the dorsal inside surface. The apparent release constants range from 60.93 $\mu g/cm^2/h^{1/2}$ for the dorsal outside orientation to 21.29 $\mu g/cm^2/h^{1/2}$ for the dorsal inside orientation. The correlation coefficients are in the range of 0.8786 to 0.9120.

The dorsal membrane of the Cape cobra with outside surface contact is not significantly different to the ventral membrane with outside surface contact. However, the two outside orientations are significantly different to the two inside orientations. The ventral inside surface is significantly different to the dorsal inside surface as a diffusion membrane. The apparent release constants range from 37.93 $\mu g/cm^2/h^{1/2}$ for the dorsal outside orientation to 9.69 $\mu g/cm^2/h^{1/2}$ for the dorsal inside orientation. The correlation coefficients are in the range of 0.8949 to 0.9882.

It can be seen from figure 4.17 that all four species of snake produce shed skins which appear to display different characteristics as diffusion membranes according to the orientation of the membrane. In all four orientations the cumulative amount of ibuprofen released per square centimetre of membrane decreases in the following order: African rock python, forest cobra, Egyptian cobra and Cape cobra. The apparent release constants (Table 4.5), obtained from regression analysis of the diffusion curves, display the same trend.

The dorsal membrane of the African rock python with the outside surface in contact with the formulation is not significantly different to the dorsal outside membrane of the forest cobra. The dorsal outside membrane of the forest cobra is significantly different to the dorsal outside membrane of the Egyptian cobra, which is significantly different to the Cape cobra. The apparent release constants range from 173.09 $\mu g/cm^2/h^{1/2}$ for the dorsal outside orientation of the African rock python to 37.93 $\mu g/cm^2/h^{1/2}$ for the dorsal outside orientation of the Cape cobra.

The dorsal membrane of the African rock python with the inside surface in contact with the formulation is not significantly different to the dorsal inside membrane of the forest cobra. The dorsal inside membrane of the forest cobra is significantly different to the dorsal inside membrane of the Egyptian cobra, which is significantly different to the Cape cobra. The apparent release constants range from 54.20 $\mu g/cm^2/h^{1/2}$ for the dorsal inside orientation of the African rock python to 9.69 $\mu g/cm^2/h^{1/2}$ for the dorsal inside orientation of the Cape cobra.

All four species of snake produce shed skins orientated with the ventral outside surface in contact with the formulation, which are significantly different from each other. The

apparent release constants range from 114.13 $\mu g/cm^2/h^{1/2}$ for the ventral outside orientation of the African rock python to 33.13 $\mu g/cm^2/h^{1/2}$ for the ventral outside orientation of the Cape cobra.

The ventral membrane of the African rock python with the inside surface in contact with the formulation is not significantly different to the ventral inside membrane of the forest cobra which is not significantly different to the ventral inside surface of the Egyptian cobra. The ventral inside surface of the Egyptian cobra is significantly different to the ventral inside surface of the Cape cobra. The apparent release constants range from 90.40 $\mu g/cm^2/h^{1/2}$ for the ventral inside orientation of the African rock python to 25.5 $\mu g/cm^2/h^{1/2}$ for the ventral inside orientation of the Cape cobra.

SPECIES OF SNAKE	APPARENT RELEASE CONSTANT ($\mu g/cm^2/h^{1/2}$)	LAG TIME ($h^{1/2}$)	r^2
African rock python Dorsal outside	173.09	1.22	0.9466
African rock python Dorsal inside	54.20	1.23	0.9342
African rock python Ventral outside	114.13	1.42	0.9138
African rock python Ventral inside	90.40	1.52	0.8789
Forest cobra Dorsal outside	116.83	1.23	0.9422
Forest cobra Dorsal inside	43.23	1.01	0.9573
Forest cobra Ventral outside	76.96	1.25	0.9301
Forest cobra Ventral inside	48.67	1.21	0.9248
Egyptian cobra Dorsal outside	60.93	1.50	0.8940
Egyptian cobra Dorsal inside	21.29	1.30	0.9120
Egyptian cobra Ventral outside	49.69	1.54	0.8786
Egyptian cobra Ventral inside	42.05	1.43	0.9015
Cape cobra Dorsal outside	37.93	1.27	0.8949
Cape cobra Dorsal inside	9.69	1.17	0.9397
Cape cobra Ventral outside	33.13	1.12	0.9336
Cape cobra Ventral inside	25.50	1.39	0.9882

Table 4.5 The release constants, lag times and r^2 values for ibuprofen diffusing through four species of shed snake skin, using the Franz diffusion cell apparatus

4.4.3 Results Obtained Using the European Pharmacopoeia Diffusion Cell

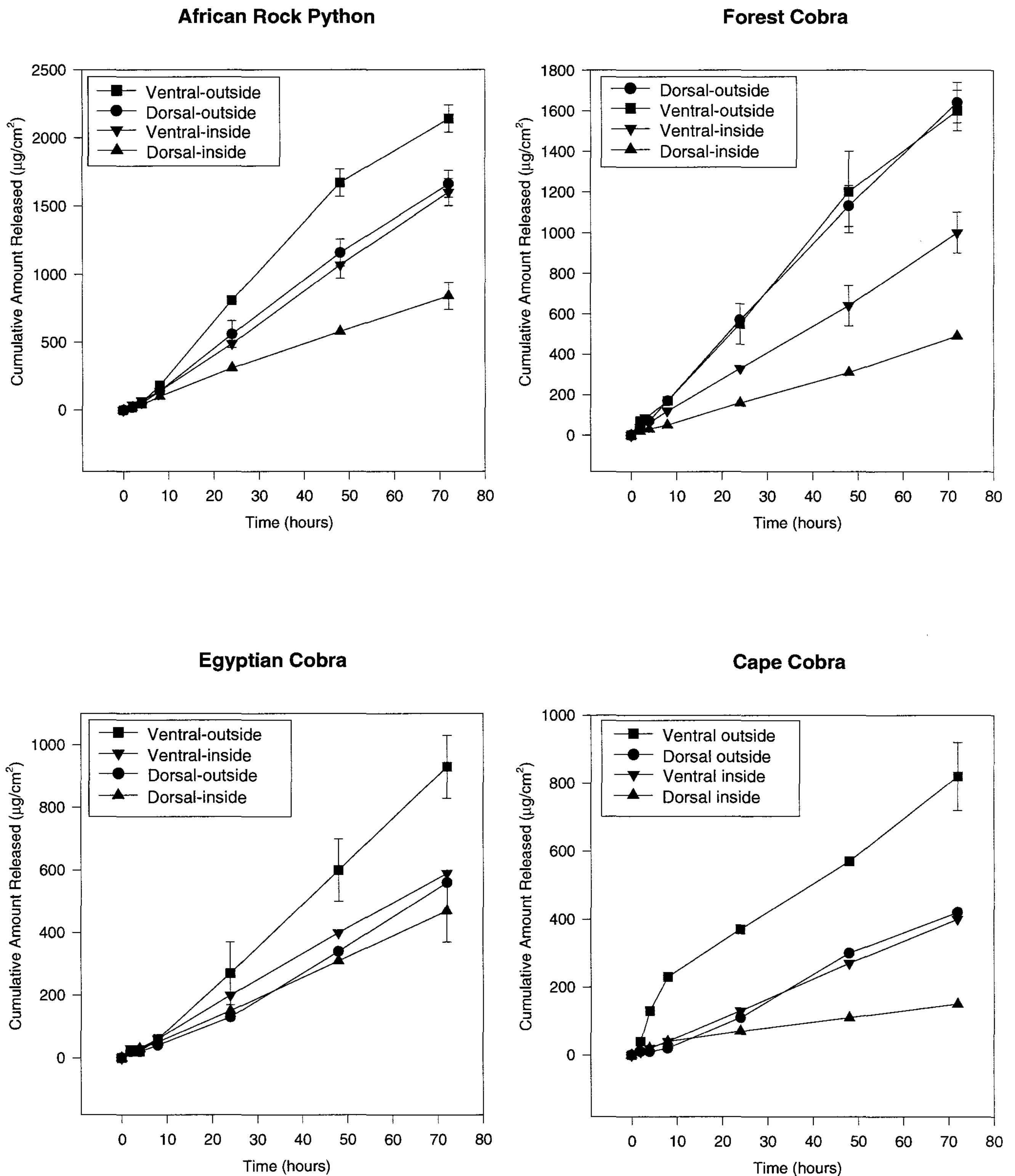


Figure 4.18 Ibuprofen diffusion profiles of four species of shed snake skin obtained using the European Pharmacopoeia diffusion cell apparatus

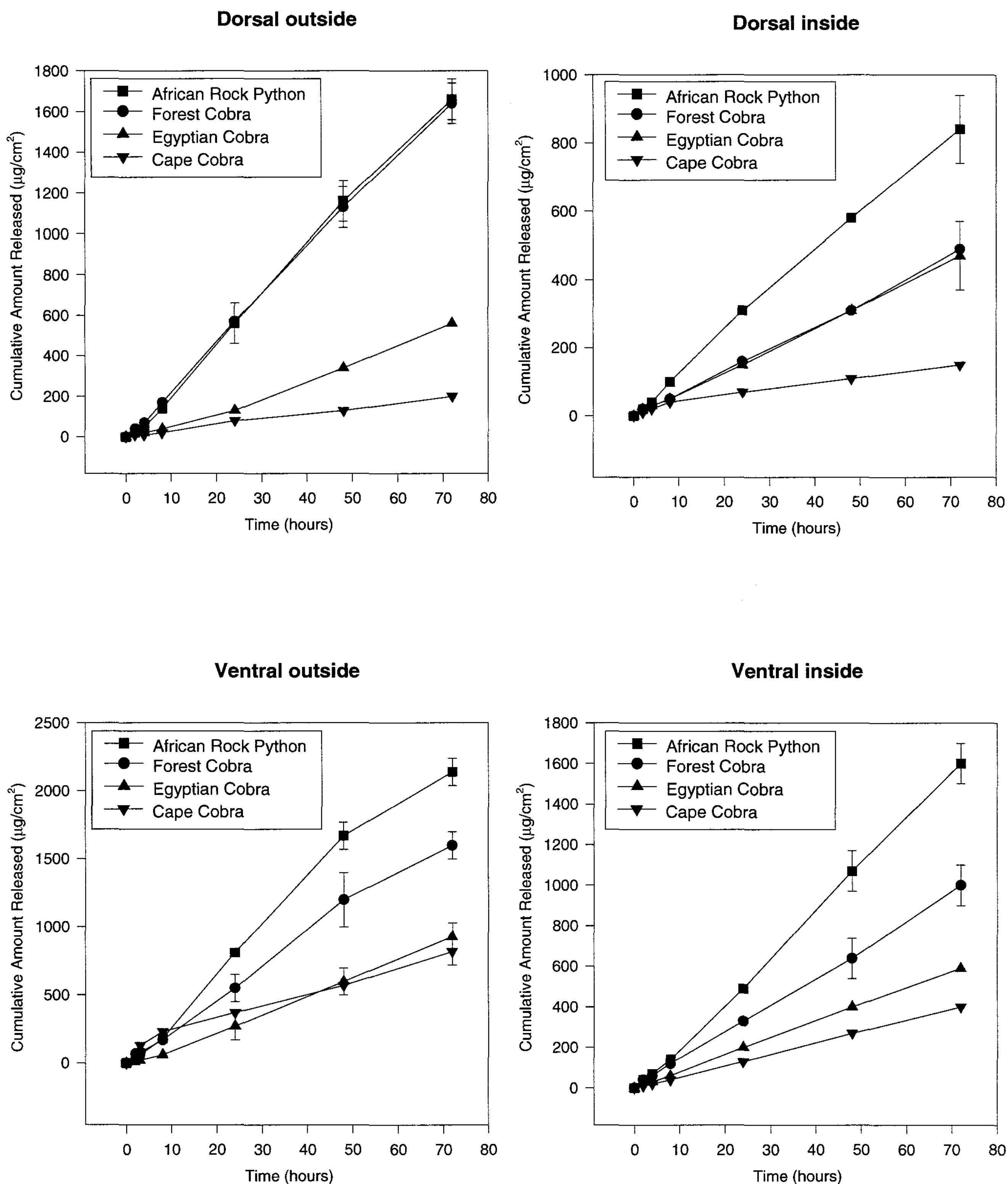


Figure 4.19 Ibuprofen diffusion profile of each shed snake skin orientation obtained using the European Pharmacopoeia diffusion cell apparatus

It can be seen from figure 4.18 that all four species of snake produce shed skins which display different characteristics as diffusion membranes. The cumulative amount of ibuprofen released per square centimetre of membrane at 72 hours does not decrease in the same order for each species of snake as was the case with the diffusion profiles obtained from the Franz diffusion cell apparatus. However, certain patterns of release have emerged. Most permeable is the ventral outside surface in contact with the formulation in three of the species investigated and least permeable is the dorsal inside membrane, which was found to be true for all four species. The apparent release constants (Table 4.6), obtained from regression analysis of the diffusion curves, display the same trends. The lag times are similar for all four species of snake regardless of the orientation of the membrane with the exception of the cape cobra dorsal inside and ventral outside orientations, which are roughly half those of the other reported values.

The ventral membrane of the African rock python with outside surface contact is significantly different at a 95% confidence interval to the dorsal membrane with outside surface contact. However, the dorsal outside and ventral inside orientations are not significantly different from one another, but are significantly different from the dorsal inside membrane in contact with the formulation. The apparent release constants range from 276.65 $\mu g/cm^2/h^{1/2}$ for the ventral outside orientation to 102.79 $\mu g/cm^2/h^{1/2}$ for the dorsal inside orientation. The correlation coefficients are in the range of 0.9226 to 0.9456.

The dorsal membrane of the Forest cobra with outside surface contact is not significantly different to the ventral membrane with outside surface contact. However, the ventral outside surface is significantly different to the ventral inside surface which is itself significantly different to the dorsal inside surface. The apparent release constants range from 202.09 $\mu g/cm^2/h^{1/2}$ for the dorsal outside orientation to 57.59 $\mu g/cm^2/h^{1/2}$ for the dorsal inside orientation. The correlation coefficients are in the range of 0.9261 to 0.9401.

The ventral membrane of the Egyptian cobra with outside surface contact is significantly different to the ventral membrane with inside surface contact which is itself significantly different to the dorsal outside surface. However, the dorsal outside surface is not significantly different to the dorsal inside surface. The apparent release constants range from 111.79 $\mu g/cm^2/h^{1/2}$ for the ventral outside orientation to 55.77 $\mu g/cm^2/h^{1/2}$ for the dorsal inside orientation. The correlation coefficients are in the range of 0.8844 to 0.9336.

All four orientations of the Cape cobra shed skins are significantly different from one another. The ventral membrane with the outside surface in contact with the formulation is most permeable followed by the dorsal outside and ventral outside orientations which display very similar diffusion profiles. The dorsal inside orientation is least permeable once again. The apparent release constants range from 95.92 $\mu g/cm^2/h^{1/2}$ for the ventral outside orientation to 17.95 $\mu g/cm^2/h^{1/2}$ for the dorsal inside orientation. The correlation coefficients are in the range of 0.9019 to 0.9840.

It can be seen from figure 4.19 that all four species of snake produce shed skins which appear to display different characteristics as diffusion membranes according to the orientation of the membrane. In all four orientations the cumulative amount of ibuprofen released per square centimetre of membrane decreases in the same order as that observed using the Franz diffusion cell apparatus: African rock python, forest cobra, Egyptian cobra and Cape cobra. The apparent release constants (Table 4.6), obtained from regression analysis of the diffusion curves, display the same trend.

The dorsal membrane of the African rock python with the outside surface in contact with the formulation is not significantly different to the dorsal outside membrane of the forest cobra. The dorsal outside membrane of the forest cobra is significantly different to the dorsal outside membrane of the Egyptian cobra, which is significantly different to the Cape cobra. The African rock python and forest cobra are very much more permeable than the Egyptian and Cape cobra. The apparent release constants range from 205.57 $\mu g/cm^2/h^{1/2}$ for the dorsal outside orientation of the African rock python to 51.89 $\mu g/cm^2/h^{1/2}$ for the dorsal outside orientation of the Cape cobra.

The dorsal membrane of the African rock python with the inside surface in contact with the formulation is significantly different to the dorsal inside membrane of the forest cobra. The dorsal inside membrane of the forest cobra is not significantly different to the dorsal inside membrane of the Egyptian cobra and in fact the two membranes display very similar permeabilities. The Cape cobra is significantly different to the Egyptian cobra. The apparent release constants range from 102.79 $\mu g/cm^2/h^{1/2}$ for the dorsal inside orientation of the African rock python to 17.95 $\mu g/cm^2/h^{1/2}$ for the dorsal inside orientation of the Cape cobra.

The ventral membrane of the African rock python with the outside surface in contact with the formulation is significantly different to the ventral outside membrane of the forest cobra which is significantly different to the ventral outside surface of the Egyptian cobra. The ventral outside surface of the Egyptian cobra is not significantly different to the ventral outside surface of the Cape cobra. The apparent release constants range from 276.65 $\mu g/cm^2/h^{1/2}$ for the ventral outside orientation of the African rock python to 95.92 $\mu g/cm^2/h^{1/2}$ for the ventral outside orientation of the Cape cobra.

All four species of snake produce shed skins orientated with the ventral inside surface in contact with the formulation, which are significantly different from each other. The apparent release constants range from 192.92 $\mu g/cm^2/h^{1/2}$ for the ventral inside orientation of the African rock python to 48.35 $\mu g/cm^2/h^{1/2}$ for the ventral inside orientation of the Cape cobra.

SPECIES OF SNAKE	APPARENT RELEASE CONSTANT ($\mu g/cm^2/h^{1/2}$)	LAG TIME ($h^{1/2}$)	r^2
African rock python Dorsal outside	205.57	1.30	0.9321
African rock python Dorsal inside	102.79	1.17	0.9456
African rock python Ventral outside	276.65	1.27	0.9407
African rock python Ventral inside	192.92	1.27	0.9226
Forest cobra Dorsal outside	202.09	1.30	0.9287
Forest cobra Dorsal inside	57.59	1.16	0.9261
Forest cobra Ventral outside	198.38	1.13	0.9401
Forest cobra Ventral inside	117.78	1.14	0.9312
Egyptian cobra Dorsal outside	64.87	1.35	0.8844
Egyptian cobra Dorsal inside	55.77	1.16	0.9286
Egyptian cobra Ventral outside	111.79	1.37	0.9098
Egyptian cobra Ventral inside	70.90	1.15	0.9336
Cape cobra Dorsal outside	51.89	1.40	0.9019
Cape cobra Dorsal inside	17.95	0.61	0.9840
Cape cobra Ventral outside	95.92	0.58	0.9773
Cape cobra Ventral inside	48.35	1.22	0.9310

Table 4.6 The release constants, lag times and r^2 values for ibuprofen diffusing through four species of shed snake skin, using the European Pharmacopoeia diffusion cell apparatus

4.4.4 Diffusion Through the Scale and Hinge Regions of Shed Snake Skin

Shed snake skin is composed of two very different regions. These comprise the scales which are separated by hinges. The scales are fairly rigid, whereas the hinge region is relatively elastic. Generally, the ventral scales are much larger than the dorsal scales resulting in a much larger hinge:scale ratio for dorsal skin than for ventral skin. Since permeation appears to be more facile through dorsal skin, it may imply that diffusion occurs mainly through the hinge region of the skin.

The *in vitro* diffusion experiments to investigate the differences, if any, between the scale and hinge regions of the skin were performed in modified Franz cells (88,169) with a reduced diffusional area ($0.44cm^2$) using African rock python ventral skin with the outside surface in contact with the formulation. The African rock python species was chosen because the ventral scales were the largest of all four species with the outside orientation giving a greater release rate than the inside orientation. Two experiments utilising five Franz cells each were performed. One experiment orientated the shed snake skin membrane so that only the scale region was in contact with the formulation and thus only the scale was used as the diffusional membrane. The second experiment orientated the shed snake skin membrane so that the hinge plus scale regions were in contact with the formulation. 100 mg of 5% ibuprofen gel (Ibuleve, South Africa) was placed in the donor compartment. The cells were maintained at 32°C (n=5) throughout the 72 hour period. The receptor fluid (0.2M phosphate buffer at pH 7.2) was analysed for ibuprofen at 2, 4, 8, 24, 48 and 72 hours by the hplc method described. The Franz cells were completely emptied at each sampling time and refilled with fresh receptor fluid. The different diffusion profiles observed for the scale and hinge regions are shown in figure 4.20.

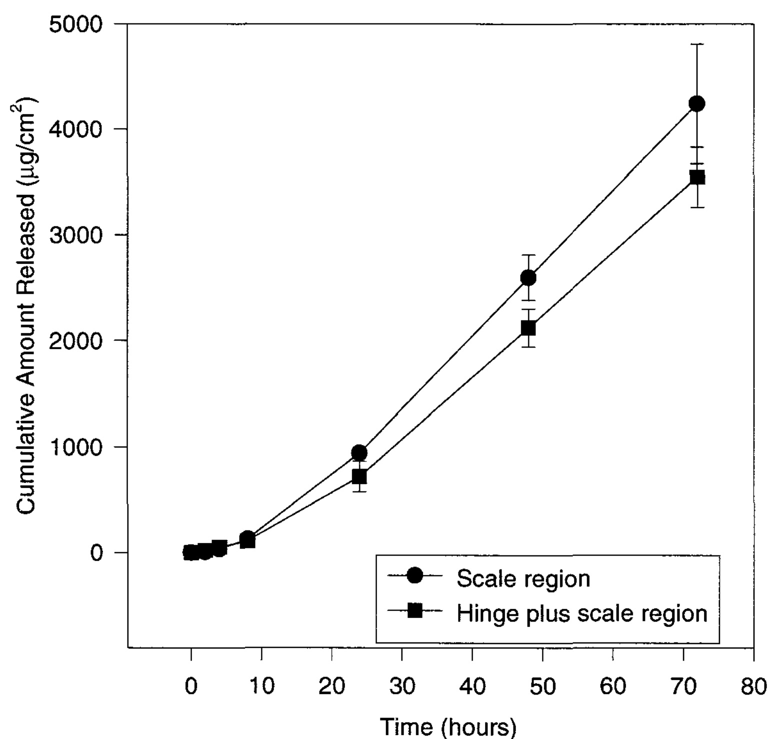


Figure 4.20 Ibuprofen diffusion profiles of the scale and hinge regions of shed snake skin

Surprisingly, the results indicate that the scale region of shed snake skin is slightly but significantly more permeable to the diffusion of ibuprofen than the hinge region of the skin. Despite the fact that permeation appears to be more facile through dorsal skin, it does not imply that diffusion occurs mainly through the hinge region of the skin.

4.4.5 Results of Scanning Electron Microscopy

The thickness of the skins (Table 4.7) shows some interesting trends. The ventral skin of all four species is always thicker than the dorsal skin. This is due to the fact that when the snake moves, the ventral surface is in contact with the ground. It would therefore have to be more robust than the dorsal surface. The hinge region was observed to be considerably thinner than the scale region. The elasticity of the hinge region is illustrated in Figure 4.23 which is contrasted with the rigidity of the scale region (Figure 4.22). It is interesting to compare and contrast the shed snake skin thicknesses to those of synthetic membranes. The 0.4μm filter membrane is closest in thickness, while the silicone diffusion membrane (Figure 4.21) is roughly five times thicker than the shed snake skins. The thicknesses of the scale area of the shed snake skins and the filter and silicone membranes were determined from the electron micrographs of the transverse sections.

MEMBRANE	DORSAL SCALE THICKNESS (μm)	VENTRAL SCALE THICKNESS (μm)
African rock python	23.8	33.1
Forest cobra	13.8	30.0
Egyptian cobra	15.6	21.3
Cape cobra	7.5	32.5
0.4μm Filter membrane	10.0	
Silastic silicone membrane	156.0	

Table 4.7 Membrane thickness

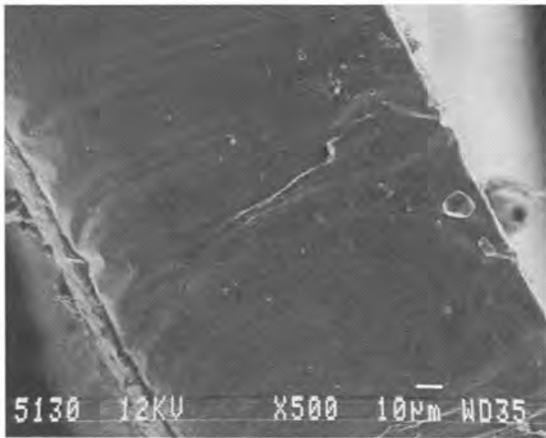


Figure 4.21 Transverse section of silastic silicone membrane

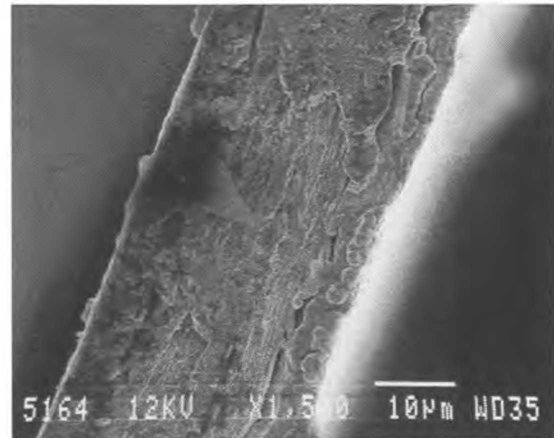


Figure 4.22 Transverse section of ventral forest cobra

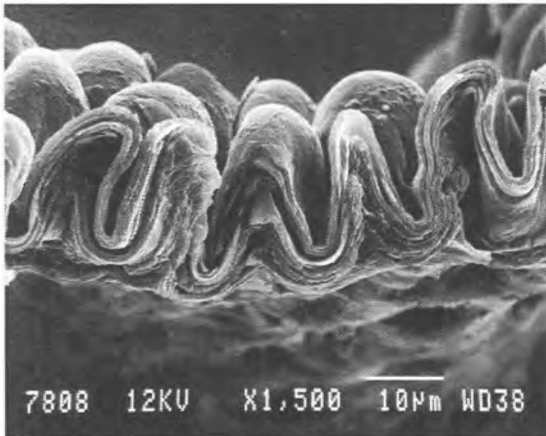


Figure 4.23 Transverse section of dorsal forest cobra hinge

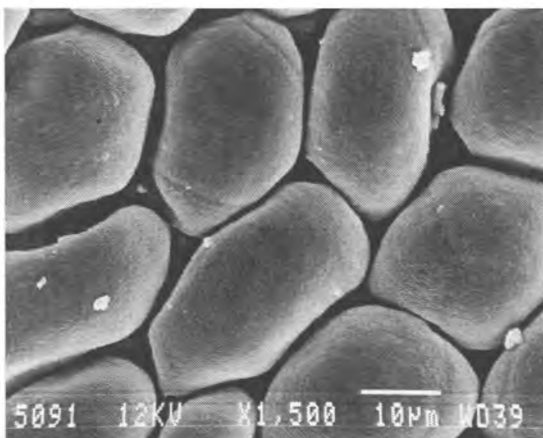


Figure 4.24 Dorsal Egyptian cobra hinge (inside surface)

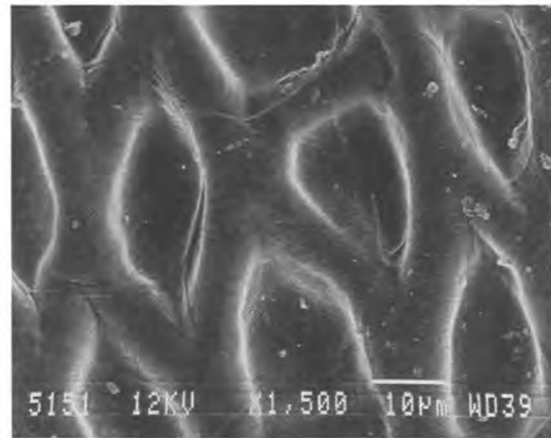
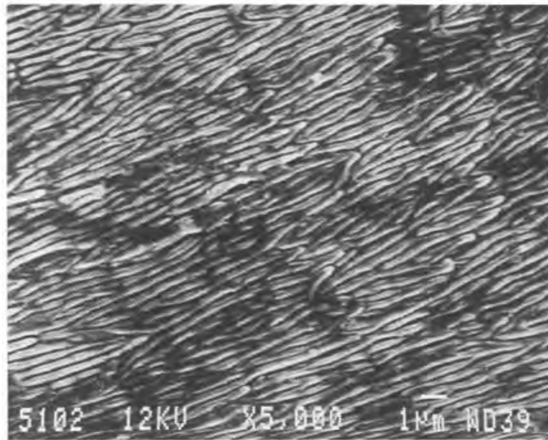


Figure 4.25 Dorsal Cape cobra hinge (outer surface)



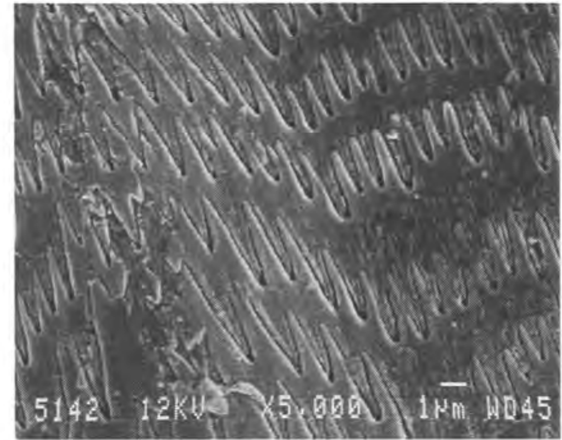
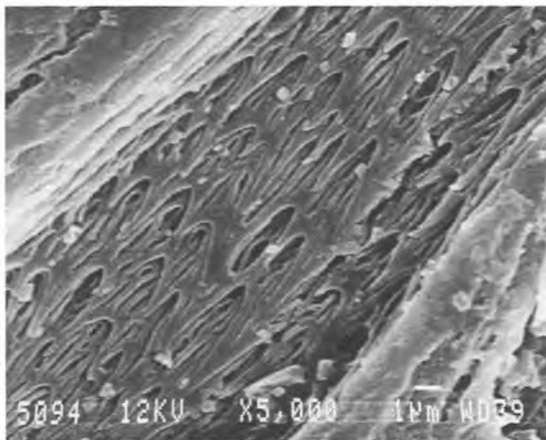


Figure 4.26 Dorsal African rock python scale (inside surface)

Figure 4.27 Dorsal forest cobra scale scale (inside surface)



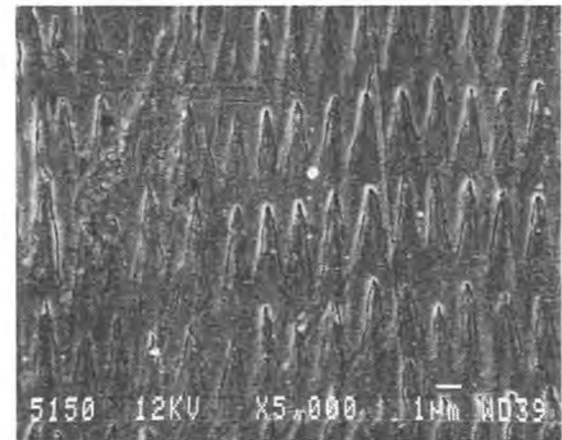


Figure 4.28 Dorsal Egyptian cobra scale (inside surface)

Figure 4.29 Dorsal Cape cobra scale (inside surface)

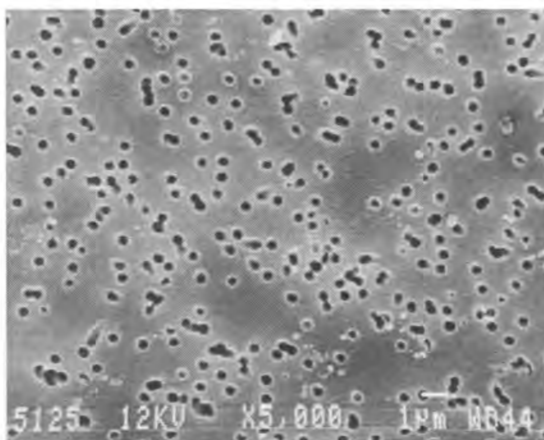


Figure 4.30 Surface region of 0.4 µm filter membrane

Typical electron micrographs of the surface topography are illustrated in Figures 4.24 to 4.30. The electron micrographs of the surface topography of the three cobra species showed the same type of structure for the hinge and scale regions respectively. The outside surface of the dorsal membrane and the ventral outside surface of the hinge areas were found to be similar in structure as were the dorsal inside and ventral inside hinge orientations. The dorsal outside and the ventral outside surfaces of the scale regions were found to be similar in structure as were the dorsal inside and ventral inside surfaces of the scales. The ventral scales are very much larger than the dorsal scales in all four snake species therefore there is a much larger hinge:scale ratio for dorsal skin than there is for ventral skin.

As the electron micrographs show, the surface topography of the African rock python skin is completely different to the cobra skins, however a pattern of similarity emerged between the different orientations of the hinge areas of the African rock python. The dorsal outside and the ventral outside surfaces of the hinge areas were found to be similar in structure as were the dorsal inside and ventral inside hinge surfaces. The dorsal outside and inside surfaces were found to be similar, while the ventral outside and inside surfaces differed not only from one another but from all the other surfaces as well.

High magnification electron microscopic examination of larger areas of the dorsal and ventral scale and hinge areas of the inner and outer surfaces of the skins of all four species indicated that there are no pores present. This may be contrasted with the filter membrane of the smallest pore size used in this study (Figure 4.30).

4.4.6 Discussion

The electron micrographs of the transverse sections of the shed snake skin show that no correlation was seen between the thickness of the ventral and dorsal skins respectively of all four species and the diffusion of ibuprofen through these membranes. Another deviation from the correlation between thickness and release of ibuprofen is that the thickness of both the dorsal and ventral sections of the African rock python skin is greater than the thickest cobra skin yet the African rock python species produces the greatest release profile of all four species. The differences in the skin composition may therefore be a reason for the different diffusion characteristics displayed by the shed snake skin membranes. The chemical composition of the different shed snake skins was not elucidated in this study.

The difference in the structure of the snake skins as illustrated by the electron micrographs of the surface topography may also be a reason for the different diffusion characteristics. The three cobra species showed similar diffusion characteristics that were different from those of the African rock python. The dorsal and ventral outside surfaces of both the hinge and scale regions of the cobra species show similar structures. These regions produce a greater rate of diffusion of ibuprofen when using both the Franz and European Pharmacopoeia diffusion cells compared to the dorsal and ventral inside surfaces of both the hinge and scale regions. The inside surfaces of these regions show similar structures but produce a lower rate of diffusion.

Permeation through shed snake skin differs depending on the orientation of the membrane with respect to the formulation and the receptor fluid. This is a difficult aspect of the results to explain but it might be that the outside surface of the membrane in contact with the formulation presents less of a barrier to the diffusing drug molecules than the inside surface in contact with the formulation. The differences in release may be explained by the structure of shed snake skin which consists of three distinctive layers (113). These are the beta-keratin-rich outermost beta layer, alpha-keratin- and lipid-rich intermediate mesos layer, and alpha-keratin-rich innermost alpha layer (149). Further, the mesos layer shows three to five layers of multilayer structure with cornified cells surrounded by intercellular lipids, which is similar to human stratum corneum. This mesos layer is also a major depot of lipids, and the mesos layer and alpha layer are considered to be the main barrier to water and thus ibuprofen penetration through the skin (172,180-186). However, when ibuprofen

diffuses through the outside surface of the membrane it diffuses through the beta and alpha protein layers as well as the mesos lipid layer before coming into contact with the inside membrane surface which appears to present no additional barrier. The differences in release may therefore be attributed to what is in contact with the membrane depending on its orientation *i.e.*, the outside surface in contact with the formulation and the inside surface in contact with the receptor fluid and *vice versa*. It is therefore important that if shed snake skin is used as a membrane, the orientation should be identical for each sample.

Shed snake skin is composed of two very different regions. These comprise the scales which are separated by hinges. The scales are fairly rigid, whereas the hinge region is relatively elastic. The scales on the dorsal surface are much smaller than the scales on the ventral surface and, in addition, the size of the scales varies considerably between species. The investigation into the difference in the diffusion of ibuprofen through the scale and hinge regions yielded interesting and unexpected results. Since permeation appears to be more facile through dorsal skin when investigated using the European Pharmacopoeia cell apparatus, it was assumed that diffusion occurred mainly through the hinge region of the skin and that a higher ratio of hinge to scale area existed for dorsal skin (111). These assumptions were found to be incorrect and in fact the opposite was true. Firstly, due to the placement of the ventral skin over the diffusional area of the Franz cell with the extended hinge occupying the central area and the adjacent scales covering the remaining diffusional area a hinge:scale area of approximately 1:1 resulted. The dorsal scales are very much smaller than the ventral scales therefore a smaller ratio of hinge:scale was found for dorsal skin than for ventral skin when placing the membrane over the diffusional area of the Franz cell. Secondly, it was found that the scale region of shed snake skin is slightly but significantly more permeable to the diffusion of ibuprofen than the hinge region of the skin. A possible explanation may be that the relative fragility of the hinge region as compared to the scale region necessitates a greater barrier to water penetration and thus ibuprofen penetration provided by a modified mesos and alpha layer.

The most permeable orientation of shed snake skin differs when the membrane is assessed using the Franz and European Pharmacopoeia diffusion cells. When assessed using the Franz diffusion cell apparatus the most permeable orientation is the dorsal outside surface in contact with the formulation as opposed to the ventral outside surface when assessed using the European Pharmacopoeia diffusion cells. This may be explained by the hinge to

scale area ratio. The cells of the European Pharmacopoeia have a surface area from which the formulation can diffuse that is five times larger than the corresponding surface area of the Franz cells. Due to the placement of the ventral skin over the diffusional area of the Franz cell with the extended hinge occupying the central area and the adjacent scales covering the remaining diffusional area a hinge:scale area of approximately 1:1 resulted. This may be contrasted with a hinge:scale area of approximately 0.5:1 when using the European Pharmacopoeia cell. It was shown that diffusion of ibuprofen occurs mainly through the scale region of the skin, more scale to hinge area of ventral skin is exposed to the formulation in the European Pharmacopoeia cells thus resulting in greater release.

The dorsal scales are very much smaller than the ventral scales therefore a smaller ratio of hinge:scale was found for dorsal skin than for ventral skin when placing the membrane over the diffusional area of the Franz cell. The dorsal outside orientation was therefore found to be more permeable than the ventral outside orientation when assessed using the Franz diffusion apparatus.

The higher permeability of the African rock python skin may be due to a reduction in integrity because of the rough manner in which this snake sheds its skin by rubbing its body against rocks and thus removing the skin in fragments (111). This is however unlikely as the replicate determinations showed good reproducibility.

High magnification electron microscopic examination of larger areas of all four species indicated that there are no pores present. The integrity of shed snake skin, as verified by electron microscopy, therefore indicates that it behaves as a diffusion membrane. The choice of the species of snake that produces shed skins that most closely mimic diffusion through a synthetic silicone diffusing membrane depends on the diffusion apparatus to be used in the experiment. When using the Franz diffusion cell it appears that the best shed snake skin of the four species investigated in terms of diffusion profile, apparent release constant, lag time and correlation coefficient is the African rock python orientated with the dorsal outside surface in contact with the formulation. When using the European Pharmacopoeia diffusion apparatus it appears that the African rock python orientated with the ventral outside surface in contact with the formulation most closely mimics a synthetic silicone diffusing membrane.

4.4.7 Conclusion

Previous reports have described shed snake skin as a 'model' membrane, *i.e.* a membrane which shows similar permeability to human stratum corneum (103,104,108). The results reported here show clearly that, for ibuprofen, the four species of snake produce shed skin with completely different diffusion characteristics when all other conditions are identical. It may well be that there is one particular species of snake which produces shed skin of identical permeability to human stratum corneum, but to describe shed snake skin in general as a model membrane seems incorrect. It is therefore important that if shed snake skin is used as a membrane, the species, skin site and orientation should be reported.

4.4.8 Comparison of the Diffusion Cells

4.4.8.1 *Python sebae natalensis* (African Rock Python)

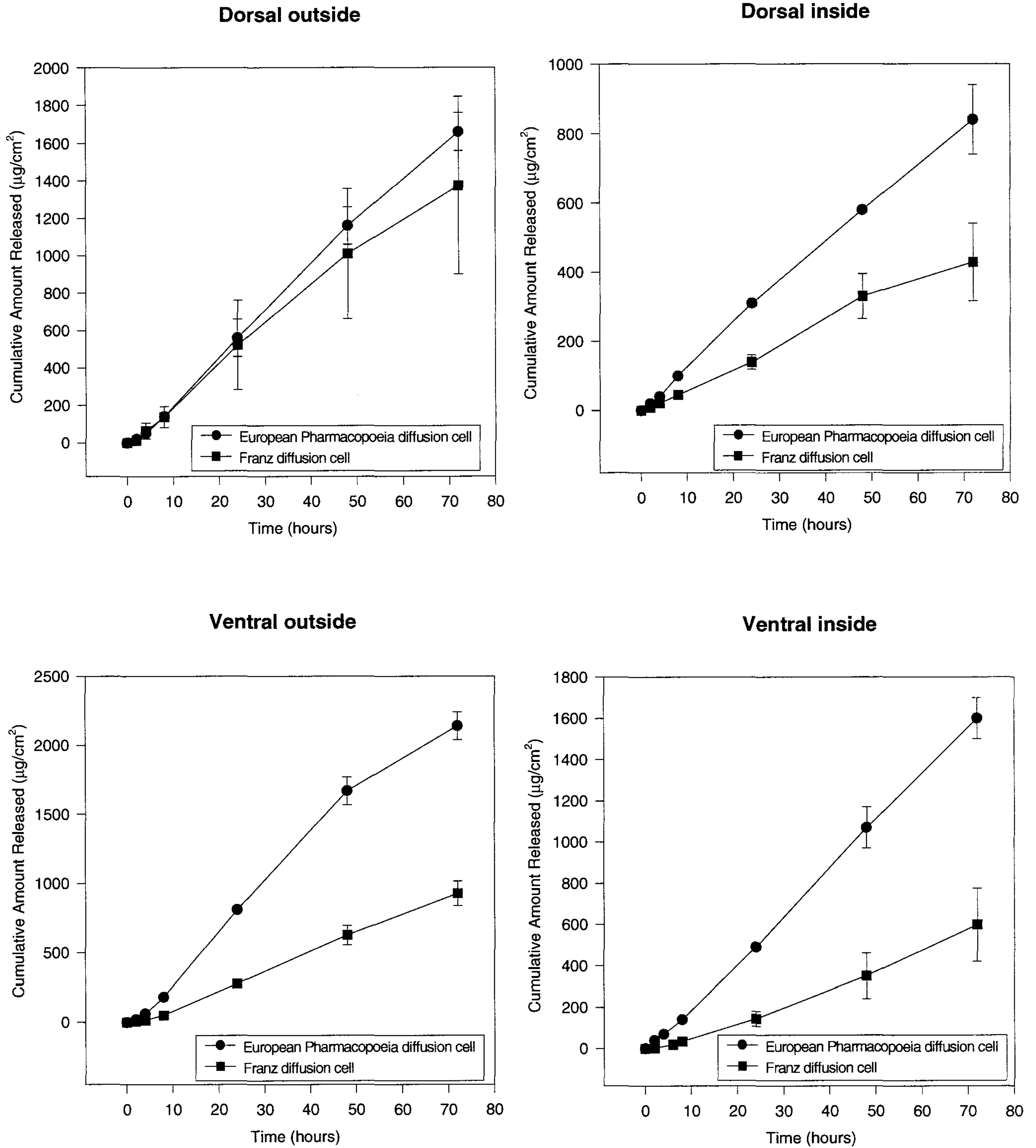


Figure 4.31 Ibuprofen diffusion profiles of the African rock python

4.4.8.2 *Naja melanoleuca* (Forest Cobra)

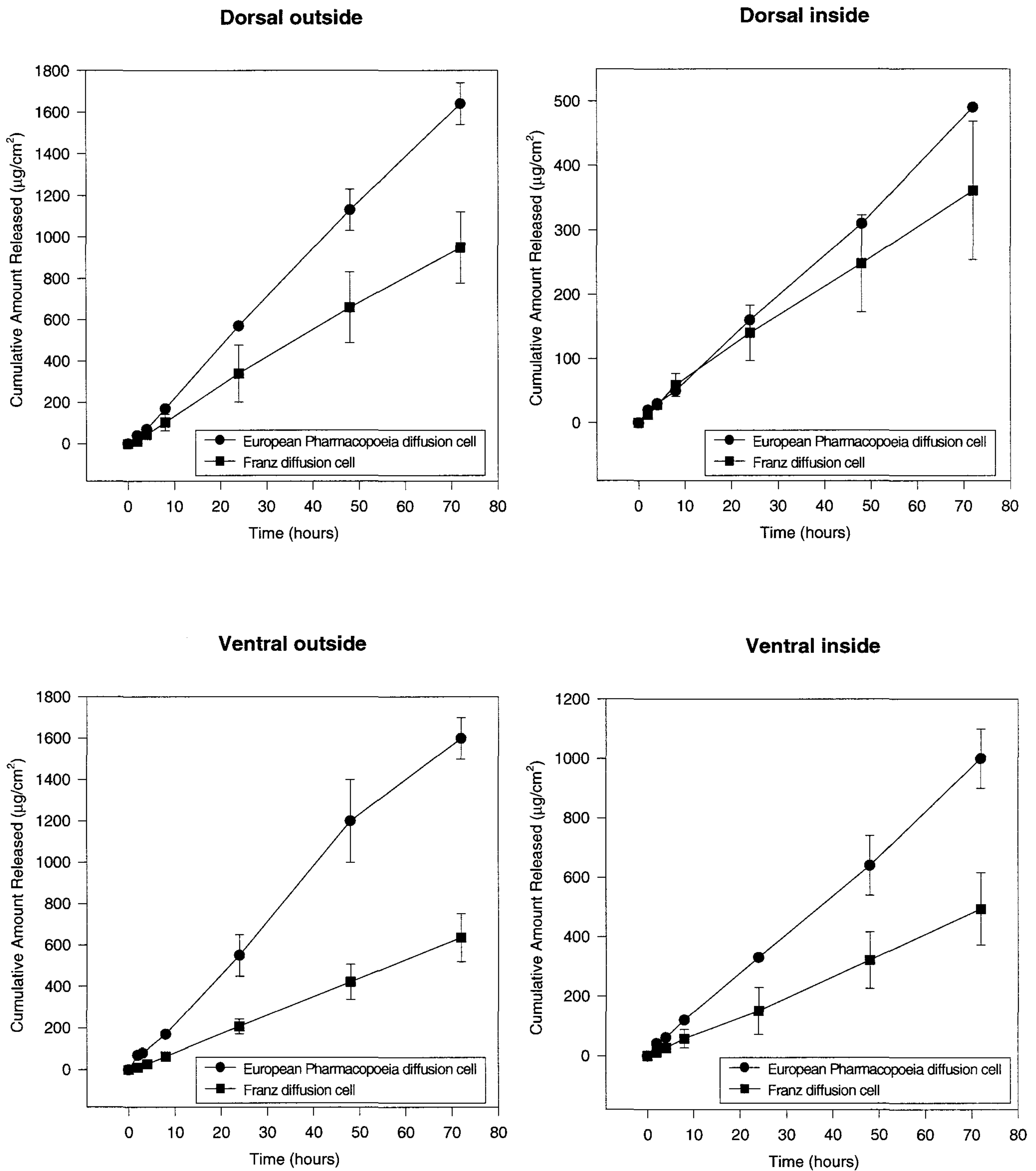


Figure 4.32 Ibuprofen diffusion profiles of the forest cobra

4.4.8.3 *Naja haje haje* (Egyptian Cobra)

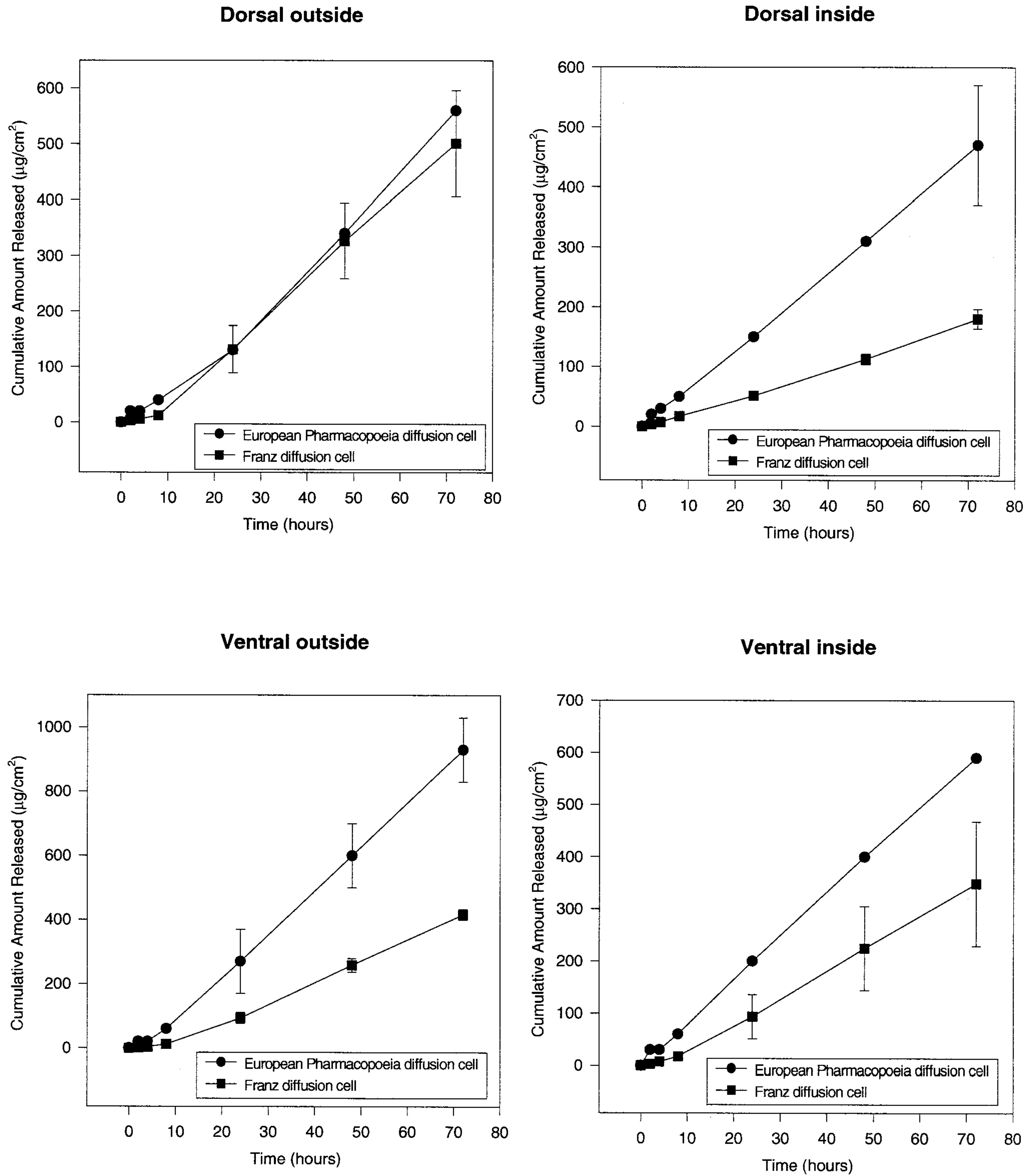


Figure 4.33 Ibuprofen diffusion profiles of the Egyptian cobra

4.4.8.4 *Naja nivea* (Cape Cobra)

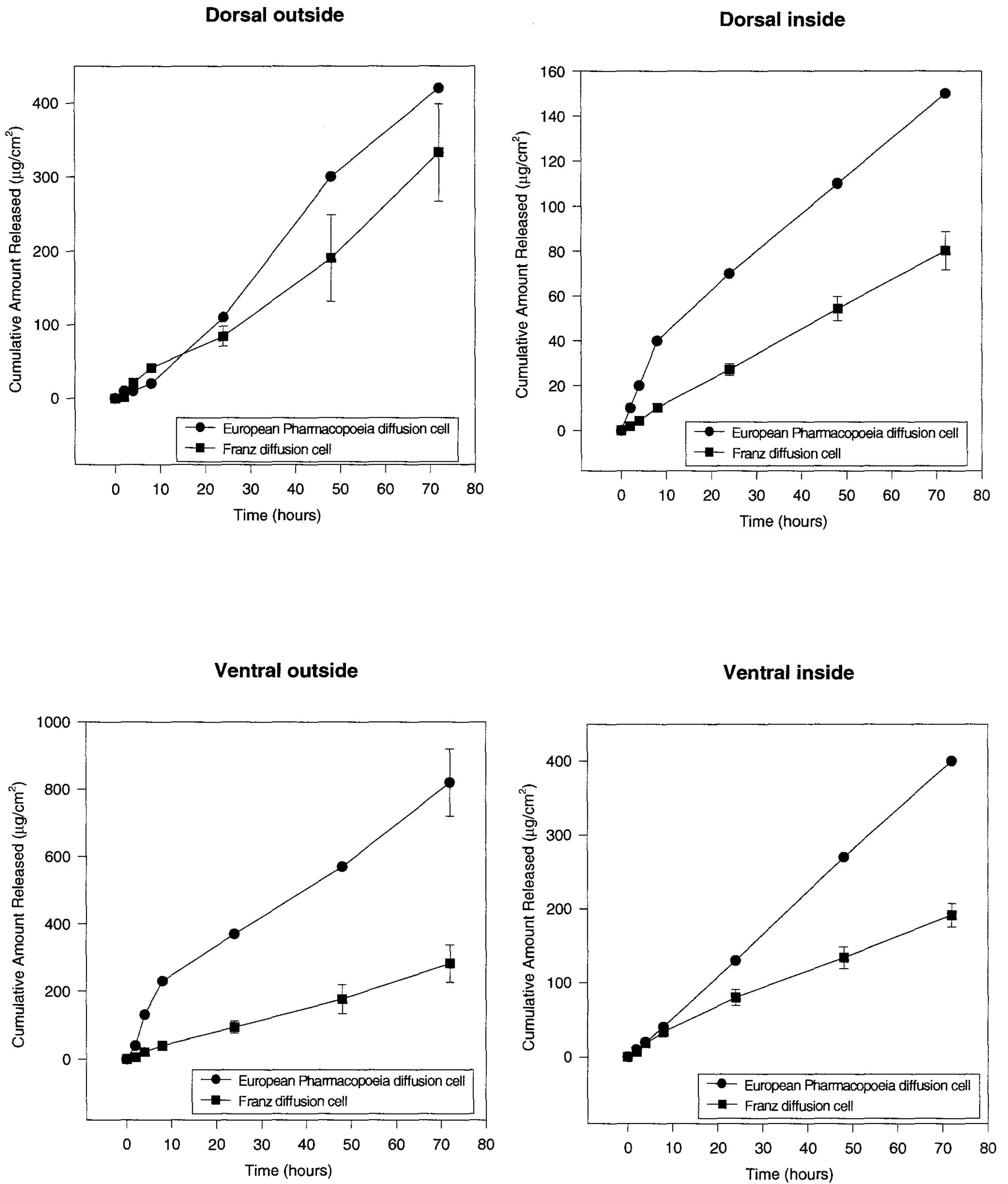


Figure 4.34 Ibuprofen diffusion profiles of the Cape cobra

4.4.8.5 Discussion

The diffusion profiles obtained for each orientation of shed snake skin, utilising the Franz and European Pharmacopoeia diffusion cells, were compared in an attempt to demonstrate an appropriate diffusion technique for the measurement of the amount of ibuprofen diffusing across a biological membrane during *in vitro* testing.

A statistically significant difference, at a 95% confidence interval, was seen between the diffusion profiles obtained using the Franz and the European Pharmacopoeia diffusion cells for all the shed snake skin orientations with the exception of the dorsal membrane of the African rock python and Egyptian cobra orientated such that the outside surface is in contact with the formulation. The percentage standard deviation using the Franz diffusion cells was greater than the European Pharmacopoeia diffusion cells. The standard deviation of the cumulative amount of ibuprofen released per centimetre squared was generally greater using the Franz diffusion cells. In all cases the use of the European Pharmacopoeia diffusion apparatus resulted in a greater apparent release constant. The possible explanations for the differences observed between the two diffusion systems have previously been discussed.

Since it is possible that most diffusion occurs through the scale region of a shed snake skin the relatively large area of the European Pharmacopoeia diffusion cell may reduce the influence the hinge:scale area ratio may have on the diffusion of a drug molecule and may therefore be the apparatus of choice when using shed snake skin as a biological membrane.

4.4.8.6 Conclusion

The European Pharmacopoeia diffusion apparatus was judged to be the better of the two diffusion techniques assessed for the measurement of the amount of ibuprofen diffusing across the ventral outside orientation of shed skin during *in vitro* testing. The European Pharmacopoeia diffusion apparatus once again shows better reproducibility. The Franz diffusion apparatus was judged to be the better for the measurement of the amount of ibuprofen diffusing across the dorsal outside orientation of shed skin during *in vitro* testing. However, the choice of this diffusion apparatus must be weighed against the relatively poor reproducibility as compared with the European Pharmacopoeia diffusion apparatus.

APPENDIX I

EXACT DRUG CONTENT OF THE FORMULATIONS AND STATISTICAL EVALUATION

PRERARATION	ACTUAL PERCENTAGE OF ACTIVE (n=3)	% RSD	PERCENT DEVIATION
Deep Relief Gel (SA)	4,36	1.86	12.8
Ibuleve Gel (SA)	4,31	1.98	13.8
Deep Relief Gel (UK)	4,31	1.75	13.8
Ibuleve Gel (UK)	4,38	1.73	12.4
Ibuleve Mousse (UK)	4,38	2.08	12.4
Nurofen Gel (UK)	4,34	1.59	13.2
Proflex Cream (UK)	4,54	2.22	9.2
Radian B Gel (UK)	4,42	1.92	11.6
Ralgex Gel (UK)	4,32	1.83	13.6
Cliptol Gel (FR)	4,41	1.49	11.8
Dolgit Cream (FR)	4,32	2.41	13.6
Ibutop Gel (FR)	4,34	1.73	13.2
Intralgis Gel (FR)	4,51	1.97	9.8
Syntofene Gel (FR)	4,34	1.48	13.2
Tiburon Gel (FR)	4,48	1.79	10.4

One-way analysis of variance (Tukey test)

Source of variation	Degrees of freedom	Sum of squares	Mean square
Treatments (between columns)	14	5.772E+11	4.123E+10
Residuals (within columns)	30	5.101E+11	1.700E+10
Total	44	1.087E+12	

$F = 2.425$

APPENDIX II

COMPOSITION OF FORMULATIONS

PREPARATION (5% m/m)	MANUFACTURED	COMPOSITION
Ibuleve Gel	South Africa	Industrial methylated spirit (BP) Carbomer Propylene glycol (BP) Diethylamine Purified water
Deep Relief Gel	United Kingdom	Levomenthol 3% m/m Carbomer Propylene glycol Di-isopropanolamine Ethanol Purified water
Ibuleve Gel	United Kingdom	Industrial methylated spirit (BP) Carbomer Propylene glycol (BP) Diethylamine Purified water
Ibuleve Mousse	United Kingdom	Carbomer Propylene glycol (BP) Phenoxyethanol (BP) Diethylamine Butane 40 Purified water
Nurofen Gel	United Kingdom	Ethanol Propylene glycol Di-isopropanolamine Carbomer Purified water
Proflex Cream	United Kingdom	Coconut oil Glyceryl stearate Polyoxyethylene fatty acid Ester Propylene glycol Xanthan gum Parabens Purified water
Radian B Gel	United Kingdom	Hydroxyethylcellulose Sodium hydroxide Benzyl alcohol Isopropyl alcohol Purified water

Ralgex Gel	United Kingdom	Isopropyl alcohol Solketal Poloxamer 407 Miglyol 812 Lavender oil Neroli oil/orange tree flower oil Purified water
Cliptol Gel	France	Levomenthol 3% Proplylene glycol Di-isopropanalamine Carbomere 980 96% ethanol Purified water
Dolgit Cream	France	Triglycerides of average carbon chain length (miglyol 812) Mixture of glyceryl stearates and monostearates Polyoxyethylene esters (Arlacel 165) Polyoxyethylene esters of fatty acids (Arlatone 983 s) Propylene glycol Xanthane gum (Keltrol F) Lavender oil Neroli oil Purified water
Ibutop Gel	France	Isopropyl alcohol Glycerol isopropylidene (Solketal) Poloxamere 407 (Pluronic F127) Neutral oil (Miglyol 812) Lavender essence Artificial essence of Orange tree flower Purified water
Intralgis Gel	France	Isopropyl alcohol Hydroxyethylcellulose Sodium hydroxide Benzyl alcohol Purified water

Syntofene Gel	France	Isopropyl alcohol Hydroxyethylcellulose Sodium hydroxide Benzyl alcohol Purified water
Tiburon Gel	France	Isopropyl alcohol Hydroxyethylcellulose Sodium hydroxide Benzyl alcohol Purified water

APPENDIX III

RAW DATA USED TO ASSESS THE PROPRIETARY IBUPROFEN-CONTAINING TOPICAL PREPARATIONS FROM THREE COUNTRIES

Franz diffusion cell apparatus:
Total amount liberated in µg/cm²
( )=standard deviation
n=5
Silastos silicone membrane

Time	Dolgit cream FR	Proflex cream UK	Ralgex gel UK	Ibutop gel FR	Ibuleve gel UK	Deep Relief gel SA	Ibuleve gel SA	Nurofen gel UK
0	0	0	0	0	0	0	0	0
2	201 (29)	170 (23)	451 (19)	447 (40)	157 (18)	167 (17)	128 (25)	132 (17)
4	374 (33)	320 (38)	700 (18)	681 (57)	260 (30)	296 (25)	201 (32)	239 (24)
8	684 (52)	594 (67)	1061 (19)	1044 (131)	387 (52)	455 (35)	311 (82)	380 (36)
24	1550 (82)	1460 (89)	1860 (48)	1816 (162)	951 (200)	650 (58)	608 (181)	734 (178)
48	2172 (49)	2138 (55)	2260 (76)	2201 (119)	1643 (160)	1126 (230)	1157 (378)	1141 (380)
72	2450 (58)	2377 (53)	2383 (82)	2295 (105)	1889 (151)	1537 (491)	1437 (355)	1399 (565)

Time	Deep Relief gel UK	Cliptol gel FR	Ibuleve Mousse UK	Tiburon gel FR	Intralgis gel FR	Radian B gel UK	Syntofene gel FR
0	0	0	0	0	0	0	0
2	182 (17)	222 (27)	124 (14)	24 (3)	127 (6)	50 (5)	76 (11)
4	338 (35)	380 (37)	202 (18)	44 (5)	177 (6)	83 (6)	104 (9)
8	499 (45)	543 (37)	298 (24)	68 (6)	207 (7)	114 (7)	130 (7)
24	718 (65)	763 (38)	487 (56)	112 (18)	231 (11)	146 (5)	155 (5)
48	871 (85)	1066 (114)	745 (92)	208 (81)	267 (14)	204 (37)	191 (26)
72	987 (137)	1247 (145)	986 (164)	315 (125)	306 (18)	267 (59)	284 (65)

European Pharmacopoeia diffusion cell apparatus:
Total amount liberated in µg/cm^2
( )=standard deviation
n=5
Silastos silicone membrane

Time	Dolgit cream FR	Proflex cream UK	Ralgex gel UK	Ibutop gel FR	Ibuleve gel UK	Deep Relief gel SA	Ibuleve gel SA	Nurofen gel UK
0	0	0	0	0	0	0	0	0
2	570 (0)	460 (0)	590 (0)	690 (100)	680 (100)	370 (0)	360 (0)	450 (0)
4	780 (0)	640 (100)	840 (0)	970 (100)	940 (0)	530 (0)	540 (100)	650 (100)
8	1110 (100)	930 (100)	1210 (0)	1340 (100)	1210 (0)	700 (100)	760 (100)	920 (0)
24	2060 (200)	1850 (300)	1970 (100)	2090 (100)	1660 (100)	1060 (100)	1250 (0)	1330 (100)
48	2810 (200)	2600 (100)	2350 (200)	2410 (100)	2120 (200)	1360 (100)	1690 (100)	1700 (0)
72	2960 (200)	2930 (100)	2480 (200)	2510 (100)	2600 (100)	1580 (100)	2080 (200)	1920 (0)

Time	Deep Relief gel UK	Cliptol gel FR	Ibuleve Mousse UK	Tiburon gel FR	Intralgis gel FR	Radian B gel UK	Syntofene gel FR
0	0	0	0	0	0	0	0
2	530 (0)	600 (0)	230 (0)	290 (0)	350 (0)	60 (0)	250 (0)
4	680 (100)	810 (100)	300 (100)	340 (0)	350 (0)	80 (0)	250 (0)
8	900 (0)	1060 (100)	410 (200)	340 (0)	370 (0)	110 (0)	290 (0)
24	1280 (100)	1530 (100)	810 (300)	430 (0)	510 (100)	210 (0)	380 (0)
48	1640 (0)	1870 (100)	1200 (300)	490 (0)	680 (100)	350 (100)	470 (100)
72	1860 (100)	2150 (100)	1620 (400)	590 (100)	790 (200)	460 (100)	550 (100)

Franz diffusion cell apparatus:
Diffusion rate in µg/h
( )=standard deviation
n=5
Silastos silicone membrane

Time	Dolgit cream FR	Proflex cream UK	Ralgex gel UK	Ibutop gel FR	Ibuleve gel UK	Deep Relief gel SA	Ibuleve gel SA	Nurofen gel UK
1	165 (23)	140 (19)	371 (15)	369 (33)	129 (15)	137 (14)	105 (20)	108 (14)
3	143 (7)	124 (12)	206 (8)	192 (15)	84 (11)	105 (9)	60 (7)	88 (7)
6	127 (9)	112 (12)	148 (6)	150 (26)	52 (10)	65 (5)	45 (22)	58 (6)
16	89 (5)	89 (4)	82 (4)	80 (5)	58 (20)	20 (3)	30 (16)	36 (20)
36	43 (4)	47 (5)	27 (5)	26 (6)	47 (9)	32 (17)	37 (16)	27 (18)
60	19 (4)	16 (6)	8 (2)	7 (2)	16 (4)	28 (18)	19 (7)	17 (14)

Time	Deep Relief gel UK	Cliptol gel FR	Ibuleve Mousse UK	Tiburon gel FR	Intralgis gel FR	Radian B gel UK	Syntofene gel FR
1	150 (14)	183 (22)	102 (11)	19 (3)	105 (5)	42 (4)	63 (9)
3	128 (20)	130 (9)	65 (4)	17 (2)	41 (2)	26 (1)	23 (2)
6	66 (5)	67 (2)	40 (7)	10 (0.5)	12 (1)	13 (0.5)	11 (2)
16	22 (3)	23 (2)	19 (4)	4 (2)	3 (0.5)	3 (0.5)	3 (0.5)
36	10 (1)	20 (6)	17 (3)	7 (5)	3 (0.5)	4 (3)	3 (1)
60	7 (4)	12 (2)	16 (5)	7 (3)	3 (0.5)	4 (2)	6 (3)

European Pharmacopoeia diffusion cell apparatus:
Diffusion rate in µg/h
( )=standard deviation
n=5
Silastos silicone membrane

Time	Dolgit cream FR	Proflex cream UK	Ralgex gel UK	Ibutop gel FR	Ibuleve gel UK	Deep Relief gel SA	Ibuleve gel SA	Nurofen gel UK
1	1934 (950)	1582 (780)	2009 (985)	2849 (210)	2317 (1157)	1519 (155)	1220 (613)	1834 (201)
3	725 (369)	608 (439)	847 (416)	1138 (205)	893 (474)	670 (91)	637 (336)	814 (270)
6	571 (291)	499 (283)	640 (316)	751 (24)	464 (248)	335 (91)	369 (202)	552 (104)
16	407 (206)	391 (204)	323 (167)	386 (30)	193 (104)	188 (14)	209 (106)	214 (43)
36	212 (113)	214 (121)	110 (58)	107 (34)	130 (82)	102 (20)	124 (66)	126 (25)
60	43 (29)	93 (52)	37 (25)	36 (10)	138 (84)	75 (24)	111 (56)	74 (19)

Time	Deep Relief gel UK	Cliptol gel FR	Ibuleve Mousse UK	Tiburon gel FR	Intralgis gel FR	Radian B gel UK	Syntofene gel FR
1	2164 (144)	2060 (1020)	786 (402)	1186 (125)	1182 (583)	177 (139)	690 (537)
3	643 (282)	691 (356)	236 (213)	215 (192)	2 (1)	34 (33)	6 (4)
6	432 (120)	443 (221)	192 (258)	11 (3)	50 (30)	47 (39)	51 (52)
16	198 (48)	200 (99)	172 (114)	49 (26)	59 (36)	32 (26)	30 (24)
36	121 (20)	95 (49)	110 (56)	21 (4)	47 (36)	33 (37)	21 (21)
60	76 (22)	80 (45)	120 (68)	33 (14)	33 (32)	25 (20)	19 (17)

APPENDIX IV

RAW DATA USED TO ASSESS DIFFUSION OF IBUPROFEN THROUGH SHED SNAKE SKIN

Franz diffusion cell apparatus:
Total amount liberated in µg/cm^2
( )=standard deviation
n=5

	AFRICAN ROCK PYTHON				FOREST COBRA			
Time	Dorsal outside	Dorsal inside	Ventral outside	Ventral inside	Dorsal outside	Dorsal inside	Ventral outside	Ventral inside
0	0	0	0	0	0	0	0	0
2	12 (12)	8 (3)	5 (1)	3 (1)	11 (3)	12 (2)	11 (2)	12 (1)
4	64 (42)	20 (3)	15 (3)	20 (5)	43 (17)	28 (6)	27 (10)	26 (2)
8	138 (57)	45 (4)	49 (8)	35 (6)	104 (40)	59 (18)	64 (22)	57 (31)
24	522 (239)	140 (21)	277 (30)	145 (36)	339 (138)	140 (43)	208 (35)	150 (79)
48	1011 (347)	331 (65)	626 (70)	352 (110)	660 (171)	248 (76)	423 (85)	321 (95)
72	1373 (472)	429 (112)	928 (90)	599 (177)	949 (173)	362 (107)	637 (116)	494 (122)

	EGYPTIAN COBRA				CAPE COBRA			
Time	Dorsal outside	Dorsal inside	Ventral outside	Ventral inside	Dorsal outside	Dorsal inside	Ventral outside	Ventral inside
0	0	0	0	0	0	0	0	0
2	3 (1)	3 (1)	1 (1)	3 (1)	1 (0)	2 (0)	6 (3)	7 (1)
4	7 (2)	7 (1)	4 (1)	7 (2)	21 (1)	4 (1)	21 (4)	19 (1)
8	12 (6)	17 (2)	12 (3)	17 (2)	41 (1)	10 (1)	40 (7)	33 (3)
24	131 (43)	51 (5)	94 (17)	94 (43)	84 (14)	27 (3)	94 (18)	80 (11)
48	326 (68)	113 (9)	258 (21)	225 (81)	190 (59)	54 (5)	177 (43)	134 (15)
72	501 (95)	180 (17)	416 (16)	349 (119)	333 (66)	80 (9)	283 (56)	192 (16)

European Pharmacopoeia diffusion cell apparatus:
Total amount liberated in μg/cm^2
( )=standard deviation
n=5

	AFRICAN ROCK PYTHON				FOREST COBRA			
Time	Dorsal outside	Dorsal inside	Ventral outside	Ventral inside	Dorsal outside	Dorsal inside	Ventral outside	Ventral inside
0	0	0	0	0	0	0	0	0
2	20 (0)	20 (0)	20 (0)	40 (0)	40 (0)	20 (0)	70 (0)	40 (0)
4	50 (0)	40 (0)	60 (0)	70 (0)	70 (0)	30 (0)	80 (0)	60 (0)
8	140 (0)	100 (0)	180 (0)	140 (0)	170 (0)	50 (0)	170 (0)	120 (0)
24	560 (100)	310 (0)	810 (0)	490 (0)	570 (0)	160 (0)	550 (0)	330 (0)
48	1160 (100)	580 (0)	1670 (100)	1070 (100)	1130 (100)	310 (0)	1200 (200)	640 (100)
72	1660 (100)	840 (100)	2140 (100)	1600 (100)	1640 (100)	490 (0)	1600 (100)	1000 (100)

	EGYPTIAN COBRA				CAPE COBRA			
Time	Dorsal outside	Dorsal inside	Ventral outside	Ventral inside	Dorsal outside	Dorsal inside	Ventral outside	Ventral inside
0	0	0	0	0	0	0	0	0
2	20 (0)	20 (0)	20 (0)	30 (0)	10 (0)	10 (0)	40 (0)	10 (0)
4	20 (0)	30 (0)	20 (0)	30 (0)	10 (0)	20 (0)	130 (0)	20 (0)
8	40 (0)	50 (0)	60 (0)	60 (0)	20 (0)	40 (0)	230 (0)	40 (0)
24	130 (0)	150 (0)	270 (100)	200 (0)	110 (0)	70 (0)	370 (0)	130 (0)
48	340 (0)	310 (0)	600 (100)	400 (0)	300 (0)	110 (0)	570 (0)	270 (0)
72	560 (0)	470 (100)	930 (100)	590 (0)	420 (0)	150 (0)	820 (100)	400 (0)

REFERENCES

1. *The British Pharmacopoeia*, The Pharmaceutical Press, London, 1998, pp 713-714

2. *The Pharmaceutical Codex*, The Pharmaceutical Press, London, 12th Edition, 1994, pp 908-911

3. *Martindale, "The Extra Pharmacopoeia"*, The Pharmaceutical Press, London, 32nd Edition, 1999, pp 44-45

4. *Therapeutic Drugs*, Dollery C. (ed.), Churchhill Livingston, UK, 1991, pp I1-14

5. American Hospital Formulary Service Drug Information, a drug information reference from the American Society of Health-System Pharmacists, 1999

6. McNeil. Motrin® (ibuprofen suspension, chewable tablets, caplets) prescribing information. Fort Washington, PA. 1994

7. *Clarke's Isolation and Identification of Drugs*, Moffet AC. (ed.), The London Pharmaceutical Press, London, 2nd Edition, 1986, pp 677-678

8. Wood J. Osteoarthritis and its management. *The Pharm. J.* **262**: 744-746 (1999)

9. Wood J. Rheumatoid arthritis: the disease and its management with NSAIDs. *The Pharm. J.* **262**: 881-884 (1999)

10. Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: Structural, cellular, and molecular biology. *Annu Rev Biochem.* **69**: 145-182 (2000)

11. *Clinical Pharmacy and Therapeutics*, Herfindal ET, Gourley DR, Lloyd Hart L. (ed.), Williams & Wilkins, Baltimore, Maryland, 4th Edition, 1988, pp 323-324.

12. Kurumbail RG, Stevens AM, Gierse JK et al. Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents. *Nature.* **384**: 644-8 (1996)

13. Riendeau D, Charleson S, Cromlish W et al. Comparison of the cyclooxygenase-1 inhibitory properties of nonsteroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors, using sensitive microsomal and platelet assays. *Can J Physiol Pharmacol.* **75**: 1088-95 (1997)

14. DeWitt DL, Bhattacharyya D, Lecomte M et al. The differential susceptibility of prostaglandin endoperoxide H synthases-1 and -2 to nonsteroidal anti-inflammatory drugs: aspirin derivatives as selective inhibitors. *Med Chem Res.* **5**: 325-43 (1995)

15. *Pharmacology*, Churchhill Livingston, UK, 3rd Edition, 1995, pp 246-255

16. Pharmacia & Upjohn Company. Motrin® (ibuprofen tablets USP) prescribing information. Kalamazoo, MI, 1998 May

17. Ryan TJ, Antman EM, Brooks NH et al. ACC/AHA guidelines for the management of patients with acute myocardial infarction. 1999. Update: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines

18. Mitchell JA, Warner TD. Cyclo-oxygenase-2: pharmacology, physiology, biochemistry and relevance to NSAID therapy. *Br J Pharmacol.* **128**: 1121-1132 (1999)

19. Perera DR, Seiffert AK, Greeley HM. Ibuprofen and meningoencephalitis. *Ann Intern Med.* **100**: 619 (1984)

20. Sandler DP, Burr FR, Weinberg CR. Nonsteroidal anti-inflammatory drugs and the risk for chronic renal disease. *Ann Intern Med.* **115**: 165-72 (1991)

21. Stillman MT, Schlesinger PA. Nonsteroidal anti-inflammatory drug nephrotoxicity: should we be concerned? *Arch Intern Med.* **150**: 268-70 (1990)

22. Murray MD, Brater DC. Adverse effects of nonsteroidal anti-inflammatory drugs on renal function. *Ann Intern Med.* **112**: 559-60 (1990)

23. Fong HJ, Cohen AH. Ibuprofen induced acute renal failure with acute tubular necrosis. *Am J Nephrol.* **2**: 28 (1982)

24. Food and Drug Administration. Labelling revisions for NSAIDs. FDA Drug Bull. **19**: 3-4 (1989)

25. Bailey CE, Stewart JT, McElroy RA. Ibuprofen-induced lithium toxicity. *South Med J.* **82**: 1197 (1989)

26. Khan IH. Lithium and non-steroidal anti-inflammatory drugs. *Br Med J.* **302**: 1537-8 (1991)

27. Rahgeb M. Ibuprofen can increase serum lithium level in lithium-treated patients. *J Clin Psychiatry.* **48**: 161-3 (1987)

28. Kristoff CA, Hayes PE, Barr WH et al. Effect of ibuprofen on lithium plasma and red blood cell concentrations. *Clin Pharm.* **5**: 51-5 (1985)

29. Children's Motrin® (ibuprofen) oral suspension, drops, and chewable tablets prescribing information (undated). Physicians' desk reference. 52nd Edition. Montvale, NJ. Medical Economics Company Inc. 1998, 1536

30. Linden CH, Townsend PL. Clinical and laboratory observations: metabolic acidosis after acute ibuprofen overdosage. *J Pediatr.* **111**: 922-5 (1987)

31. Menzies DG, Conn AG, Williamson IJ et al. Fulminant hyperkalaemia and multiple complications following ibuprofen overdosage. *Med Toxicol Adverse Drug Exp.* **4**: 468-71 (1989)

32. Le HT, Bosse GM, Tsai YY. Ibuprofen overdose complicated by renal failure, adult respiratory distress syndrome, and metabolic acidosis. *Clin Toxicol.* **32**: 315-20 (1994)

33. Dowine A, Ali A, Bell D. Severe metabolic acidosis complicating massive ibuprofen overdose. *Postgrad Med J.* **69**: 575-7 (1993)

34. Primos W, Bhatnager A, Bishop P et al. Acute metabolic acidosis due to ibuprofen overdose. *J MS State Med Assoc.* **28**: 233-4 (1987)

35. Advanced Chemical Development Inc., Toronto, Canada (version 3.5)

36. Yano T, Nakagawa A, Tsuji M, Noda K. Skin permeability of various non steroidal anti-inflammatory drugs in man. *Life Sci.* **39**: 1043-1050 (1986)

37. Foye WO, Lemke TL, Williams DA. *Principles of Medicinal Chemistry.* Williams & Wilkins, 1995, pp 121, 562

38. Mondino A, Zanolo G, Giachetti C. *Medwelt.* **34**: 1052 (1983)

39. Berner G, Engels B, Vogtle-Junkert U. *Drugs Exp Clin Res.* **XV**: 559 (1989)

40. Seth PL. Percutaneous absorption of ibuprofen from different formulations / Comparative study with gel, hydrophilic ointment and emulsion cream. *Arzneim-Forsch/Drug Res.* **43(II)**: 919-921 (1993)

41. Kleinbloesem CH, Ouwerkerk M, Spitznagel W, Wilkinson FE, Kaiser RR. Pharmacokinetics and bioavailability of percutaneous ibuprofen. *Arzneim-Forsch/Drug Res.* **10**: 117-1121 (1995)

42. Lenhard G, Kieferndorf U, Berner G, Engels B, Vogtle-Junkert U. Pharmakokinetik und bioaquivalenz von zwei ibuprofen-formulierungen. *Arzneim-Forsch/Drug Res.* **40(II)**: 1358-1362 (1990)

43. Chlud K, Berner G, Wagener HH. *Therapiewoche.* **38**: 2872 (1985)

44. Peters H, Chlud K, Berner G. *Akt Rheumatol.* **12**: 208 (1987)

45. Schaefer H, Zesch A, Stuttgen G. *Skin Permeability.* Springer-Verlag, Berlin, 1982, pp 733-734, 739-740

46. *Physical Pharmacy*, Martin A (ed.), Lea and Febiger, London, 4th Edition, 1993, pp 324-329, 335-342, 346-356

47. Higuchi T. Physical chemical analysis of percutaneous absorption process from creams and ointments. *J Soc Cosmet. Chem.* **11**: 85-97 (1960)

48. Barry BW, El Eini DID. Influence of non-ionic surfactants on permeation of hydrocortisone, dexamethasone, testosterone and progesterone across cellulose acetate membrane. *J Pharm Pharmacol.* **28**: 219-227 (1976)

49. Bronaugh RL, Stewart RF, Congdon ER. Differences in permeability of rat skin related to sex and body site. *J Soc Cosmet Chem.* **34**: 127-135 (1983)

50. Elias PM, Brown BE, Ziboh VA. The permeability barrier in essential fatty acid deficiency: evidence for a direct role for linoleic acid in barrier function. *J Invest Dermatol.* **74**: 230-233 (1980)

51. Elias PM, Cooper ER, Korc A, Brown BE. Percutaneous transport in relation to stratum corneum structure and lipid composition. *J Invest Dermatol.* **76**: 297-301 (1981)

52. Bronaugh RL, Maibach HI. *In vitro* models for human percutaneous absorption. In *Models in Dermatology 2*, Maibach HI, Lowe (eds.), Karger, Basel. 1985, pp 178-188

53. Bronaugh RL, Stewart RF, Congdon ER. Methods for *in vitro* percutaneous absorption studies II. Animal models for human skin. *Toxicol Appl Pharmacol.* **62**: 481-488 (1982)

54. Sato S, Wan Kim S. Macromolecular diffusion through polymer membranes. *Int J Pharm.* **22**: 229-255 (1984)

55. Stehle RG, Higuchi WI. *In vitro* model for transport of solutes in three-phase system II: experimental considerations. *J Pharm Sci.* **61**: 1931-1935 (1972)

56. Hadgraft J, Walters KA, Wotton PK. Facilitated percutaneous absorption: a comparison and evaluation of two *in vitro* models. *Int J Pharm.* **32**: 257-263 (1986)

57. Beastall J, Guy RH, Hadgraft J, Wilding I. The influence of urea on percutaneous absorption. *Pharm Res.* **3**: 294-297 (1986)

58. Kazmi S, Kennon L, Sideman M, Plakogiannis FM. Medicament release from ointment bases: I. Indomethacin. *In vitro* and *in vivo* release studies. *Drug Dev Ind Pharm.* **10**: 1071-1083 (1984)

59. Parikh NH, Babar A, Plakogiannis FM. Medicament release from ointment bases: II. Testosterone: *in vitro* release and effects of additives on its release. *Drug Dev Ind Pharm.* **12**: 2493-2509 (1986)

60. Ayres JW, Laskar PA. Diffusion of benzocaine from ointment bases. *J Pharm Sci.* **63**: 1402-1406 (1974)

61. Kneczke M, Landersjo L, Lundgren P, Fuhrer C. *In vitro* release of salicylic acid from two different quantities of white petrolatum. *Acta Pharma Suec.* **23**: 193-204 (1986)

62. Touitou E, Abed L. The permeation behaviour of several membranes with potential use in the design of transdermal devices. *Pharm Acta Helv.* **60**: 193-198 (1985)

63. Sun Y, Tojo K, Chien YW. Kinetics and thermodynamics of drug permeation through silicone elastomers (II). Effect of penetrant lipophilicity. *Drug Dev Ind Pharm.* **12**: 327-348 (1986)

64. Lee C-L, Ulman KL, Larson KR. Kinetics and thermodynamics of drug permeation through silicone elastomers (IV). Effect of polymer backbone structure. *Drug Dev Ind Pharm.* **12**: 369-383 (1986)

65. Garrett ER, Chemburkar PB. Evaluation, control, and prediction of drug diffusion through polymeric membranes I. Methods and reproducibility of steady state diffusion studies. *J Pharm Sci.* **57**: 944-949 (1968)

66. Flynn GL, Roseman TJ. Membrane diffusion II. Influence of physical adsorption on molecular flux through heterogeneous dimethylpolysiloxane barriers. *J Pharm Sci.* **60**: 1788-1796 (1971)

67. Bottari F, Di Colo G, Nannipieri E, Saettone MF, Serafini MF. Influence of drug concentration on *in vitro* release of salicylic acid from ointment bases. *J Pharm Sci.* **63**: 1779-1783 (1974)

68. Lovering EG, Black DB, Rowe ML. Drug permeation through membranes IV: effect of excipients and various additives on permeation of chlordiazepoxide through polymethylsiloxane membranes. *J Pharm Sci.* **63**: 1224-1227 (1974)

69. Carelli V, Di Colo G, Nannipieri E. Effect of water-soluble additives on drug release from silicone rubber matrices. III. A study of release mechanisms by differential scanning calorimetry. *Int J Pharm.* **30**: 9-16 (1986)

70. Berenson GS, Burch GE. Studies of diffusion of water through dead human skin: the effect of different environmental states and of chemical alterations of the epidermis. *Am J Trop Med Hyg.* **31**: 842-853 (1951)

71. Galey WR, Lonsdale HK, Nacht S. The *in vitro* permeability of skin and buccal mucosa to selected drugs and tritiated water. *J Invest Dermatol.* **67**: 713-717 (1976)

72. Ainsworth M. Methods for measuring percutaneous absorption. *J Soc Cosmet Chem.* **11**: 69-78 (1960)

73. Bronaugh RL, Stewart RF, Congdon ER, Giles AL. Methods for *in vitro* percutaneous absorption studies I. Comparison with *in vivo* results. *Toxicol Appl Pharmacol.* **62**: 474-480 (1982)

74. Rochefort A, Druot P, Leduc M, Vassalet R, Agache P. A new technique for the evaluation of cosmetics effect on mechanical properties of stratum corneum and epidermis *in vitro*. *Int J Cosmet Sci.* **8**: 27-36 (1986)

75. Southwell D, Barry BW, Woodford R. Variations in permeability of human skin within and between species. *Int J Pharm.* **18**: 299-309 (1984)

76. Langguth P, Spahn H, Mutschler E. Variations of *in vitro* nitroglycerine permeation through human epidermis. *Pharm Res.* **3**: 61-63 (1986)

77. Billups NF, Patel NK. Experiments in physical pharmacy V. *In vitro* release of medicament from ointment bases. *Am J Pharm Educ.* **34**: 190-196 (1970)

78. Wester RC, Maibach HI. Animal models for percutaneous absorption. *Models in dermatology 2*, Maibach and Lowe (eds.) Karger, Basel, 1985, pp 159-169

79. Hawkins GS, Reifenrath WG. Influence of skin source, penetration cell fluid, and partition coefficient on *in vitro* skin penetration. *J Pharm Sci.* **75**: 378-381 (1986)

80. Haigh JM, Smith EW. The selection and use of natural and synthetic membranes for *in vitro* diffusion experiments. *Eur J Pharm Sci.* **2**: 311-330 (1994)

81. Marzulli FN, Brown DWC, Maibach HI. Techniques for studying skin penetration. *Toxicol Appl Pharmacol* (suppl.). **3**: 76-83 (1969)

82. Bronaugh RL, Stewart RF. Methods for in vitro percutaneous absorption studies IV. The flow through diffusion cell. *J Pharm Sci.* **74**: 64-67 (1985)

83. Scott RC, Walker M, Dugard PH. A comparison of the in vitro permeability properties of human and some laboratory animal skins. *Int J Cosmet Sci.* **8**: 189-194 (1986)

84. Ackerman C, Flynn GL. Ether-water partitioning and permeability through nude mouse skin in vitro I. Urea, thiourea, glycerol and glucose. *Int J Pharm.* **36**: 61-66 (1987)

85. Touitou E. Transdermal delivery of anxiolytics: *in vitro* skin permeation of midazolam maleate and diazepam. *Int J Pharm.* **33**: 37-43 (1986)

86. Touitou E, Abed L. Effect of propylene glycol, azone and N-decylmethyl sulphoxide on skin permeation kinetics of 5-fluorouracil. *Int J Pharm.* **27**: 89-98 (1976)

87. Liang W-Q, Petersen RV, Wan Kim S. Drug permeation through polymer-skin membranes. *Acta Pharm Technol.* **31**: 210-214 (1985)

88. Chien YW, Keshary PR, Huang YC, Sarpotdar PP. Comparative controlled skin permeation of nitroglycerin from marketed transdermal delivery systems. *J Pharm Sci.* **72**: 968-970 (1983)

89. Flynn GL, Durrheim H, Higuchi WI. Permeation of hairless mouse skin II: membrane sectioning techniques and influence on alkanol permeabilities. *J Pharm Sci.* **70**: 52-56 (1981)

90. Behl CR, Flynn GL, Linn EE, Smith WM. Percutaneous absorption of corticosteroids: age, site, and skin-sectioning influences on rates of permeation of hairless mouse skin by hydrocortisone. *J Pharm Sci.* **73**: 1287-1290 (1984)

91. Michniak BB. Advances in topical corticosteroid therapy. *Int J Cosmet Sci.* **5**: 277-291 (1983)

92. Michniak-Mikolajczak BB, Bodor N. A study of the penetration of five novel synthetic steroids through hairless mouse skin *in vitro*. *Int J Cosmet Sci.* **7**: 175-179 (1985)

93. Dugard PH, Scott RC, Ramsey JD, Mawdsley SJ, Rhodes C. Percutaneous absorption of phthalate esters: *in vitro* experiments on human and rat epidermal membranes. *Hum Toxicol.* **2**: 561-562 (1983)

94. Marzulli FN, Maibach HI. Relevance of animal models: The hexachlorophene story. In: *Animal models in dermatology*, Maibach HI (ed.), Churchhill Livingstone, New York, 1975, pp 156-167

95. Foreman MI, Clanachan I, Kelly IP. Diffusion barriers in skin-a new method of comparison. *Br J Dermatol.* **108**: 549-553 (1983)

96. Bronaugh RL, Stewart RF, Congdon ER. Differences in permeability of rat skin related to sex and body site. *J Soc Cosmet Chem.* **34**: 127-135 (1983)

97. Picton W, Devitt H, Forgie MA. Practical applications of the Quantimet 720, image analysing computer, in the field of investigative dermatology. *Br J Dermatol.* **95**: 341-345 (1970)

98. Creasey NH, Battensby J, Fletcher J. Factors affecting the permeability of the skin. *Curr Prob Dermatol.* **7**: 95-106 (1978)

99. Walker M, Dugard PH, Scott RC. *In vitro* percutaneous absorption studies: a comparison of human and laboratory species. *Hum Toxicol.* **2**: 561-562 (1983)

100. Wester RC, Noonan PK, Maibach HI. Variations in percutaneous absorption of testosterone in the rhesus monkey due to anatomic site of application and frequency of application. *Arch Dermatol Res.* **267**: 229-235 (1980)

101. Washitake M, Takashima Y, Tanaka S, Anmo T, Tanaka I. Drug permeation through egg shell membranes. *Chem Pharm Bull.* **28**: 2855-2861 (1980)

102. Rigg PC, Barry BW. Shed snake skin and hairless mouse skin as model membranes for human skin during permeation studies. *J Invest Dermatol.* **94**: 235-240 (1990)

103. Takahashi K, Tamagawa S, Katagi T, Rytting JH, Nishihata T, Mizuno N. Percutaneous permeation of basic compounds through shed snake skin as a model membrane. *J Pharm Pharmacol.* **45**: 882-886 (1993)

104. Itoh T, Magavi R, Casady RL, Nishihata T, Rytting JH. A method to predict the percutaneous permeability of various compounds – shed snake skin as a model membrane. *Pharm Res.* **7**: 1302-1306 (1990)

105. Smith EW, Haigh JM. *In vitro* systems for the assessment of drug release from topical formulations and transmembrane permeation. *Percutaneous Absorption. Mechanisms – Methodology – Drug Delivery.* Bronaugh RL and Maibach HI, (eds.), Marcel Dekker, New York, 1989, pp 465-508

106. Smith EW and Haigh JM. *In vitro* diffusion cell design and validation. I. A stability-indicating high-performance liquid chromatographic assay for betamethasone 17-valerate in purified isopropyl myristate receptor phase. *Pharm Res.* **6**: 431-435 (1989)

107. Smith EW and Haigh JM. *In vitro* diffusion cell design and validation. II. Temperature, agitation and membrane effects on betamethasone 17-valerate permeation. *Acta Pharm Nord.* **4**: 171-179 (1992)

108. Harada K, Murakami T, Kawasaki E, Higashi Y,Yamamoto S, Yata N. *In vitro* permeability to salicylic acid of human, rodent and shed snake skin. *J Pharm Pharmacol.* **4**: 414-418 (1993)

109. Craane-van Hinsberg WHM, Verhoef JC, Bax LJ, Junginger HE, Bodde HE. Role of appendages in skin resistance and ionotophoretic peptide flux: human versus snake skin. *Pharm Res.* **12**: 1506-1512 (1995)

110. Kuramoto M, Tanaka T, Makita H, Nakamura Y, Yata N. Characteristics of shed snake skin permeability to indomethacin and fatty alcohols. *J Pharm Pharmacol.* **48**: 680-684 (1996)

111. Haigh JM, Beyssac E, Chanet L, Aiache J-M. In vitro permeation of progesterone from a gel through the shed skin of three different snake species. *Int J Pharm.* **170**: 151-156 (1998)

112. Fleeker C, Wong O, Rytting JH. Facilitated transport of basic and acidic drugs in solutions through snake skin by a new enhancer- dodecyl *N,N*-dimethylamino acetate. *Pharm Res.* **6**: 443-448 (1989)

113. Wong O, Huntington J, Nishihata T, Rytting JH. New alkyl *N,N*-dialkyl-substituted amino acetates as transdermal penetration enhancers. *Pharm Res.* **6**: 286-295 (1989)

114. Bhatt PP, Rytting JH, Topp EM. Influence of azone and lauryl alcohol on the transport of acetaminophen and ibuprofen through shed snake skin. *Int J Pharm.* **72**: 219-226 (1991)

115. Hiroven J, Rytting JH, Paronen P, Urtti A. Dodecyl *N,N*-dimethylamino acetate and azone enhance drug penetration across human, snake and rabbit skin. *Pharm Res.* **8**: 933-937 (1991)

116. Bhattachar SN, Rytting JH, Itoh T, Nishihata T. The effects of complexation with hydrogenated phospholipid on the transport of salicylic acid, diclofenac and indomethacin across snake stratum corneum. *Int J Pharm.* **79**: 263-271 (1992)

117. Fu Lu M, Lee D, Subba Rao G. Percutaneous absorption enhancement of leuprolide. *Pharm Res.* **9**: 1575-1579 (1992)

118. Buyuktimkin S, Buyuktimkin N, Rytting JH. Synthesis and enhancing effect of 2-(*N,N*-dimethyllamino)propionate on the transdermal delivery of indomethacin, clonidine and hydrocortisone. *Pharm Res.* **10**: 1632-1637 (1993)

119. Hirvonen J, Kontturi K, Murtomaki L, Paronen P, Urtti A. Transdermal iontophoresis of sotalol and salicylate; the effect of skin charge and penetration enhancers. *J Control Release.* **26**: 109-117 (1993)

120. Turunen TM, Buyuktimkin S, Buyuktimkin N, Urtti A, Paronen P, Rytting JH. Enhanced delivery of 5-fluorouracil through shed snake skin by two new transdermal penetration enhancers. *Int J Pharm.* **92**: 89-95 (1993)

121. Suh H, Jun HW. Effectiveness and mode of action of isopropyl myristate as a permeation enhancer for naproxen through shed snake skin. *J Pharm Pharmacol.* **48**: 812-816 (1996)

122. Katz M, Poulsen BJ. Corticoid, vehicle, and skin interaction in percutaneous absorption. *J Soc Cosmet Chem.* **23**: 565-590 (1972)

123. Zesch A, Schaefer H. Penetration kinetics of four drugs in the human skin. *Acta Derm Venereol (Stockh.).* **54**: 91-98 (1974)

124. Idson B. Percutaneous absorption. *J Pharm Sci.* **64**: 901-921 (1975)

125. Ostrenga J, Haleblian J, Poulsen B, Ferrell B, Mueller N, Shastri S. Vehicle design for a new topical steroid, fluocinonide. *J Invest Derm.* **56**: 392-399 (1971)

126. Idson B. Vehicle effects in percutaneous absorption. *Drug Metab Rev.* **14**: 207-222 (1983)

127. Bath BD, White HS, Scott ER. Visualization and analysis of electroosmotic flow in hairless mouse skin. *Pharm Res.* **17**: 471 (2000)

128. Behl CR, Char H, Patel SB, Mehta DB, Piemontese D, Malick AW. *In vivo* and *in vitro* skin uptake and permeation studies. Critical considerations and factors which affect them. In: *Topical Drug Bioavailability, Bioequivalence, and Penetration.* Shah VP, Maibach HI (eds.), Plenum Press, New York and London, 1993, pp 225-252

129. Wurster DE, Kramer SF. Some factors affecting percutaneous absorption. *J Pharm Sci.* **50**: 288 (1961)

130. Marzulli FN, Brown DWC, Maibach HI. Techniques for studying skin penetration. *Toxicol Appl Pharmacol Suppl.* **3**: 76 (1969)

131. Bartec MJ, La Budde JA. Percutaneous absorption in vitro. In: Maibach (ed.), *Animal Models in Dermatology*, New York, Churchhill-Livingstone, 1975

132. Garcia B, Marty JP, Wepierre J. Effect of Tween 80 on the permeation rates of alkanols through the hairless rat skin. *Int J Pharmaceut.* **4**: 205 (1980)

133. Sugibayashi K, Hosoya KI, Morimoto Y, Higuchi WI. Effect of absorption enhancer, Azone, on the transport of 5-fluorouracil across hairless rat skin. *J Pharm Pharmacol.* **35**: 578 (1985)

134. Feldman RJ, Maibach HI. Percutaneous absorption of steroids in man. *J Invest Dermatol.* **52**: 89 (1969)

135. McKenzie AW. Percutaneous absorption of steroids. *Arch Dermatol.* **86**: 611 (1962)

136. Scheuplein RJ, Ross L. Effects of surfactants and solvents on the permeability of the epidermis. *J Soc Cosmet Chem.* **21**: 853 (1970)

137. Dempski RE, Portnoff JB, Wase AW. *In vitro* release and *in vivo* penetration studies of a topical steroid from non-aqueous vehicles. *J Pharm Sci.* **58**: 579 (1969)

138. Stoughton RB, Clendenning WE, Kruse D. Percutaneous absorption of nicotinic acid and derivatives. *J Invest Dermatol.* **35**: 337 (1960)

139. Shahi V, Zatz JL. Effect of formulation factors on penetration of hydrocortisone through mouse skin. *J Pharm Sci.* **67**: 789 (1978)

140. Spruance SL, McKeough MB, Cardinal JR. Dimethylsulfoxide as a vehicle for topical antiviral chemotherapy. *Ann NY Acad Sci.* **411**: 28 (1983)

141. Lorenzetti OJ. Propylene glycol gel vehicles. *Cutis.* **23**: 747 (1979)

142. Kumar S, Malick AW, Meltzer NM, Mouskountakis JD, Behl CR. *In vitro* skin permeation and retention of a leukotriene antagonist from topical vehicles using hairless guinea pig model. *J Pharm Sci.* **69**: 631 (1992)

143. Barry BW. Vehicle effect: what is an enhancer. In: *Topical Drug Bioavailability, Bioequivalence, and Penetration.* Shah VP, Maibach HI (eds.), Plenum Press, New York and London, 1993, pp 261-276

144. Flynn GL. Topical and transdermal drug delivery systems. In: *Topical Drug Bioavailability, Bioequivalence, and Penetration.* Shah VP, Maibach HI (eds.), Plenum Press, New York and London, 1993, pp 370-391

145. Idson B. Percutaneous absorption enhancers. *Drug and Cosmet Ind.* **137**: 30-33 (1985)

146. Cooper ER. Increased skin permeability for lipophilic molecules. *J Pharm Sci.* **73**: 1153-1156 (1984)

147. Goodman M, Barry BW. Lipid-protein-partitioning (LPP) theory of skin enhancer activity: finite dose technique. *Int J Pharm.* **57**: 29-40 (1989)

148. Obata Y, Takayama K, Machida Y, Nagai T. Combined effect of cyclic monoterpenes and ethanol on percutaneous absorption of diclofenac sodium. *Drug Des Discov.* **8**: 137-144 (1991)

149. Banerjee TK, Mittal AK. Histochemistry of snake epidermis. In: *The Skin of Invertebrates.* Spearman RIC, Riley PA (eds.), Academic Press, New York, 1980, pp 23-32

150. Scheuplein RJ, Blank IH. Permeability of the skin. *Physiol Rev.* **51**: 702-747 (1971)

151. Roberts JB, Lillywhite HB. Lipid barrier to water exchange in reptile epidermis. *Science.* **207**: 1077-1079 (1980)

152. Smith E.W. Ph.D. Thesis: Aspects of the Transdermal Permeation and Analysis of Betamethasone 17-Valerate, School of Pharmaceutical Sciences, Rhodes University, Grahamstown, South Africa, 1987

153. Bronaugh RL. Diffusion cell design. In: *Topical Drug Bioavailability, Bioequivalence, and Penetration.* Shah VP, Maibach HI (eds.), Plenum Press, New York and London, 1993, pp 117-125

154. Scheuplein RJ. Mechanisms of percutaneous absorption. I. Routes of penetration and the influence of solubility. *J Invest Dermatol.* **45**: 334-346 (1965)

155. Franz TJ. On the relevance of *in vitro* data. *J Invest Dermatol.* **64**: 190-195 (1975)

156. Barry BW. Methods for studying percutaneous absorption. In: *Dermatological Formulations: Percutaneous Absorption.* Marcel Dekker, New York, 1983, pp 234-295

157. Lovering EG, Black DB. Diffusion layer effects on permeation of phenylbutazone through polydimethylsiloxane. *J Pharm Sci.* **63**: 1399-1402 (1974)

158. Bottari F, Di Colo G, Nannipieri E, Saettone MF, Serafini MF. Release of drugs from ointment bases II: *In vitro* release of benzocaine from suspension-type aqueous gels. *J Pharm Sci.* **66**: 926-931 (1977)

159. Scott RC, Leahy DE, Evans JR, Bole E. *In vitro* percutaneous model for quantitative structure-absorption studies. *J Pharm Pharmacol.* (suppl.). 66 (1986)

160. Valia KH, Chien YW. Long-term skin permeation kinetics of estradiol: (I) effect of drug solubilizer-polyethylene glycol 400. *Drug Dev Ind Pharm.* **10**: 951-981 (1984)

161. Bronaugh RL, Stewart RF. Methods for in vitro percutaneous absorption studies. III. Hydrophobic compounds. *J Pharm Sci.* **73**: 1255-1258 (1984)

162. Sloan KB, Siver KG, Koch SAM. The effect of vehicle on the diffusion of salicylic acid through hairless mouse skin. *J Pharm Sci.* **75**: 744-749 (1986)

163. Southwell D, Barry BW. Penetration enhancement in human skin: effect of 2-pyrrolidone, dimethylformamide and increased hydration on finite dose permeation of aspirin and caffeine. *Int J Pharm.* **22**: 291-298 (1984)

164. Akhter SA, Barry BW. Flurbiprofen penetration and enhancement through cadaver skin: value of "*in vivo* mimic" approach with low enhancer concentrations. *J Pharm Pharmacol.* (suppl.). **35**: 30 (1983)

165. Akhter SA, Barry BW. Penetration enhancers in human skin - effect of oleic acid and azone on flurbiprofen permeation. *J Pharm Pharmacol.* (suppl.). **36**: 7 (1984)

166. Akhter SA, Barry BW, Meyer MC. Absorption through cadaver skin of ibuprofen, applied as dry films; effects of solvents. *J Pharm Pharmacol.* (suppl.). **34**: 34 (1982)

167. Akhter SA, Barry BW. Absorption through human skin of ibuprofen and flurbiprofen; effect of dose variation, deposited drug films, occlusion and the penetration enhancer N- methyl-2-pyrrolidone. *J Pharm Pharmacol.* **37**: 27-37 (1985)

168. Franz TJ. The finite dose technique as a valid *in vitro* model for the study of percutaneous absorption. *Curr Prob Dermatol.* **7**: 58-68 (1978)

169. Keshary PR, Chien YW. Mechanisms of controlled nitroglycerin administration (I): development of a finite-dosing skin permeation system. *Drug Dev Ind Pharm.* **10**: 883-913 (1984)

170. Chien YW, Valia KH. Development of a dynamic skin permeation system for long-term permeation studies. *Drug Dev Ind Pharm.* **10**: 575-599 (1984)

171. Fares HM, Zatz JL. Measurement of drug release from topical gels using two types of apparatus. *Pharm Tech.* **Jan**: 52-58 (1995)

172. Itoh T, Xia J, Magavi R, Nishihata T, Rytting JH. Use of shed snake skin as a model membrane for *in vitro* percutaneous penetration studies: comparison with human skin. *Pharm Res.* **7**: 1042-1047 (1990)

173. Lippold BC. Selection of the vehicle for topical administration of drugs. *Pharm Acta Helv.* **59**: 166-171 (1984)

174. Mohammed FA. Evaluation of the usefulness of sodium carboxymethylcellulose as a vehicle for transdermal delivery of ibuprofen. Éditions De Santé. 1999. Submitted for publishing

175. Twist JN, Zatz JL. Interaction of vehicles with model skin membranes in permeation process. *In: Percutaneous Absorption: Mechanisms-Methodology-Drug Delivery.* Bronaugh RL and Maibach HI (eds.), Marcel Dekker, New York, 1999, pp 147-147

176. ECETOC (European Centre for Ecotoxicology and Toxicology of Chemicals). Percutaneous Absorption, Monogram 20, Brussels, 1993, pp 20

177. Pongjanyakul T, Prakongpan S, Priprem A. Permeation studies comparing cobra skin with human skin using nicotine transdermal patches. *Drug Dev Ind Pharm.* **26**: 635-642 (2000)

178. Qvist MH, Hoeck U, Kreilgaard B, Madsen F, Frokjaer S. Evaluation of Gottingen minipig for transdermal *in vitro* permeation studies. *Eur J Pharm.* **11**: 59-68 (2000)

179. Higuchi T, Konishi R. Proceedings of the 2nd Transdermal Therapeutic Symposium. *Therapeutic Res.* **6**: 280-288 (1987)

180. Roberts JB. Use of squamate epidermis in percutaneous absorption studies: A review. *J Toxicol – Cut & Ocular Toxicol.* **5**: 319-324 (1986)

181. Roberts JB, Lillywhite HB. Lipids and the permeability of epidermis from snakes. *J Exp Zool.* **228**: 1-9 (1983)

182. Burken RR, Wertz PW, Downing DT. The effects of lipids on transepidermal water permeation in snakes. *Comp Biochem Physiol.* **81**: 213-216 (1985)

183. Roth SI, Jones WA. The ultrastructure of epidermal maturation in the skin of the Boa constrictor. *J Ultrastruct Res.* **32**: 69-93 (1970)

184. Landmann L. Keratin formation and barrier mechanisms in the epidermis of Natrix natrix (Reptilia:Serpentes): An ultrastructural study. *J Morphol.* **162**: 93-126 (1979)

185. Landmann L. Lamellar granules in mammalian, avian and reptilian epidermis. *J Ultrastruct Res.* **72**: 245-263 (1980)

186. Landmann L, Stolinski C, Martin B. The permeability barrier in the epidermis of the grass snake during the resting stage of the sloughing cycle. *Cell Tissue Res.* **215**: 369-382 (1981)

187. European Pharmacopoeia, Addendum 2001, Dissolution test for transdermal dosage forms. Conseil de l'Europe, Strasbourg, France, Section 2.9.4, pp 110-111

188. Instrumental Methods of Analysis. Willard HH, Merritt LL, Dean JA, Settle FA (eds.), Wadsworth Publishing Company, Belmont, California, 7th Edition, 1988, pp 513-539, 580-655

189. Adams SS, Cliffe EE. *J Pharm Pharmacol.* **17**: 173 (1965)

190. Midha KK, Cooper JK, Hubbard JW, McGilveray IJ. Rapid and simple GLC procedure for determinations of plasma concentrations of ibuprofen. *J Pharm Sci.* **12**: 29-31 (1977)

191. Hofffman DJ. Rapid GLC determination of ibuprofen in serum. *J Pharm Sci.* **66**: 749-750 (1977)

192. Vangaissen GJ, Kaiser DG. GLC determination of ibuprofen enantiomers in biological specimens. *J Pharm Sci.* **64**: 798 (1975)

193. Kaiser DG, Vangaissen GJ. GLC determination of ibuprofen in plasma. *J Pharm Sci.* **63**: 219-221 (1974)

194. Heikkinen L. Silica capillary gas chromatographic determination of ibuprofen in serum. *J Chromatgr.* **307**: 206209 (1984)

195. Pettersen JE, Ulsaker GA, Jellum E. Studies on the metabolism of ibuprofen by gas chromatography and mass spectrometry: dialysis fluid, a convenient medium for studies on drug metabolism. *J Chromatogr.* **145**: 413-420 (1978)

196. Lalande M, Wilson DL, McGilveray IJ. Rapid high performance liquid chromatographic determination of ibuprofen in human plasma. *J Chromatogr.* **377**: 410-414 (1986)

197. Minkler PE, Hopple CL. Determination of ibuprofen in human plasma by high performance liquid chromatography. *J Chromatogr.* **428**: 388-394 (1988)

198. Haikala VE, Heimonen IK, Vuorela HJ. Determination of ibuprofen in ointments by reversed phase liquid chromatography. *J Pharm Sci.* **80**: 456-458 (1991)

199. Avgerinos A, Hutt AJ. High performance liquid chromatographic determination of ibuprofen in human plasma and urine by direct injection. *J Chromatogr.* **380**: 468-471 (1986)

200. Sodhi RA, Chawla JL, Sane RT. Simultaeous determination of paracetamol, ibuprofen and chlorzoxazone by HPLC, HPTLC and GC methods. *Indian Drugs.* **33**: 280-285 (1996)

201. Sochor J, Klimes J, Sedlacek J, Zahradnicek M. Determination of ibuprofen in erythrocytes and human plasma by high performance liquid chromatography. *J Pharm Biomed Anal.* **13**: 899-903 (1995)

202. Naidong W, Lee JW. Development and validation of a liquid chromatographic method for the quantitation of ibuprofen enantiomers in human plasma. *J Pharm Biomed Anal.* **12**: 551-556 (1994)

203. Van Overbeke A, Baeyens W, Van Den Bossche W, Dewaele C. Enantiomeric separation of amide derivatives of some 2-arylpropionic acids by HPLC on a cellulose-based chiral stationary phase. *J Pharm Biomed Anal.* **12**: 911-916 (1994)

204. Lemko CH, Caille G, Foster RT. Stereospecific high performance liquid chromatographic assay of ibuprofen: improved sensitivity and sample processing efficiency. *J Chromatogr Biomed Appl.* **130**: 330-335 (1993)

205. Castillo M, Smith PC. Direct determination of ibuprofen and ibuprofen acyl glucuronide in plasma by high performance liquid chromatography using solid phase extraction. *J Chromatogr Biomed Appl.* **125**: 109-116 (1993)

206. Wright MR, Sattari S, Brocks DR, Jamali F. Improved high performance liquid chromatographic assay method for the enantiomers of ibuprofen. *J Chromatogr Biomed Appl.* **121**: 259-265 (1992)

207. Blagbrough IS, Daykin MM, Doherty M, Pattrick M, Shaw PN. *J Chromatogr Biomed Appl.* **116**: 251-257 (1992)

208. Pettersson KJ, Olsson A. Liquid chromatographic determination of the enantiomers of ibuprofen in plasma using a chiral AGP column. *J Chromatogr Biomed Appl.* **563**: 414-418 (1991)

209. Menzel-Soglowek S, Geisslinger G, Brune K. Stereoselective high performance liquid chromatographic determination of ketoprofen, ibuprofen and fenoprofen in plasma using a chiral alpha-acid glycoprotein column. *J Chromatogr Biomed Appl.* **532**: 295-303 (1990)

210. Rustum AM. Measurement of ibuprofen in human whole blood by reversed phase ion pair high performance liquid chromatography using a pH-stable polymeric column. *J Chromatogr.* **526**: 246-253 (1990)

211. Satterwhite JH, Boudinot FD. High performance liquid chromatographic determination of ibuprofen in rat and human plasma. *J Chromatogr.* **497**: 330-335 (1989)

212. Geisslinger G, Dietzel K, Loew D, Schuster O, Brune K. High performance liquid chromatographic determination of ibuprofen, its metabolites and enantiomers in biological fluids. *J Chromatogr.* **491**: 139-149 (1989)

213. Albert KS, Raabe A, Garry M, Antal EJ, Gillespie WR. Determination of ibuprofen in capillary and venous plasma by high performance liquid chromatography with ultraviolet detection. *J Pharm Sci.* **73**: 1487-1489 (1984)

214. Spraul M, Hofmann M, Dvortsak P, Nicholson JK, Wilson JD. Liquid chromatography coupled with high-field proton NMR for profiling human urine for endogenous compounds and drug metabolites. *J Pharm Biomed Anal.* **10**: 601-605 (1992)

215. Unterhalt B. Analysis of antiphlogistics from the series of the arylpropane acids. *Pharm Ztg.* **123**: 1801-1803 (1978)

216. Muktadir A, Babar A, Cutie AJ, Plakogiannis FM. Medicament release from ointment bases III. Ibuprofen: *in vitro* release and *in vivo* absorption in rabbits. *Drug Dev and Ind Pharm.* **12**: 2521-2540 (1986)

217. Irwin WJ, Sanderson FD, Li Wan Po A. Percutaneous absorption of ibuprofen: Vehicle effects on transport through rat skin. *Int J Pharm.* **66**: 193-200 (1990)

218. Irwin WJ, Sanderson FD, Li Wan Po A. Percutaneous absorption of ibuprofen and naproxen: Effect of amide enhancers on transport through rat skin. *Int J Pharm.* **66**: 243-252 (1990)

219. Verner JJ. M.Sc. Thesis: Formulation and Assessment of a Novel Repeat Action Tablet Containing a Decongestant and an Antihistamine. School of Pharmaceutical Sciences, Rhodes University, Grahamstown, South Africa, 2000

220. Swartz M, Krull IS. *Analytical Method Development and Validation.* Marcel Dekker, New York, 1997, pp 17-83

221. Lang JR, Bolton S. A comprehensive method validation strategy for bioanalytical applications in the pharmaceutical industry-1. Experimental considerations. *J Pharm and Biomed Anal.* **9**: 357-361 (1991)

222. Requirements for initial assay validation and publication in the Journal of Chromatography B. *Analytical Separation News*, 1998

223. Shah VP, Elkins JS, Williams RL. Role of *in vitro* release measurements in semisolid dosage forms. *In: Percutaneous Absorption: Mechanisms-Methodology-Drug Delivery.* Bronaugh RL and Maibach HI (eds.), Marcel Dekker, New York, 1999, pp 555-570.

224. Guidance for Industry: SUPAC-SS Nonsterile Semisolid Dosage Forms. Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls; In Vitro Release Testing and In Vivo Bioequivalence Documentation. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, May 1997

225. Bonnabry C. Mise Au Point et Validation de Modèles D'Étude In Vitro du Passage Percutané. L'Université d'Auvergne, Sciences Pharmaceutiques, France, 1999

226. Meyer E. A Critical Evaluation of the Human Skin Blanching Assay and Comparative Bioavailability Studies on Topical Corticosteroid Preparations. School of Pharmaceutical Sciences, Rhodes University, Grahamstown, South Africa, 1988

227. Tuomi A, Turakka L, Raty T, Lehmussaari K. Release of hydrocortisone from topical semisolid preparations using three different *in vitro* methods. *Acta Pharm Fenn.* **98**: 93-99 (1989)

228. Rahman MS, Babar A, Patel NK, Plakogiannis FM. Medicament release from ointment bases. V. Naproxen *in vitro* release and *in vivo* percutaneous absorption in rabbits. *Drug Dev Ind Pharm.* **16**: 651-672 (1990)

229. Guy RH, Hadgraft J. On the determination of drug release rates from topical dosage forms. *Int J Pharm.* **60**: R1-R3 (1990)

230. Higuchi T. Rate of release of medicaments from ointment bases containing drugs in suspension. *J Pharm Sci.* **50**: 874-875 (1961)

231. Higuchi WI. Analysis of data on the medicament release from ointments. *J Pharm Sci.* **51**: 802-804 (1962)

232. Koizumi T, Higuchi WI. Analysis of data on drug release from emulsions. II. Pyridine release from water-in-oil emulsions as a function of pH. *J Pharm Sci.* **57**: 87-92 (1968)

233. Turakka L, Ala-Fossi N. Influence of propylene glycol on the release of hydrocortisone and its acetate from carbopol hydrogel. *Acta Pharm Fenn.* **96**: 15-21 (1987)

234. Arellano A, Santoyo S, Martin C, Ygartua P. Influence of propylene glycol and isopropyl myristate on the *in vitro* percutaneous penetration of diclofenac sodium from carbopol gels. *Eur J Pharm Sci.* **7**: 129-135 (1989)

235. Sarpotdar PP, Zatz JL. Evaluation of penetration enhancement of lidocaine by nonionic surfactants through hairless mouse skin *in vitro. J Pharm Sci.* **75**: 176-181 (1986)

236. Barry BW, Bennet SL. Effect of penetration enhancers on the permeation of mannitol, hydrocortisone and progesterone through human skin. *J Pharm Pharmacol.* **39**: 535-546 (1987)

237. Bendas B, Schmalfuss U, Neubert R. Influence of propylene glycol as cosolvent on mechanisms of drug transport from hydrogels. *Int J Pharm.* **116**: 19-30 (1995)

238. Bailik W, Walters KA, Brain KR, Hadgraft J. Some factors affecting the *in vitro* penetration of ibuprofen through human skin. *Int J Pharm.* **92**: 219-223 (1993)

239. Lippold BC. Selection of the vehicle for topical administration of drugs. *Pharm Acta Helv.* **59** :166-171 (1984)

240. Green PK. Overseas Containers (UK Agencies) Ltd., Central House, 25 Camperdown Street, London, Communications of E. Meyer, 1985

241. McIntosh I. P&O Containers (SA) Pty. Ltd., PO Box 27, Cape Town, South Africa, Communications of E. Meyer, 1987

242. Prozesky JG. Director (Avionics), South African Airways Technical, Jan Smuts Airport, Johannesburg, South Africa, Communications of E. Meyer, 1987

243. Electron Microscopy Unit, Rhodes University, Grahamstown, South Africa. http://www.ru.ac.za/administrative/emu/. Retrieved 4 June 2001

244. Takahashi k, Rytting JH. Novel approach to improve permeation of ondansetron across shed snake skin as a model membrane. *J Pharm and Pharmacol.* **53**: 789-793 (2001)