Formulation, in vitro release and transdermal diffusion of anti-inflammatory gel preparations containing diclofenac salts.

by

HEIDI STEYN

(B.Pharm.)

Dissertation approved in the partial fulfilment of the requirements of the degree

MAGISTER SCIENTIAE

in the

Faculty of Health Sciences, School of Pharmacy (Pharmaceutics)

at the

North-West University

Supervisor: Prof. J.L. du Preez

Co-Supervisor: Prof. J. du Plessis

Co-Assistant supervisor: Dr. J.M. Viljoen

Potchefstroom

2010

This dissertation was written in the article format. Heidi Steyn was the primary author of this dissertation which includes an introductory chapter with sub-chapters, a full length article for publication in a pharmaceutical journal and annexure containing relevant experimental data. The work was carried out under the supervision and assistance of Prof. J.L. du Preez, Prof. J. du Plessis and Dr. J.M. Viljoen. The article contained in this dissertation is to be published in the International Journal of Pharmaceutics of which the complete guide for authors is contained in the annexure.

"Knowledge is a rich storehouse for the glory of the Creator and the relief of man's estate."
Francis Bacon

Abstract

Most individuals are influenced by pain at some stage in their lives. It can either be of acute or chronic nature. An acute pain condition initiates and is treated within a time span of 12 weeks. Chronic pain can, however, take substantially longer to treat. Chronic pain may last up to 6 months after the original injury was sustained. The after effects of chronic pain can, however, take years to heal, but physical and emotional scars may even last much longer than the initial chronic ailment.

In this study the skin was chosen as an area for delivery of non-steroidal anti-inflammatory drugs for the treatment of pain at the joint and muscle tissue regions. The stratum corneum (the topmost horny layer of the skin), however bars the effective movement of chemical substances across the skin as it forms part of the skin's function to protect the superficial tissue of the body against the external environment. It furthermore plays an important role in regulation of the movement of chemicals across the skin. Sweat pores and hair follicles can be utilised as pathways for the movement of chemical substances through the stratum corneum. Physical deformation ie, hydration of the top layer of the skin, may also enhance the movement of chemicals

The non-steroidal anti-inflammatory drug, diclofenac, has been evaluated for transdermal diffusion. Three different diclofenac salts were evaluated, namely diclofenac diethylamine, diclofenac hydroxyethyl pyrrolidine and diclofenac sodium. These salts have the potential to relieve systemic pain conditions. Diclofenac salts, however, possess physicochemical characteristics that are unfavourable for transdermal diffusion.

Pheroid™ delivery technology, as patented by the Northwest-University, was implemented as a method to enhance transdermal delivery of the diclofenac salts. During the study each of the diclofenac salts was formulated in a Pheroid™ and non-Pheroid™ formulation. All the formulations as well as corresponding retail products containing similar diclofenac salts were evaluated in order to determine which preparation had the most effective transdermal diffusion.

High performance liquid chromatograhphy was implemented in order to determine the concentration of each salt in their various preparations. The Pheroid™ and non-Pheroid™ formulations were also compared to retail products currently available. An active ingredient flux was determined by means of Franz cell diffusion studies. Membrane diffusion studies were utilised in order to determine whether the active ingredients were effectively released from the formulated preparations and market products.

Membrane diffusion studies determined that Arthruderm® (the retail product containing diclofenac sodium) had the most potential to effectively release the active ingredient from the formulation (median flux 28.36 ± 0.26 $\mu g/cm^2.h^{-1}$). Franz cell diffusion studies showed no flux values for any of the evaluated preparations, including the retail products. Concentrations obtained within the epidermis and dermis were determined through tape stripping of these areas. The largest concentration of active ingredient within the epidermis was obtained from the studies done on Voltaren® (the retail product containing diclofenac diethylamine) which was 7.27 $\mu g/cm^2.h^{-1}$ the largest value in the dermis was obtained from a non-Pheroid™ formulation containing diclofenac sodium (4.47 µg/ml).

Confocal laser scanning microscopy was utilised and the micrographs where evaluated to ensure that the diclofenac salts were effectively entrapped in the Pheroid™ delivery system.

Keywords: Transdermal delivery, Stability testing, Pheriod™ formulations and Diclofenac salts.

Opsomming

Pyn is 'n toestand wat meeste individue op een of ander stadium in hulle leeftyd affekteer. Dit kan van 'n akute of kroniese aard wees. Akute pyn klaar gewoonlik binne 12 weke na die besering op. Kroniese pyn kan egter baie langer neem voordat dit genees. Kroniese pyn kan maande tot jare neem voordat dit genees en die finansiele en emosionele implikasies wat dit agter laat kan veel langer neem om te genees as die oorspronklike pyntoestand.

In hierdie studie is die vel gekies as 'n area vir aflewereing van nie-steroiede anti-inflammatoriese middels vir die behandeling van pyn in die gewrigte en spierweefsel. Die stratum corneum (die boonste horingagtige deel van die vel) bied egter weerstand teen die effektiewe deurgang van chemiese stowwe as deel van sy rol om liggaamsweefsel te beskerm teen die eksterne omgewing. Die vel beheer die deurgang van chemiese stowwe tot die onderliggende weefsel. Bystrukture van die vel (sweetporiëe en haarfollikelskagte) kan gebruik word as weë vir die effektiewe deurvoer van chemiese stowwe deur die stratum corneum. Fisiese aanpassing van die velvorm kan ook gebruik word om die deurgang van stowwe te vergemaklik. Dit kan fisies aangepas word deur die boonste lae van die vel te hidreer om sodoende die deurlaatbaarheid te verbeter.

Die aktiewe bestanddeel wat bestudeer is vir transdermale deurgang is die nie-steroiede anti-inflammatoriese middel diklofenak. Drie verskillende diclofenaksoute is tydens die studie geëvalueer vir transdermale effektiwiteit, naamlik diklofenakdietielamien, diklofenak-hydroksieetielpirrolidien en diklofenaknatrium. Die soute het almal die potensiaal om effektiewe sistemiese verligting van pyn teweeg te bring. Die diklofenak soute toon egter ongunstige fisies-chemiese einskappe wat effektiewe deurgang van die soute deur die vel belemmer.

Die Pheroid™ afleweringstegnologie, soos gepatenteer deur die Noordwes Universiteit is geïmplementeer om die transdermale deurgang van die diklofenaksoute te verbeter. Tydens die studie is elkeen van die diklofenaksoute in 'n Pheroid™ en 'n nie-Pheroid™ preparaat geformuleer. Elkeen van hierdie preparate tesame met die kommersieel beskikbare produkte wat 'n soorteglyke diklofenaksout bevat, is geëvalueer om te bepaal wat die mees effektiewe formulering asook diklofenaksout is om the gebruik in transdermale produkte.

Elke sout se konsentrasie is bepaal deur middel van hoë verrigting vloeistof chromatografie. Flukse is bepaal deur van Franz-sel diffusiestudies gebruik te maak. Membraandiffusiestudies is gebruik om vrystelling van diklofenak uit die verskillende formulerings te evalueer.

Membraandiffusiestudies het getoon dat Arthruderm© (die handelsproduk wat diklofenak-natruim bevat) die potensiaal het om die aktiewe bestanddeel die effektiefste uit die preparaat uit vry te stel (mediaanfluks 28.36 ± 0.26 µg/cm^2.h). Franz-sel transdermale diffusie studies het geen transdermale fluks vir enige van die preparate getoon nie. Dit sluit al die geformuleerde preparate en die handelsprodukte in. Konsentrasies van elke aktiewe bestanddeel in die verskillende preparate, wat vasgevang is in die epidermis en die dermis van die vel is bepaal deur middel van kleefbandafstropingsstudies. Die grootste konsentrasie vasgevang in die epidermis was verkry vanaf Voltaren® (die handelsproduk wat dickofenakdietielamien bevat) nl. 7.27 µg/ml. Die grootste konsentrasie in die dermis was verkry vanaf 'n nie-Pheroid™ preperaat wat diklofenaknatruim bevat het (4.47 µg/ml).

Daar is van konfokaallaseraftastingsmikroskopie gebruik gemaak om te verseker dat die dickofenak soute wel vasgevang is in die Pheroid™.

Sleutelwoorde: Transermale aflewering, stabiliteits toesting, Pheroid™-formulerings en diklofenak soute.

Acknowledgements

To the **Lord my Saviour** the Father and son Jesus thank you for providing the opportunities that has brought me on this path up until now. Thank you for being my rock and my safe haven to turn to, only You can truly make free. Psalm 18:1-2.

My **parents** (Franscois and Olga) thank you for believing and supporting me in every possible way, I am also deeply grateful for the support and encouragement of my **siblings** (Linda, Anemie and Rian), I could always look up to you and gain wisdom from the diversely special ways that you lead your lives.

Linda Boddington thank you for your help, support and encouragement to follow a life of research and thank you for being my "Ousus".

Willem Smith you were there to face every setback and every tear with me and I am eternally grateful, thank you for you love, understanding, support and encouragement it means the world to me.

My friends, colleagues and comrades (Juanita, Sarita and Tizané) thank you for all the support you are the flowers of my soul.

Professor J.L. du Preez, my supervisor, thank you for the support and guidance. It was an honour to learn from someone with so much experience in life as in research.

Professor Jeanetta du Plessis, my co-supervisor, thank you for sharing your knowledge and encouragement, thank you for the friendly environment and time devoted to the wellbeing of the "cosmo-girls".

Doctor J.M. Viljoen, thank you for the encouragement and the hours spent proofreading my literature.

Doctor M. Gerber, thank you for your help, and being involved in every aspect of this study, thank you for trying your best to accommodate me and help me as quickly as possible.

Professor Jan du Plessis, thank you for the friendly advice and support in the statistical analysis of my data.

Ms. L Nieuwoudt thank you for the help and support in producing Pheroid™ formulations.

Mr. F Viljoen, thank you for your support and friendship in the ATL.

The **National Research Foundation** (NRF) and the **Unit for Drug Research and Development** at the North-West University, Potchefstroom campus for the funding of this project.

Table of contents

Abstract	I
Opsomming	III
Acknowledgments	VI
Table of contents	VII
List of figures	XX
List of tables	XXII
CHAPTER 1: Introduction and problem statement	1
1.1 Introduction	1
1.2 Study objectives	2
1.3 Project design	2
1.4 *References*	4
CHAPTER 2: PHYSIOLOGY OF PAIN AND PAIN MANAGEMENT	5
2.1 Introduction	5
2.2 Pathogenesis of pain	5
2.2.1 Neural pathways of the somatosensory system	6
2.2.1.1 Anatomy of the somatosensory system	6
2.2.1.2 Neurotransmitters in the somatosensory pathway	6
2.2.2 Inflammation (peripheral sign of pain)	7
2.2.3 The role of mediators in the inflammatory response	7
2.2.3.1 Vasodilatation and increased permeability to protein	8

2.2.3.2 Chemotaxis 8

2.4 Overview of the management of pain 9

2.4.1 Opium 9

2.4.2 Morphine 9

2.4.3 Acetanilide and the salicylates 10

2.5 Modern pain relief regimes 10

2.5.1 Mild to moderate pain (1-3) 10

2.5.2 Moderate to severe pain (4-6) 10

2.5.3 Severe pain (7-10) 11

2.6 Pain treatment utilising non-steroidal anti-inflammatory drugs (NSAIDS) 11

2.6.1 Physiological enzymes involved in anti-inflammatory treatment 11

2.6.1.1 Phospholipase A2 12

2.6.1.2 Physiological effects of prostaglandin 12

2.7 Classification of analgesic and anti-inflammatory drugs 13

2.7.1 Drugs that have an analgesic and weak anti-inflammatory effect 13

2.7.2 Drugs that have an analgesic and mild anti-inflammatory effect 13

2.7.3 Drugs that have an analgesic and strong anti-inflammatory effect 13

2.7.4 DMARD (Disease modifying anti rheumatic drugs) 13

2.8 The current topical treatment of pain utilising NSAIDs 14

2.9 Treatment of pain using diclofenac 14

2.9.1 Introduction 14

2.9.2 Pharmacological properties 14

2.9.3 Pharmacokinetics and metabolism 15

2.9.4 Toxic effects 15

2.10 Physicochemical character of diclofenac salts 15

2.10.1 Physicochemical and biopharmaceutical character of diclofenac diethylamine 16

2.10.1.1 Molecular structure 16

2.10.1.2 Chemical characteristics 16

2.10.1.3 Physical characteristic 16

2.10.2 Physicochemical and biopharmaceutic character of diclofenac hydroxyethylpyrrolidine 17

2.10.2.1 Molecular structure 17

2.10.2.2 Chemical characteristics 17

2.10.2.3 Biopharmaceutic characteristics 18

2.10.3 Physicochemical and biopharmaceutic character of diclofenac sodium 19

2.10.3.1 Molecular structure 19

2.10.3.2 Chemical characteristics of diclofenac sodium 19

2.10.3.3 Biopharmaceutical parameters 20

2.11 Transdermal delivery of diclofenac salts 20

2.12 Transdermal delivery of NSAIDs 21

2.12.1 Advantages of transdermal delivery 21

2.13 Structure and barrier function of the skin 22

2.13.1 Anatomy of the skin 22

2.13.2 Percutaneous absorption 22

2.13.2.1 Transappendageal - diffusion through sweat ducts 23

2.13.2.2 Diffusion through hair follicles 23

2.13.2.3 Transcellular pathway 23

2.13.2.4 Intercellular pathway 24

2.13.3 Functions of the skin 24

2.13.3.1 Containment 24

2.13.3.2 Microbial barrier 24

2.13.3.3 Chemical barrier 25

2.13.3.4 Radiation barrier 25

2.13.3.5 Electrical barrier 25

2.13.3.6 Thermal barrier and body temperature regulation 26

2.14 Physiological factors affecting transdermal drug delivery 26

2.14.1 Healthy skin 26

2.14.2 Diseased skin 28

2.14.3 Compromised skin 28

2.14.4 Skin age 29

2.14.5 Site on the body 30

2.14.6 Race 30

2.15 *Other factors that influence transdermal drug delivery* 30

2.15.1 Ideal properties of a molecule that effectively penetrates the skin 31

2.16 Mathematics of skin permeation 31

2.16.1 An infinite dose at steady state 31

2.16.2 A finite dose deposited on the membrane 32

2.17 Penetration enhancement 33

2.17.1 Developing absorption-enhanced topical products 33

2.17.2 Chemical enhancers 33

2.17.3 Physical delivery enhancement 34

2.17.3.1 Iontophoresis 34

2.17.3.2 Electroporation 35

2.17.3.3 Phonophoresis 35

2.17.3.4 PowderJect 35

2.17.3.5 Intraject 36

2.17.4 Drug delivery vehicles 36

2.17.4.1 Liposomes 36

2.17.4.2 Transferosomes 36

2.17.4.3 Ethosomes 36

2.17.4.4 Niosomes 36

2.18 Possible solutions to the problem of transdermal delivery of diclofenac 37

2.18.1 Advantages of Pheroid™ incorporation 37

2.19 Summary 38

2.20 References 39

CHAPTER 3: ARTICLE FOR PUBLICATON IN THE INTERNATIONAL JOURNAL OF PHARMACEUTICS 42

Abstract 43

1. Introduction 44

2. Materials and Methods 45

2.1. Reagents and raw materials 45

2.2. Methods 45

2.2.1. Preparation of gel formulations containing different diclofenac salts 45

2.2.1.1. Pheroid™ gel preparation 45

2.2.1.2. Non-Pheroid™ gel preparation 46

2.2.2. Stability determinations 46

2.2.2.1. High performance liquid chromatography method for stability testing (Assay) 46

2.2.2.1.1. Sample preparation for assay determination 47

2.2.2.1.2. Standard solution preparation 47

2.2.2.2. Aesthetics 47

2.2.2.3. Confocal laser scanning microscopy 47

2.2.2.4. Mass variation 47

2.2.2.5. pH variation 48

2.2.2.6. Viscosity 48

2.2.3. Physicochemical properties 48

2.2.3.1. High performance liquid chromatography method for the determination of physical properties and skin diffusion 48

2.2.3.1.1. Standard preparation 48

2.2.3.2. Aqueous solubility determinations 48

2.2.3.3. Octanol-buffer partition coefficient determinations 49

2.2.4. Diffusion determinations 49

2.2.4.1. Human skin samples 49

2.2.4.2. Method of skin diffusion 49

2.2.5. Tape stripping method 50

2.2.3.4.3. Statistical determinations 51

3. Results and discussion 52

3.1. Stability testing 52

3.1.1. Assay 52

3.1.2 pH Variation 53

3.1.3. Viscosity variation 53

3.1.4. Aesthetics 54

3.1.5. Confocal laser scanning microscopy 54

3.1.6. Mass variation 54

3.2. Physicochemical properties 55

3.2.1. Aqueous solubility and Log D determinations 55

3.3. Skin diffusion 55

3.3.1. *In vitro* permeation studies 55

3.3.2. Tape stripping data 55

3.3.3. Statistical analysis of data 56

4. Conclusions 58

Acknowledgments 59

References 60

CHAPTER 4: FINAL CONCLUSIONS AND FUTURE PERSPECTIVES 66

4.1 Study conclusions 66

References 68

ANNEXURE A: Method development and validation for the chromatographic determination of diclofenac salts 69

A.1 Introduction 69

A.2 HPLC (High performance liquid chromatography) method validation for diclofenac 69

A.2.1 Methods and Reagents 69

A.2.2 Standard preparation 70

A.3.1 Validation of test procedure and acceptance criteria 71

A.3.1.1 Linearity 71

A.3.1.2 Accuracy 72

A.3.1.3 Precision 73

A.3.1.4 Inter-day precision 74

A.3.1.4.1 Day 2 inter-day precision 75

A.3.1.4.2 Day 3 inter-day precision 75

A.3.1.5 Ruggedness 76

A.4 HPLC (High performance liquid chromatography) method validation for Pheroid™ and non-Pheroid™gel preparations containing diclofenac 77

A.4.1 Methods and Reagents 77

A.4.2 Validation of test procedure and acceptance criteria 79

A.4.2.1 Linearity 79

A.4.2.1.1 Linearity of BHA 79

A.4.2.1.2 Linearity of BHT 81

A.4.2.1.3 Linearity of diclofenac 82

A.4.2.1.4 Linearity of methyl paraben 83

A.4.2.1.5 Linearity of vitamin E 84

A.4.2.2 Accuracy 85

A.4.2.2.1 Accuracy of BHA 85

A.4.2.2.2 Accuracy of BHT 86

A.4.2.2.3 Accuracy of diclofenac 87

A.4.2.2.4 Accuracy of methyl paraben 88

A.4.2.2.5 Accuracy of vitamin E 89

A.4.2.3.1 Precision of BHA 90

A.4.2.3.2 Precision of BHT 91

A.4.2.3.3 Precision of diclofenac 92

A.4.2.3.4 Precision of methyl paraben 93

A.4.2.3.5 Precision of vitamin E 94

A.4.2.4 Inter-day precision 95

A.4.2.4.1 Day 2 inter day precision of BHA 95

A.4.2.4.2 Day 3 inter-day precision BHA 96

A.4.2.4.3 Day 2 inter-day precision of BHT 97

A.4.2.4.4 Day 3 inter-day precision BHT 98

A.4.2.4 5 Day 2 inter-day precision of diclofenac 99

A.4.2.4.6 Day 3 inter-day precision of diclofenac 100

A.4.2.4.7 Day 2 inter-day precision of methyl paraben 101

A.4.2.4.8 Day 3 inter-day precision methyl paraben 102

A.4.2.4.9 Day 2 inter-day precision of vitamin E 103

A.4.2.4 9 Day 3 inter-day precision of vitamin E 104

A.4.2.5 Ruggedness 104

A.4.2.5.1 Ruggedness of BHA 105

A.4.2.5.2 Ruggedness of BHT 106

A.4.2.5.3 Ruggedness of diclofenac 107

A.4.2.5.4 Ruggedness of methyl paraben 108

A.4.2.5.5 Ruggedness of vitamin E 109

A.5 Conclusion 110

A.6 References 111

ANNEXURE B: Method for the determination of solubility and Log D

in phosphate buffer 112

B.1 Introduction 112

B.2 Preparation of phosphate buffer pH 7.4 (PBS) 112

B.3 Method for the determination of the solubility of diclofenac in PBS 112

B.4 Method for determining octanol-buffer partitioning coefficient (log D) 113

B.4.1 Saturation of n-octanol and PBS 113

B.4.2 Method for saturation of octanol and PBS 113

B.4.3 Method for determining log D values 113

B.5 Results 114

B.6 Conclusion 115

B.7 References 116

ANNEXURE C: Formulation of Pheroid™ and non-Pheroid™ formulations containing diclofenac salts 117

C.1 Introduction 117

C.2 Methods and materials 117

C.2.1 Ingredients of Pheroid™ preparations containing a diclofenac salt 117

C.2.2 Method for the preparation of Pheroid™ gel containing diclofenac salts 118

C.3.1 Ingredients of non-Pheroid™ preparations containing a diclofenac salt 119

C.3.2 Method for the preparation of non-pheroid™ gel containing diclofenac salts 119

C.4 Summary 120

ANNEXURE D: Transdermal and membrane diffusion studies of preparations containing diclofenac salts 121

D.1 Introduction 122

D.2 Membrane diffusion studies 122

D.2.1.1 Standard solution preparation 122

D.2.1.2 Membrane diffusion preparation 122

D.2.1.3 Membrane diffusion 122

D.3 Results 123

3.10 Membrane diffusion discussion 134

D.4 Skin diffusion 135

D.4.1 Method for skin diffusion studies 135

D.4.1.1 Standard solution preparation 135

D.4.1.2 Preparation for diffusion 135

D.4.1.3 Diffusion 136

D.4.1.4 Tape stripping 136

D 5.1 Results 137

D.5.1.1 Skin diffusion flux values 137

D.5.1.2 Tape stripping results 137

D 6 Discussion 139

D.7 References 140

ANNEXURE E: Guide for authors 141

List of figures

CHAPTER 2

Figure 2.1: Brain arachidonic acid metabolic pathway via cyclooxygenases. 12

Figure 2.2: Structure of Diclofenac diethylamine. 16

Figure 2.3: Structure of diclofenac hydroxyethylpyrrolidine. 17

Figure 2.4: Structure of diclofenac sodium. 19

Figure 2.5: The functional layers of the skin. 22

Figure 2.6: Typical permeation profile for an infinite dose application to human skin membranes. 32

CHAPTER 3

Figure 1: The tape stripping epidermis and the dermis concentration values (µg/ml) for **(a)** diclofenac diethylamine, **(b)** diclofenac hydroxyethylpyrrolidine and **(c)** diclofenac sodium; obtained after skin diffusion studies 65

ANNEXURE A

Figure A1: Linear regression curve of diclofenac. 72

Figure A2: Curve indicating linearity of BHA. 80

Figure A3: Curve indicating linearity of BHT. 81

Figure A4: Curve indicating linearity of diclofenac. 82

Figure A5: Curve indicating linearity of methyl paraben. 83

Figure A6: Curve indicating linearity of vitamin E. 84

ANNEXURE D

Figure D1: Average membrane cumulative concentration amounts of diclofenac diethylamine Pheroid™ gel. 123

Figure D2: Average membrane cumulative amounts diffused of diclofenac diethylamine *non-Pheroid™ gel.* 124

Figure D3: Box plots of the Pheroid™ and non-Pheroid™ preparations containing diclofenac diethylamine show black lines to illustrate median flux and red lines to illustrate the average flux values. 125

Figure D4: Cumulative amounts diffused of diclofenac hydroxyethylpyrrolidine Pheroid™ gel. 126

Figure D5: Cumulative amounts diffused of diclofenac hydroxyethyl non-Pheroid™ gel.

Figure D6: *Cumulative amounts diffused of Panamor® gel.* 127

Figure D7: Box plots of the Pheroid™, non-Pheroid™ and retail product, containing diclofenac hydroxyethylpyrrolidine show black lines to illustrate median flux and red lines to illustrate the average flux values. 128

Figure D8: Cumulative amounts diffused of diclofenac sodium Pheroid™ gel. 129

Figure D9: Cumulative amounts diffused of diclofenac sodium non-Pheroid™ gel. 130

Figure D10: Cumulative amounts diffused of Arthruderm® lotion. 131

Figure D11: Box plots of the Pheroid™, non-Pheroid™ and retail product, containing diclofenac sodium show black lines to illustrate median flux and red lines to illustrate the average flux values. 132

List of tables

CHAPTER 2

Table 2.1: *Chemical characteristic of diclofenac diethylamine.* 16

Table 2.2: Table indicating physical characteristic of diclofenac diethylamine. 17

Table 2.3: Chemical characteristics of diclofenac hydroxyethylpyrrolidine. 18

Table 2.4: Biopharmaceutical characteristics of diclofenac hydroxyethylpyrrolidine. 18

Table 2.5: Chemical character of diclofenac sodium. 19

Table 2.6: *Biopharmaceutical parameters of diclofenac sodium.* 20

CHAPTER 3

Table 1: p-Values depicting statistical significant variance between the epidermal values obtained. 63

Table 2: p-Values depicting statistical significant variance between the dermal values obtained. 64

ANNEXURE A

Table A1: Peak area values of diclofenac. 71

Table A2: Table indicating accuracy values of diclofenac samples in methanol and water. 73

Table A3: Precision values of diclofenac sample in methanol and water. 74

Table A4: Inter day precision values of a diclofenac sample in methanol and water on day 2. 75

Table A5: Inter-day precision values of a diclofenac sample in methanol and water on day 3. 75

Table A6: Table indicating ruggedness values of a diclofenac sample in methanol and water. 76

Table A7: Gradient table of mobile phase ingredient ratios. 78

Table A8: Linearity values of BHA in preparation sample. 79

Table A9: Linearity values of BHT in preparation sample. 81

Table A10: Linearity values of diclofenac salt in preparation sample. 82

Table A11: Linearity values of methyl paraben in preparation sample. 83

Table A12: Linearity values of vitamin E in preparation sample. 84

Table A13: Accuracy values of BHA in the preparation sample. 85

Table A14: Accuracy values of BHT in the preparation sample. 86

Table A15: Accuracy values of diclofenac in the preparation sample. 87

Table A16: Accuracy values of methyl paraben in the preparation sample. 88

Table A17: Accuracy values of vitamin E in the preparation sample. 89

Table A18: Precision values of BHA. 90

Table A19: Precision values of BHT. 91

Table A20: Precision values of diclofenac. 92

Table A21: Precision values of methyl paraben. 93

Table A22: Precision values of vitamin E. 94

Table A23: Inter-day precision of BHA day 2. 95

Table A24: Inter-day precision of BHA on day 3. 96

Table A25: Inter-day precision values of BHT on day 2. 97

Table A26: Inter-day precision values of BHT on day 3. 98

Table A27: Inter-day precision of diclofenac on day 2. 99

Table A28: Inter-day precision of diclofenac on day 3. 100

Table A29: Inter-day precision of methyl paraben on day 2. 101

Table A30: Inter-day precision of methyl paraben on day 3. 102

Table A31: Inter-day precision of vitamin E on day 2. 103

Table A32: Inter-day precision of vitamin E on day 3. 104

Table A33: Ruggedness of BHA. 105

Table A34: Ruggedness of BHT. 106

Table A35: Ruggedness of diclofenac. 107

Table A36: Ruggedness of methyl paraben. 108

Table A37: Ruggedness of vitamin E. 109

ANNEXURE B

Table B1: Solubility and log D values of diclofenac diethylamine, diclofenac hydroxyethylpyrrolidine and diclofenac sodium. 114

ANNEXURE C

Table C1: Percentages of ingredients in Pheroid™ formulations containing a diclofenac salt. 118

Table C2: Percentages of ingredients in a diclofenac containing non-Pheroid™ gel preparation. 119

ANNEXURE D

Table D1: Average and median flux values of preparations containing various diclofenac salts. 134

Table D2: Concentration of diclofenac in epidermis and dermis after application of various preparations containing diclofenac salts. 138

CHAPTER 1

Introduction and problem statement

1.1 Introduction

The most commonly used drugs to reduce inflammation and pain are the non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs inhibit the cyclooxygenase-2 pathway at the area of pain. Unfortunately it inhibits the cyclooxygenase-1 pathway which is responsible for the protection of the stomach mucosa. This drug therefore inhibits the biosyntheses of prostaglandin in vivo and in vitro. This leads to the main side effect of the NSAIDs, damage of the stomach wall (Mitchell and Warner, 1999:1122).

The skin can be used for drug delivery in order to overcome the above mentioned problem in two ways, namely transdermally and dermally (in this study the focus will be on the transdermal route). Transdermal delivery involves percutaneous delivery of the drug across the skin into the systemic circulation (i.e. the blood stream) (Brown *et al.*, 1995:135).

Transdermal administration offers several important advantages over other routes of delivery. Some advantages are: ease of use, ease of withdrawal, and the bypass of the first pass metabolism. The skin is, however, a barrier against the penetration of external chemicals and drugs (Ghafourian *et al.,* 2004:114). The main barrier of absorption in the skin is the stratum corneum. This is the outermost layer of the skin and is rich in keratin. Penetration enhancers are usually utilised to overcome the barring effect of the stratum corneum.

An improved diclofenac formula with a high degree of skin permeation could be useful for the treatment of not only locally inflamed skin tissues, but also inflammatory and painful states of the supporting structures of the body – bones, ligaments, joints, tendons, and muscles (Galer *et al.,* 2000:293).

The efficacy of a topical formulation can be evaluated using two subdivisions namely the amount of drug that penetrates the skin (biopharmaceutics) and the demonstration of the

localised effect (pharmacological). In this study the focal point will be the biopharmaceutic study.

1.2 Study objectives

The objectives of this study were to:

- compare the *in vitro* release and transdermal diffusion of three different diclofenac salts (diclofenac sodium, diclofenac diethylamine and diclofenac hydroxyethylpyrrolidine).
- investigate the influence of the physiochemical characteristics of the three diclofenac salts on the transdermal absorption.
- evaluate the different formulations for the transdermal delivery of the three salts.
- compare the formulated preparations to commercially available formulations.

1.3 Project design

The main objective of the study was to determine if Pheroid™ technology could be incorporated to enhance the weak skin permeation of diclofenac salts. The study was undergone in the following order. A literature study was done to determine the physiochemical character and factors governing the absorption and formulation of the three diclofenac salts. Thereafter a high performance liquid chromatography method was developed which could be implemented in determining flux obtained from membrane and skin diffusion studies.

The *in vitro* release (from within formulation) of the three diclofenac salts was determined by means of a membrane diffusion study. The membrane diffusion study was done using a similar Franz cell experimental setup as in the transdermal studies fitted with a cellulose acetate membrane. These samples were then analysed making use of high performance liquid chromatography. Peak area values obtained were then used to determine the average flux of active ingredient from within each formulation. Thereafter Franz cell diffusion studies, using human skin samples, were done to determine absorption through the skin samples into the systemic circulation. High performance liquid chromatographic analysis of the receptor cell fluid was done to determine the amount of flux into the systemic circulation using values obtained from human skin sample diffusion.

After completion of the Franz cell diffusion studies tape stripping was performed to determine the concentration contained within the epidermal and dermal layers of the skin. The final objective of the study was to determine stability of diclofenac containing preparations when subjected to accelerated stability conditions. The following properties were evaluated over a period of 6 months; pH, rheological properties, mass variation, visual assessment, particle size (utilising confocal laser scanning microscopy) and assay of adjuvants and active ingredients.

1.4 References

MITCHELL, J.A. & WARNER, T.D. 1999 Cyclooxygenase-2: pharmacology, Physiology, biochemistry and relevance to NSAID therapy. *British Journal of Pharmacology.* 128:1121-1132 [In Science direct: http://www.sciencedirect.com] [Date of use: 23 Feb. 2008]

GHAFOURIAN, T., ZANDASRAR, P., HAMISHEKAR, H. & NOKHOCHI, A. 2004. The effect of penetration enhancers of drug delivery through skin: a QSAR study. *Journal of Controlled Release.* 99(1):113-125 [In Science direct: http://www.sciencedirect.com] [Date of use: 23 Feb. 2008]

GALER, B.S., ROWBOTHAM, M., PERANDER, A., DEVERS, A. & FRIEDMAN, E. 2000 Topical diclofenac path relieves minor sports injury pain: results of a mulitcentre controlled clinical trail. *Journal of Pain Symptoms Management.* 19:287-294 [In Science direct: http://www.sciencedirect.com] [Date of use: 23 Feb. 2008]

BROWN, M.B., MARRIOTT, C. & MARTIN, G.P. 1995 The effect of hyaluronan on the in vitro deposition of diclofenac within the skin. *International Journal of Tissue reactions.* XVII:133-140 [In Science direct: http://www.sciencedirect.com] [Date of use: 23 Feb. 2008]

CHAPTER 2

PHYSIOLOGY OF PAIN AND PAIN MANAGEMENT

2.1 Introduction

Pain is a very complex phenomenon that is defined by the international association for the study of pain as the unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (International association for the study of pain, 2009:1). For the purpose of this study simple acute pain will be considered and discussed. Acute pain is defined as pain that is usually temporary and results from something specific, such as a surgery, an injury, or an infection. The acute pain reaction acts as a measure to kick-start healing through occluding the injured area by means of an inflammatory process. The inflammatory process is, however, preceded by a sensory neuron cascade (Woolf, 1995:169).

2.2 Pathogenesis of pain

Pain differs from other somatosensory modalities due to a set of reactions that occur in the pain pathway. Directly after the transduction of the first stimuli in the afferent neuron, a series of changes could occur in the pain pathway, both excitatory as well as inhibitory. Excitatory changes lead to an increased painful experience known as hyperalgesia and can last for hours in the absence of the original stimulus. Hyperalgesia is more frequently found in chronic pain conditions than in an episode of acute pain (Widmaier *et al.,* 2004:219).

Analgesia, however, is the selective suppression of pain without effects on consciousness or other sensations. Analgesia can be achieved by electrical stimulation of specific areas of the central nervous system that form part of the inhibitory pain pathways and could produce a profound reduction in pain (Widmaier *et al.,* 2004:220). Non-steroidal anti-inflammatory drugs mediate analgesia by inhibition of the enzymes that synthesise prostaglandins on a peripheral level; and by altering the peripheral response of the body by acting on the somatosensory system (Cashman, 1996:513).

2.2.1 Neural pathways of the somatosensory system

2.2.1.1 Anatomy of the somatosensory system

Two major somatosensory pathways exist, organised differently in the spinal cord as well as the brain. The ascending anterolateral pathway makes its first synapse between the sensory receptor neuron and a second neuron located in the gray matter of the spinal cord. A second neuron projects through the anterolateral column of the cord to the thalamus where it synapses on cortically projecting neurons. The anterolateral pathway processes pain and temperature information (Widmaier *et al.,* 2004:221).

The second major pathway is the dorsal column pathway. This pathway moves through the section of white matter where the first synapse occurs. In the somatosensory cortex, the endings of the axons of the specific pathways are grouped according to the peripheral location of the receptors that give input to the pathways. The parts of the body where the fibres are densely grouped together are the fingers, thumbs and lips (Widmaier *et al.,* 2004:221).

2.2.1.2 Neurotransmitters in the somatosensory pathway

Stimuli interpreted by the primary somatosensory cortex of the brain as being painful, will be initiated in the periphery by the stimulation of a nociceptor. The stimulus could be mechanical (pin prick mechanical deformation), thermal or chemical (inflammatory peptides). Inflammatory agents are released in the inflammatory cascade when a cell membrane is disrupted. One of the mediators that play an important role in the inflammatory cascade is phospholiphase A2 (Widmaier *et al.,* 2004:219). These transmitters can also be released by immune system cells that migrate into the injured area. These transmitters causes activation by binding to the specific ligand-sensitive ion channels on the nociceptor plasma membrane. Nociceptors are free nerve endings without any form of specialization. When a stimulus is triggered in the nociceptor the impulse travels from the receptor in the afferent neuron of the peripheral nerve to a synapse with a projection neuron (Widmaier *et al.,* 2004:219).

Two of these neurotransmitters released at the synapses in the central nervous system are glutamate and substance P. Activation of glutamate and substance P receptors results in excessive sensitivity and a heightened experience of pain sensation. Non-steroidal anti-

inflammatory drugs, however, play an important role in blocking the increased sensitivity towards pain (Mamberg & Yaskh, 1992:1276).

Some of the neurons in these inhibitory pathways of the central nervous system release endogenous opioids. The analgesic effects of opioids arise from their ability to directly inhibit the ascending transmission of nociceptive information from the spinal cord dorsal horn and to activate pain control circuits that descend from the midbrain, to the spinal cord. The μ-opioid receptor mRNA is seen throughout the peri-aqueductal grey (PAG), pontine reticular formation, median raphne magnus and adjacent gigantocellular reticular nucleus in the rostral ventromedial medulla (RVM) and spinal cord. Thus, infusion of morphine which binds to and stimulates the opioid receptors in the spinal cord at the level of entry of the active nociceptor fibres, can provide relief in many cases of intractable pain. This is separate from the effects of morphine on the brain (Widmaier *et al.,* 2004:220). The following sections will discuss the inflammatory process as a result of the central nervous system pain cascade.

2.2.2 Inflammation (peripheral sign of pain)

The inflammatory response of the body is defined as the fundamental response by the body to disease and injury; characterised by the classical signs of (pain) dolor, (warmth) calor, (rubor) redness and (tumor) swelling. The functions of the inflammatory response are to destroy or inactivate foreign invaders and to set the stage for tissue repair. The main mediators of inflammation are the cells that function as phagocytes. The most important phagocytes are neutrophils, macrophages and magrophage-like cells. Chemical mediators also play an important additional role in regulating the inflammatory process, resulting in a complex cascade of physiological events (Widmaier *et al.*, 2004:699).

2.2.3 The role of mediators in the inflammatory response

Mediators fall into two basic categories: peptides generated in the infected/inflamed area and substances secreted into the extracellular fluid from cells that exist in the inflamed area or have migrated into the area (Widmaier *et al.,* 2004). The inflammatory response will be described in the following sections.

2.2.3.1 Vasodilatation and increased permeability to proteins

Chemical mediators dilate most of the micro-circulation vessels in a damaged area. Mediators cause the local capillaries and venules to become permeable to proteins by inducing their endothelial cells to contract, opening spaces between them, through which protein then moves (Widmaier *et al.,* 2004b:699).

The above mentioned process has two functions:

- Increased blood flow to the inflamed area increases the delivery of proteins and leukocytes.
- Increased permeability to protein ensures that the plasma proteins that participate in inflammation can gain entry to the interstitial fluid.

The net filtration of plasma into the interstitial fluid causes oedema. Oedema accounts for the swelling in an enflamed area (Widmaier *et al.,* 2004b:700).

2.2.3.2 Chemotaxis

When inflammation occurs, circulating neutrophils begin to move out of the blood across the endothelium of capillaries and venules to enter the inflamed area. This process is known as chemotaxis. It involves a variety of protein and carbohydrate adhesion molecules on both the endothelial cell and the neutrophil. Chemotaxis also regulates the release of messenger molecules in the inflamed area. These messengers are collectively termed chemoattractants (Widmaier *et al.,* 2004:700).

In the first stage, the neutrophil is loosely bound to the endothelial cells by adhesion molecules. This event, known as margination, is associated with rolling of the neutrophil along the vessel surface. It permits the neutrophil to be exposed to chemoattractants in the injured area. These chemoattractants act on the neutrophil to induce the rapid appearance of another class of adhesion molecules in its plasma membrane. Thus, the neutrophils collect along the site of injury rather than being washed away with the flowing blood (Widmaier *et al.,* 2004:700).

The next stage is named diapedesis. In this stage a narrow projection of the neutrophil is inserted into the space between the endothelial cells and the entire neutrophil moves into the interstitial

fluid. This mechanism helps with the migration of large numbers of neutrophils into the inflamed area. Damaged cells release chemoattractants which attract the neutrophils to the inflamed area (Widmaier *et al.,* 2004:700).

Movement of leukocytes from the blood into the damaged area is not limited to neutrophils. Monocytes follow later, and once in the tissue, they undergo anatomical and functional changes that transform them into macrophages. During chemotaxis multiple types of leukocytes migrate to the inflamed area. Multiple adhesion molecules that are relatively distinct for the different leukocytes are controlled by different sets of chemoattractants. The type of chemoattractant and responding leukocyte depend on the type of inflammation (Widmaier *et al.,* 2004b:701).

2.4 Overview of pain management

Due to the change in the concepts of pain, changes in the treatment have also occurred resulting in a multidisciplinary approach. Currently treatment of pain involves a combination of medical treatment, physical therapy and psychological approaches such as cognitive behavioural group therapy. Methods for modulation inputs have also been developed, for example relaxation, hypnosis, acupuncture, TENS (transcutaneous electrical nerve stimulation) and heat packs (Grant, 1998:6).

Pain is a product of science and science is product of culture; culture is constantly changing. Pain is not a fixed concept, but an evolving thing (Grant, 1998:6). The following sections depict the active ingredients utilised in pain management.

2.4.1 Opium

One of the most commonly used substances for the treatment of pain was opium derived from the poppy flower, *Papaver somniferum* (Liebeskind, 1998).

2.4.2 Morphine

Friedrich Wilhelm Serturner isolated crystals of a powerful analgesic agent in 1803. These crystals where obtained from crude opium. The chemical was named morphine, after Morpheus, the Greek

god of dreams. According to Meldrum, (2003:2740) it would have been introduced on the point of a lancet or a solution could be washed into a wound.

2.4.3 Acetanilide and the salicylates

Towards the end of the eighteenth century, German chemical companies introduced new commercially formulated products containing the compounds: acetanilide and the salicylates, which effectively relieved moderate pain, although not without side effects. The salicylates, for example, might induce gastric pain, even ulcers. In 1899, the Bayer® Company introduced a stable, easily tolerated salicylate, acetylsalicylic acid, which under the name Aspirin quickly became the best selling medication in the world (Meldrum, 2003:2470).

2.5 Modern pain relief regimes

After the development of different drugs for the treatment of pain, the modern health care practitioner has proposed the following regimes used for the treatment of pain.

2.5.1 Mild to moderate pain (1-3)

For pain at the low end of the visual analogue scale (1-10), over the counter medications, such as acetaminophen (also known as paracetamol) or a non-steroidal anti-inflammatory drug (NSAID), such as ibuprofen would be prescribed (Meldrum, 2003:2470).

2.5.2 Moderate to severe pain (4-6)

If over-the-counter medication did not relieve your pain, or you had moderate to severe pain, one of the many types of opioids would be prescribed. Also known as narcotics, these drugs are similar to natural substances produced by the body to control pain, called endorphins, and are the strongest pain relievers available. Opioids prescribed for moderate to severe pain include dihydrocodeine, propoxyphene and tramadol. Tylenol® or a NSAID may be added to the opioid in a treatment regime (Meldrum, 2003:2470).

2.5.3 Severe pain (7-10)

For severe pain or pain that was not relieved by one of the above opioids and/or over-the-counter medication, a stronger opioid would be prescribed. Opioids for severe pain include morphine, oxycodone, hydromorphone, methadone, levophanol or fentanyl. A non-opioid analgesic and/or adjuvant drug may be added as circumstances require (Meldrum, 2003:2470).

2.6 Pain treatment utilising non-steroidal anti-inflammatory drugs (NSAIDs)

Non-steroidal anti-inflammatory drugs are used for the treatment of pain due to their action on cyclooxygenase (COX), an enzyme group. There are two forms of COX termed COX-1 and COX-2. COX-1 is found in most normal cells and tissues. COX-2 is induced in settings where inflammation is mediated by cytokines and inflammatory mediators. COX-2 is constitutively expressed in certain areas of the brain and kidneys. COX-1 but not COX-2 is constitutively expressed in the stomach. This accounts for markedly reduction of gastric toxicity with the use of selective inhibitors of COX-2. NSAIDs are excreted either by glomerular filtration or by tubular secretion. These inhibitors could roughly be divided into 2 groups, those with a short half-life (< than 6 hours) and those with long half-lifes (>10 hours) (Fiorucci, 2001:232).

2.6.1 Physiological enzymes involved in anti-inflammatory treatment

To understand how the anti-inflammatory agents work the following cascade illustrated in section 2.6.1.1 has been included.

2.6.1.1 Phospholipase A2

The phospholiphase A2 cascade is only set off once the enzyme is triggered by trauma, ischemia, hormones or bradykinin.

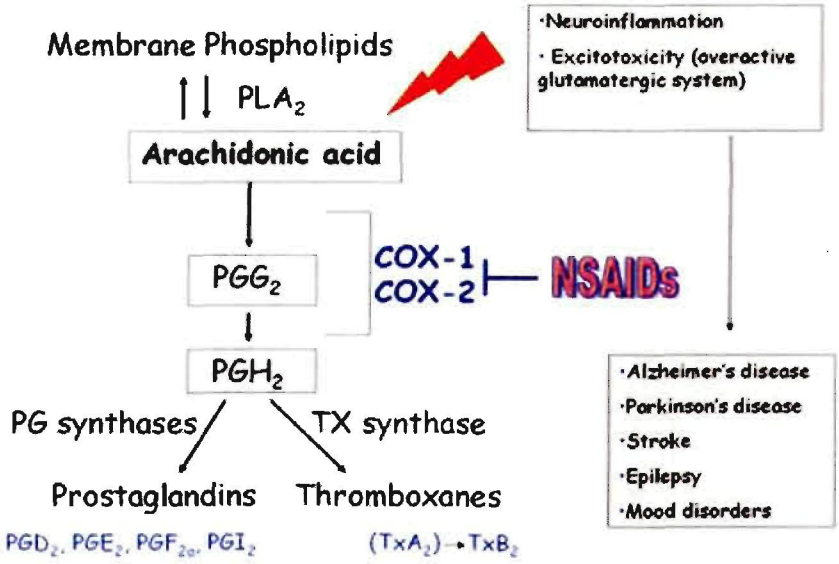

Figure 2.1: Brain arachidonic acid metabolic pathway via cyclooxygenases (www.neuroscience.nih.gov).

COX-1 is constitutive, in other words it carries out "housekeeping functions" and is always present, whereas COX-2 is induced by inflammatory cells.

2.6.1.2 Physiological effects of prostaglandin

Prostaglandins acts by inhibiting the formation of stomach ulcers. It increases the mucus content and lowers the acid content of the stomach. Prostaglandins are enzymatically derived from fatty acid compounds, and the blockage of COX-1 results in the loss of all of the above mentioned constitutive functions of this enzyme.

2.7 Classification of analgesic and anti-inflammatory drugs

The following groups of drugs have different mechanisms of action utilised in the treatment of pain.

2.7.1 Drugs that have an analgesic and weak anti-inflammatory effect

The following drugs fall into a group that shows analgesic and weak anti-inflammatory effects: Ibuprofen contained within Brufen® and Nurofen®, ketoprofen and mefanamic acid (Ponstan®) (Hardman & Limbird, 2001:712).

2.7.2 Drugs that have an analgesic and mild anti-inflammatory effect

The drugs in this subgroup show a larger degree of anti-inflammatory action. The following drugs show analgesic and mild anti-inflammatory properties: naproxen, diclofenac, peroxicam, meloxicam and lornoxicam (Hardman & Limbird., 2001:709).

2.7.3 Drugs that have an analgesic and strong anti-inflammatory effect

The following drugs are indicated for the treatment of acute inflammatory conditions: Aspirin, phenylbutazone and indomethacin (Inodcid®). Indomethacin is used as the gold standard in the treatment of rheumatic anti-inflammatory conditions, especially inflammation of the joints.

2.7.4 DMARD (Disease modifying anti rheumatic drugs)

The following group of drugs that do not have the same mechanism of action as the NSAIDs, are also used in the treatment of rheumatic inflammatory conditions: gold, penicillamine chloroquine and suphasalazine.

2.8 The current topical treatment of pain utilising NSAIDs

Various NSAIDs that have been used in the topical treatment of pain:

- Ketoprofen
- Piroxicam
- Diclofenac
- Indomethacin
- Combination diclofenac and misoprostil
- Salicylic acid
- Ibuprofen

2.9 Treatment of pain using diclofenac

2.9.1 Introduction

Diclofenac sodium is approved in the United States for the long-term symptomatic treatment of rheumatoid arthritis, osteoarthritis, and ankylosing spondylitis. Three formulations are available: an intermediate release formulation (Cataflam®), a delayed-release formulation (Voltaren®), and an extended release formulation (Voltaren XR®). The recommended daily dose for rheumatic conditions is 100 to 200 mg, given in several divided doses. It may also be useful in the short-term treatment of acute musculoskeletal injury, acutely painful shoulder, postoperative pain, and dysmenorrhoea. Diclofenac and misoprostol, a prostaglandin E analog (200 µg), have been formulated together in a preparation (Arthrotec®). This preparation is designed to retain the efficacy of diclofenac while reducing the frequency of gastrointestinal ulcers and erosions. In addition, an ophthalmic solution is available for the treatment of postoperative inflammation after cataract extraction (Hardman *et al.,* 2001:710).

2.9.2 Pharmacological properties

Diclofenac has analgesic, antipyretic, and anti-inflammatory activities. It is an inhibitor of cyclooxygenase and its potency is substantially greater than that of indomecthacin, naproxen, or several other agents. In addition, diclofenac appears to reduce intracellular concentrations of free arachidonate in leukocytes, perhaps by altering the release or uptake of the fatty acid (Hardman *et al,* 2001:709).

2.9.3 Pharmacokinetics and metabolism

Diclofenac is rapidly and completely absorbed after oral administration reaching peak concentrations in plasma within 2 to 3 hours. Administration with food slows the rate but does not alter the absorption. There is a substantial first pass effect, such that only approximately 50% of diclofenac is systemically available. The drug is extensively bound to plasma proteins (99%) and its half-life in plasma is 1 to 2 hours. Diclofenac accumulates in synovial fluid after oral administration which may explain the duration of therapeutic effect that is considerably longer than the plasma half-life. Diclofenac is metabolised in the liver by a cytochrome P450 isozyme of the CYP2C subfamily to 4-hydroxydiclofenac, the principal metabolite, 4-hydroxydiclofenac and other hydroxylated forms; form after glucoronidation and sulphation. The metabolites are excreted in the urine and bile (Hardman *et al,.* 2001:709).

2.9.4 Toxic effects

Diclofenac produces side effects in approximately 20% of patients, and approximately 2% of patients discontinue therapy as a result of this. Gastrointestinal effects are the most common; bleeding and ulceration or perforation of the intestinal wall has been observed. Elevation of hepatic aminotransferase activity in plasma occurs in approximately 15% of patients. Although usually moderate, these values may increase more than threefold in a small percentage of patients, often those who are being treated for osteoarthritis. The elevations in aminotransferase are usually reversible. Other adverse responses to diclofenac include central nervous system effects, skin rashes, allergic reactions, fluid retention, oedema and rarely, impairment of renal function. The drug is not recommended for use in children, nursing mothers, or pregnant women (Hardman *et al.,* 2001:710).

2.10 Physicochemical characteristics of diclofenac salts

The physicochemical characteristics of an active ingredient plays an important role in predicting the outcomes of a topical delivery enhancement study.

2.10.1 Physicochemical and biopharmaceutical charactereristics of diclofenac diethylamine

2.10.1.1 Molecular structure

Figure 2.2 illustrates the molecular structure of diclofenac diethylamine.

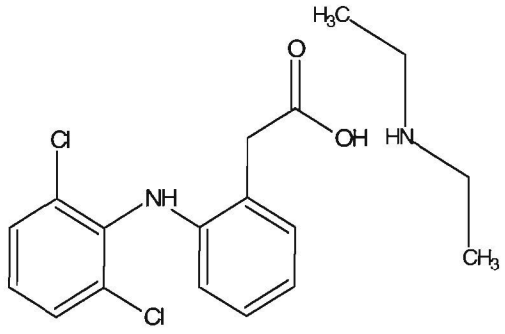

Figure 2.2: Structure of Diclofenac diethylamine (Fini *et al.*, 1999:164).

2.10.1.2 Chemical characteristics

Table 2.2 indicates the relevant chemical characteristics of diclofenac diethylamine.

Table 2.2: Chemical characteristic of diclofenac diethylamine (O'Connor & Corrigan., 2001:286).

Chemical characteristics	**Value**
Dissociation constant	pK_a 4.2
Partition coefficient	Log P (octanol/water), 4.5

2.10.1.3 Physical characteristics

The following table 2.3 indicates relevant physical characteristics of diclofenac diethylamine.

Table 2.3: Table indicating physical characteristics of diclofenac diethylamine (O'Connor & Corrigan., 2001:286).

Physical characteristics	**Value**
Molecular weight	369.38
Melting point	145-148 °C
Physical appearance	Creamed white to pale yellow powder
Clearance	Metabolites are excreted in urine

2.10.2 Physicochemical and biopharmaceutic characteristics of diclofenac hydroxyethyl-pyrrolidine

2.10.2.1 Molecular structure

Figure 2.3 illustrates the structure of diclofenac hydroxyethylpyrrolidine.

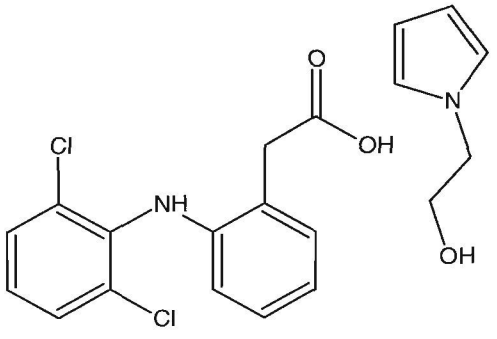

Figure 2.3: Structure of diclofenac hydroxyethylpyrrolidine (Fini *et al.*, 1999:164).

2.10.2.2 Chemical characteristics

Table 2.4 indicates the relevant chemical characteristics of diclofenac hydroxyethylpyrrolidine.

Table 2.4: Chemical characteristics of diclofenac hydroxyethylpyrrolidine (O'Connor & Corrigan., 2001:284).

Physicochemical characteristics	**Value**
Solubility in 1-octanol	8.395% w/w
Solubility in water	1.89% w/w

2.10.2.3 Biopharmaceutical characteristics

Table 2.5 indicates the biopharmaceutical characteristics of diclofenac hydroxyethylpyrrolidine.

Table 2.5: Biopharmaceutical characteristics of diclofenac hydroxyethylpyrrolidine (O'Connor & Corrigan., 2001:284).

Biopharmaceutical characteristics	**Value**
Bioavailability	Approximately 10% of the applied dose
Half-life	Approximately 12h after application of dose
Volume of distribution	0.17 L/kg
Clearance	Excretion in urine and bile

2.10.3 Physicochemical and biopharmaceutical characteristics of diclofenac sodium

2.10.3.1 Molecular structure

Figure 2.6 illustrates the structure of diclofenac sodium.

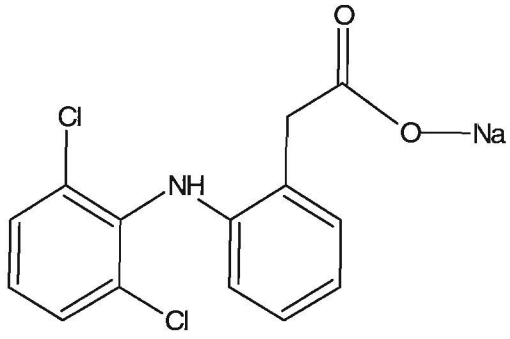

Figure 2.6: Structure of diclofenac sodium (Kenawi, 2006:152).

2.10.3.2 Chemical characteristics of diclofenac sodium

Table 2.6 indicates the relevant chemical characteristics of diclofenac sodium.

Table 2.6: Chemical characteristics of diclofenac sodium (O'Connor & Corrigan., 2001:285).

Physicochemical parameters	**Value**
Dissociation constant	pK_a 4.2
Partition coefficient	Log P (octanol/water), 4.5

2.10.3.3 Biopharmaceutical parameters

Table 2.7 indicates the biopharmaceutical parameters of diclofenac sodium.

Table 2.7: Biopharmaceutical parameters of diclofenac sodium (Hardman *et al.*, 2001:709).

Biopharmaceutical parameters	**Value**
Bioavailability	50 to 60 % (systemically)
Half-life	Plasma half-life of 3-6 hours
Volume of distribution	0.17 L/kg
Clearance	Plasma clearance, about 4 ml/min/kg.

2.11 Transdermal delivery of diclofenac salts

The physicochemical characteristics of various diclofenac salts were evaluated and it was apparent that certain physicochemical properties would result in ineffective permeation across the skin. Diclofenac has a molecular mass of 296 g/mole which is considerably higher than the most effective value for transdermal diffusion of 135 (Yosida & Roberts, 2002:239), this results in diclofenac having very low or negligible fluxes. The melting point of diclofenac is also relatively high; this factor also contributes to insufficient transdermal flux. Decreasing the melting point may result in a more effective flux. Evaluation of properties revealed that diclofenac diethylamine showed the lowest melting point of the evaluated preparations. Predicted log P values for diclofenac according to Hadgraft *et al.* (2004:2) was found to be 3.28, which was higher than the optimum effective range of 1 to 3. Taking these chemical characteristics into consideration, the importance of an effective formulation is highlighted in order to obtain a formulation that yields more effective transdermal diffusion than the active ingredient physically allows.

2.12 Transdermal delivery of NSAIDs

The literature study has indicted that the oral use of diclofenac for the treatment of pain and inflammation results in various side effects. Therefore the skin has been chosen as an alternative pathway in the delivery of diclofenac.

2.12.1 Advantages of transdermal delivery

Transdermal delivery of drugs that require low dosages for long periods can be more effective, less costly and less painful than traditional alternatives such as injections, intravenous infusions or oral ingestion (Barry, 1983:18).

Topical NSAIDs show four major advantages over the oral route:

- Higher concentrations of the drug reach the site of action.
- Lower systemic blood levels decreases the chance of drug interactions.
- Low risk of skin irritation at the site of application.

2.13 Structure and barrier function of the skin

2.13.1 Anatomy of the skin

The human skin consists of three different layers that differ in form and function. Evaluation of the characteristics of each layer of the skin is important when formulating preparations meant for topical application. Figure 2.7 gives an illustration of the functional layers of the skin and the appendages.

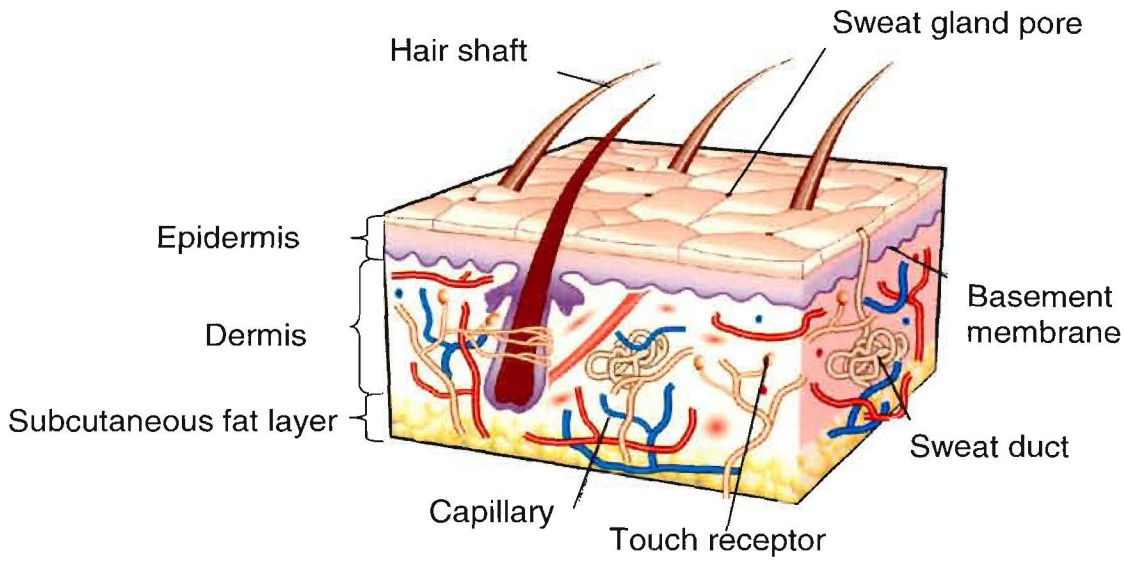

Figure 2.7: *The functional layers of the skin (Encarta encyclopedia, 2004).*

2.13.2 Percutaneous absorption

The extent and rate at which penetration through the skin occurs, depends upon a large number of biological and environmental variables. The skin can function as a barrier, has some metabolic activity, contains multiple appendages and cell types and is the largest organ in the body. Skin absorption is dictated by skin physiology, site of exposure and exposure area. It is site and species-specific (Wester and Maibach, 1984:760).

Tanojo *et al.* (2000:135) stated that utilisation of a combination of techniques can be applied in different ways. These techniques can be applied to protect the skin from the absorption of unwanted compounds and, on the other hand, to enhance the permeation of compounds or medicaments to reach specified target locations, either inside the skin or in the deeper tissues beneath the skin, or to be achieved by way of the systemic circulation. Two important factors which

should be taken into consideration when predicting skin absorption are the method of application and the condition of the skin (Tanojo *et al.*, 2000:139). The following sections discuss the transdermal pathways accessible for transdermal diffusion through the functional layers of the skin as illustrated in figure 2.7.

2.13.2.1 Transappendageal - diffusion through sweat ducts

Chemical permeation through sweat ducts is modelled as a single-phase aqueous diffusion process. For simplicity, the region around the sweat duct is assumed to be impermeable (no interactions), and the sweat duct is assumed to be filled with water. The region around the sweat duct is included in the control volume to represent a larger unit area of skin for normalisation with the other transport pathways. This pathway is an important pathway to take into consideration when the active ingredient in the preparation is hydrophilic. Active ingredients that tend to dissolve easily in water would favour this transdermal pathway (Barry, 1983:18).

2.13.2.2 Diffusion through hair follicles

During transdermal studies diffusion through hair follicles is modelled in a similar fashion as diffusion through sweat ducts. The primary difference is that the follicle is assumed to be filled with an oil phase instead of an aqueous phase. This route plays a role in the delivery of larger polar molecules and ionic substances (Feldmann & Maibach, 1970:399). The route can also act as a site of delivery for liposomal carrier molecules, microspheres and crystals (Barry, 1983:18).

In conclusion, despite the lower oil-phase molecular diffusion coefficient of the hair follicles, diffusion through the hair follicle is more significant than diffusion through the sweat ducts, because of the larger simulated porosity of hair follicles (Barry, 1983:18).

2.13.2.3 Transcellular pathway

Movement of drug molecules takes place directly across the stratum corneum. This route offers a method for polar molecules to cross the stratum corneum. Highly hydrated keratin offers a route of movement through the membrane by providing an aqueous environment. A hydrophilic molecule, however, still has a few obstacles to face when crossing the stratum corneum. In the initial step the

molecule partitions into the keratin in order to move from one keratin cell to the next. The drug should partition into the neighbouring bilipid layers. Highly polar drugs could utilise this pathway as a method to cross the stratum corneum, but it should be taken into consideration that transversing the multiple lipid layers remains the rate limiting step in diffusion processes (Williams, 2003:32).

2.13.2.4 Intercellular pathway

Lipids provide the only continuous phase within the stratum corneum. This forms the route for most small, uncharged molecules to cross the stratum corneum. The major barrier to drug flux comes in the form of the lipid bilayer. Intercellular transport takes place through the lipid domain. When a drug molecule moves via the intercellular pathway, the route is longer and more tortuous. Drug molecules move through the continuous lipid domains between keratinocytes. The path length chosen by the drug molecule depends largely on the physicochemical properties of the active drug molecule (Williams, 2003:33).

2.13.3 Functions of the skin

The functions of the skin provide insight into the barrier function and integrity of the stratum corneum and why this layer is so important.

2.13.3.1 Containment

The most important function of the skin is the containment of the underlying tissue. Muscles underneath the skin move and are constantly in motion. This motion places tension on the skin. Collagen fibres contained in the skin can help the skin deform and withstand tension giving it form and stability (Banker & Rhodes, 2002:194)

2.13.3.2 Microbial barrier

The stratum corneum is a dense molecular structure only penetrable by transdermal diffusion. This layer of the skin is an absolute barrier to microbial penetration from the outside environment. The environment directly beneath the skin is an ideal substrate for the growth of microorganisms. Beyond physical protection, the stratum corneum also contains substances making it an unfavourable area for bacteria to grow and reproduce. Secretions of the skin, i.e., sweat or sebum

are both acidic, lowering the surface pH of the skin and making it less susceptible to pathogen infection. Pilosebaceous glands on the back, face and forehead, on the other hand, are prone to form infected occlusions and can result in acne, pimples and blackhead breakouts (Banker & Rhodes, 2002:194).

2.13.3.3 Chemical barrier

The stratum corneum acts as a barrier to chemicals that are brought into contact with the skin. Resistance towards diffusion through this layer is more significant than any other barrier in the human body. External chemicals can, however, gain entry to the underlying layers of the skin by diffusion through sweat ducts or hair follicles. These two pathways can, therefore, be utilised in transdermal formulation enhancement (Banker & Rhodes, 2002:195).

2.13.3.4 Radiation barrier

Rays of the sun have the potential to cause serious tissue damage and one in six South Africans is affected by skin cancer in his/her lifetime. Fortunately, skin has a natural defence mechanism for dealing with the harsh UV rays of the sun. Melanin, a pigment in the skin, is responsible for the protection of the underlying tissue. It acts as a protective barrier absorbing the harmful rays of the sun before they cause any damage. Successful tanning is an indication of the amount of melanin present in the skin. Every time the skin is exposed to the sun's UV rays the melanin cells increase (Banker & Rhodes, 2002:195).

2.13.3.5 Electrical barrier

The dry external layer of the skin, the stratum corneum, protects the skin against possible flow of electric current. Research has shown that by stripping away the stratum corneum, the electrical resistance of the skin lowers. In the evaluation of the above mentioned concept, it is understood that the skin is the body's prime electrical insulator which protects the human body from electrical charges in the external environment (Banker & Rhodes, 2002:195).

2.13.3.6 Thermal barrier and body temperature regulation

The body is fine tuned to function at 37 □C. Skin plays a very important role in the maintenance of body temperature by the following mechanism: as soon as a change is detected in the external conditions, the skin physiologically changes to conserve or release heat. Thus, tampering with the heat regulation of the skin by means of water-impermeable occlusion can lead to a life-threatening lowering in body temperature (hypothermia) (Banker & Rhodes, 2002:196).

2.14 Physiological factors affecting transdermal drug delivery

The following sections indicate how different conditions and states of the skin influence transdermal delivery.

2.14.1 Healthy skin

Normal, disease-free skin provides excellent protection against the permeation of many compounds. To reach the viable layer of the skin and, beyond that, the systemic circulation or deeper tissues, a compound has to overcome various obstacles. Some known obstacles are depicted in the following list (Tanojo *et al.,* 2000:137).

Obstacles to skin absorption:

- Movement of hairs,
- excretion of sebum/covering of the skin surface,
- desquamation of outermost corneocytes (stratum corneum cells),
- compact flat corneocytes with hardened/cornified cell envelopes,
- dynamic intercellular lipid bilayers,
- constant upward pressure from cell regeneration,
- water vapour flow,
- lateral distribution,
- multistrata and multilayer partitioning,
- change from lipophilic to hydrophilic environment,
- pH (acidity) and pI (isoelectric point) gradient,
- interlayer differences in skin components and ion distribution,
- electrical impedance,
- chemical reactions with skin components (including anti-oxidants),
- molecular binding/retention with cell surface or components,
- activities of keratinocytes and other skin cells (such as Langerhans cells) and
- cutaneous metabolism.

In special cases, obstacles can actually "enhance" permeation. Cutaneous metabolism may transform a slow-permeating compound into a fast-permeating molecule. A number of skin barrier components have been thoroughly investigated, not only the physical macroscopic characteristics but also at molecular levels, because they determine the quality of the barrier function. In particular, composition of the lipid components (cholesterol, long-chain free fatty acids, ceramides and cholesterol sulphate) in the intercellular space will determine barrier capacity. (Tanojo *et al.,* 2000:137).

Form the increasing amount of data, percutaneous absorption kinetics can be described more accurately with a number of theoretical and mathematical models. The models are of particular importance to predict the absorption of compounds, especially new ones with inadequate toxicity data, on the basis of the molecular structure. Thus, physicochemical characteristic assumptions can be made about projected transdermal diffusion outcomes (Tanojo *et al.,* 2000:137).

General predictive approaches have been formulated that relate the permeability coefficient to the molecular weight and to the partitioning between lipid and water, but they have recently been adjusted also for molecular size and hydrogen bonding. Larger molecules and molecules that can accommodate increasing amounts of hydrogen bonds tend to show less favourable transdermal diffusion (Tanojo *et al.,* 2000:137).

For the purpose of evaluating a new formulation and to gain a better understanding of certain factors in percutaneous absorption, *in vitro* models are created. *In vitro* skin diffusion models take mainly two processes into consideration, namely intercellular permeation and permeation through shunts. In the process of intercellular permeation, the differences in the permeation through the stratum corneum compared with the other layers in the epidermis are elaborated and result in more specific calculations for the stratum corneum (Tanojo *et al.,* 2000:137). Due to the differences in the quality of the skin barrier between individuals, permeation is determined by the skin type, not by race or sex. Percuataneous absorption also differs by anatomic region in individuals. The condition of the skin is an important consideration to take into account (Tanojo *et al.*, 2000:137). The following sections show how different conditions and anatomical changes influence transdermal diffusion.

2.14.2 Diseased skin

Absorption through the skin will be enhanced in areas affected by dermatitis. This increased absorption can, however, not be fully explained due to the variation in the extent of the disease or affliction. Models consisting of tape-stripping or delipidisation have been used to mimic diseased skin. Trichloroacetic acid and sodium hydroxide have been used to alter the skin barrier function to mimic the diseased skin condition and to assess the percutaneous absorption of compounds through impaired skin (Tanojo *et al.,* 2000:138).

2.14.3 Compromised skin

To decrease the absorption of harmful objects, skin barrier capacity can be enhanced. The enhancement of the skin barrier capacity is generally achieved by applying a protective formulation that contains a permeation retardant to the skin surface. On the other hand, to increase the absorption of favourable drugs, either for application in the top layers of the skin or systemic, it is necessary to decrease the skin barrier capacity in a reversible fashion. This can be achieved by various methods. Occlusion is considered one of the simplest and most effective methods,

although it is not always successful, especially in the case of hydrophilic compounds (Tanojo *et al.,* 2000:138).

An additional challenge in the interpretation of dermal dose relates to the way absorption is assessed. A general approach used to calculate absorption has been to calculate it as a percentage of the total applied dose, but this value is highly dependent upon exposure conditions. Obviously, percentage of an infinite source would be meaningless. On the other hand, percentage of a limited source depends on the concentration and length of exposure and necessitates that the exposed dosage remains constant over the length of exposures or is accounted for by using a mathematical transformation. In order to express absorption as a percentage, the exposure conditions should mimic a real-world scenario as closely as possible (Tanojo *et al.,* 2000:138).

2.14.4 Skin age

The skin acts as a barrier to the outside environment, with time this barrier shows clear structural and functional changes (Fenske and Lober, 1986:571). Damage to the membrane can be a result of overexposure to UV light or the misuse of chemical substances on the skin, for example soaps and cosmetics. With age the moisture content of the skin decreases, this factor may play a minor role in altered absorption of medication applied on the skin (Williams, 2003:14).

The theoretical amount of topically applied drug which enters the systemic circulation can be influenced by age-related alterations of the skin, namely when blood flow decreases with age and along with this also the movement of pharmaceuticals through the skin.

There are still important differences between the skin of an adult and that of a new-born baby. Approximately a week after conception a single layer of epithelial tissue cells has formed. This layer develops to form an epidermis and dermis. At birth the dermis is only 60% as thick as that of an adult. The stratum corneum of a fetus is remarkably different from that of an adult and remains a very thin membrane until a week before birth. The very delicate nature of the neonatal skin causes difficulty in thermoregulation, transepidermal water loss, infection and absorption of exogenous compounds (Williams, 2003:15).

However, it holds potential for the treatment of disease in neonates by using the transdermal route of delivery. The stratum corneum of a neonate only develops post delivery which causes difficulty in effectively utilising this route as a means to deliver medication (Williams, 2003:15).

2.14.5 Site on the body

Skin structure varies over the human body; the stratum corneum is thicker on the hands and feet than it is on the eyelids. Thickness of the site of administration is not, however, an accurate determinant of the tempo of absorption through that area of the skin. Williams (2003:16) stated that areas with similar thickness can show different absorption values and vice versa. The general order of site permeation will now be listed from least to most: Genitals > head and neck > trunk > arm > leg.

It is valuable to take into consideration the variation of the same site in different patients. The influence of regional variations of absorption through different areas has been reviewed by Williams (2003:16).

2.14.6 Race

The literature on differences in transdermal absorption in different racial groups is limited, although many formulations are used around the world. Water content within the stratum corneum of different races vary and it would therefore be expected that the absorption values are different for the various racial groups (Williams, 2003:16).

2.15 Other factors that influence transdermal drug delivery

Keratinocytes tend to be larger in females than in males this factor, however, does not have a significant effect on drug absorption at the same site in individuals from different sexes. The level of hydration is important for permeation through the skin and this parameter also plays an important role in transdermal diffusion. Occlusive dressings and patches can be an effective measure of enhancing moisture retention within a certain area (Williams, 2003:16).

Diffusion through the stratum corneum is a passive process; an increase in temperature increases the permeant diffusion. A large increase in temperature can structurally alter the stratum corneum and enhance diffusion (Williams, 2003:16).

2.15.1 Ideal properties of a molecule that effectively penetrates the skin

The partitioning coefficient is crucially important in establishing a high initial penetration concentration in the first layer of the stratum corneum. For the maximum penetration rate, the drug should be at its highest thermodynamic activity. Thus, theoretically maximum flux could significantly increase (Barry, 2001:112).

Physicochemical characteristics of molecules that effectively penetrate the skin include:

- A low molecular weight.
- Balanced solubility in both oil and water.
- A high, but balanced partition coefficient.
- A low melting point, correlating with ideal solubility (Barry, 2001:112).

2.16 Mathematics of skin permeation

Mathematics of skin diffusion is an important factor that should be taken into consideration when initiating a diffusion study. The equations and linear equation of importance in transdermal diffusion calculations will be discussed in the following sections.

2.16.1 An infinite dose at steady state

The amount of a material passing through a unit per area per unit time is termed the flux. Fick's first law of diffusion states that the rate of transfer of diffusing substance through unit area of a section is proportional to the concentration gradient measured and which is normal to the section (Williams, 2003:43).

Equation 2.1: $J = (DPC)/h$

- Where J is the steady state flux,
- D the diffusion coefficient through the membrane,
- P the partition coefficient, and
- C the donor concentration and h is the membrane thickness (Barry, 2001:112).

Figure 2.3 indicates the permeation profile for an infinite dose applied on human skin membranes.

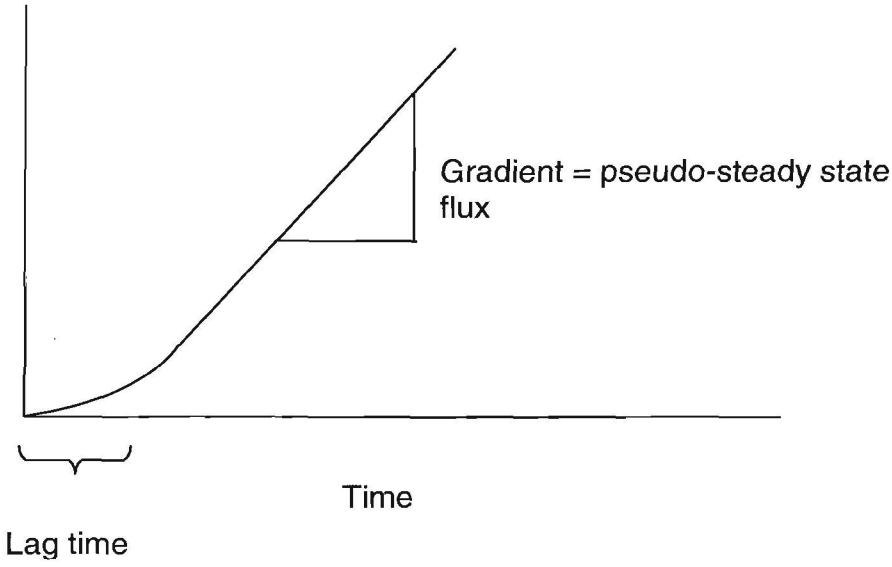

Figure 2.3: Typical permeation profile for an infinite dose application to human skin membranes (Williams, 2003:44).

The pseudo-steady-state flux is simply obtained as the gradient of the linear portion of the permeation profile, and if the concentration of the permeant in the applied vehicle is known, then the permeability coefficient can be determined (Williams, 2003:45).

2.16.2 A finite dose deposited on the membrane

The cumulative permeation when a finite dose has been applied increases to a plateau beyond which the amount permeated remains constant unless further doses are applied on the membrane surface (Williams, 2003:45).

The maximum flux, J_{max}, is given approximately by:

Equation 2.2: $J_{max} = (1.85DPC\delta)/h^2$

Where δ is the thickness of a thin film deposited on the surface of the skin (Barry 2001:113).

2.17 Penetration enhancement

2.17.1 Developing absorption-enhanced topical products

The ideal pharmacological topical enhancing agent should have the following characteristics.

- It should be pharmacologically inert,
- safely promote transdermal drug penetration with rapid onset of action,
- work with a wide variety of active pharmaceutical ingredients, and
- metabolise safely into breakdown product(s) of known safety.
- They should work unidirectionally,
- When removed from the skin the barrier ability of the skin should be able to return to normal,
- They should be compatible with active ingredients and other exipients and
- They should have an acceptable cosmetic skin feel (Williams and Barry, 2003:603).

2.17.2 Chemical enhancers

Chemical enhancers act on the skin by various mechanisms, the first of these are extraction of intercellular lipids and dilation of the spaces between the cornified cells permitting percutaneous passage of polar substances. Enhancers can be listed from highest to lowest activity as follows; azone> n-octanol> d-limonen > oleic acid > cineol (Williams and Barry, 2003:604).

Some compounds when applied to the skin, are capable of significant degrees of unenhanced penetration through the lipid matrix. To enhance the movement of penetration through the lipid matrix one would want to scramble the lipid structure. Chemical enhancers can give way to change in the arrangement of lipids in the matrix and in this manner penetration is enhanced (Williams and Barry, 2003:604)

Chemical enhancers include some very powerful organic solvents that literally strip lipids off the stratum corneum thereby allowing drugs to go through. Other early enhancers include some fatty acids, alcohols, detergents and surfactants. A partial list depicting some of the chemical enhancers follows (Williams and Barry, 2003:606).

- Ethanol,
- glyceryl monoehtyl ether,
- monglycerides,
- isopropylmyristate,
- lauryl alcohol,
- terpinol,
- d-limonene,
- beta- cyclodextrin,
- DMSO (dimethyl suphoxide),
- Polysorbates,
- fatty acids,
- bile salts,
- essential oils,
- urea,
- phospholipids,
- solvents at high concentrations and
- water.

2.17.3 Physical delivery enhancement

Chemical enhancement of transdermal diffusion has been discussed in the sections above. The following sections will discuss physical enhancement procedures.

2.17.3.1 Iontophoresis

Iontophoresis can be defined as the movement of ions through skin as a result of the application of a low-intensity electrical current as a driving force. Current densities generally do not exceed 0.5 mA/cm^2 and the application may last minutes to hours. Unlike electroporation, which acts directly on the skin and causes transient changes in tissue permeability, iontophoresis acts primarily on the ionic drug. Iontophoresis has been used not only to deliver drugs across the skin but also to extract molecules from the skin, for monitoring purposes. An example of this is the glucose monitoring device (Tanojo *et al.,* 2000:138).

2.17.3.2 Electroporation

Electroporation or electropermeabilisation involves the creation of transient aqueous pathways across lipid bilayer membranes by application of a short, high-voltage pulse. The biological composition and structure of the stratum corneum with a continuous extracellular matrix of lipids, highly ordered in bilayers, make it particularly attractive for electroporation. Electroporation may enhance percutaneous transport of fentanyl, calcein and flurbiprofen, to name but a few as well as an array of neutral compounds (Tanojo *et al.,* 2000:138).

Electroporation experiments with skin generally use exponential-decay electric field pulses of 100 to 1500 V applied voltage, 30 to 100 V transdermal voltages, and microseconds to hundreds of milliseconds pulse duration. According to Tanojo *et al.*, (2000:138) within a few minutes of high-voltage pulsing, molecular transport across skin increased by several orders of magnitude, mainly because of electrophorectic movement and diffusion through newly created aqueous pathways. This technique is also used for gene therapy and DNA transfection (Tanojo *et al.,* 2000:138).

2.17.3.3 Phonophoresis

Phonophoresis or sonophoresis can be defined as the movement of drugs through skin under the influence of ultrasound perturbation. Ultrasound is labelled as sound having a frequency beyond 20 kHz. Several authors have reported weakened skin barrier properties and increased transdermal transport of compounds such as estradiol, salicylic acid, lidocaine, insulin and erythropo erythropoietin under these conditions (Tanojo, *et al.* 2000:139).

2.17.3.4 PowderJect

The PowderJect system is a fascinating development that fires solid particles through the stratum corneum into the lower skin layers using a supersonic shock wave. This form of therapy shows significant advantages, but problems arise with bruising and particles bouncing off the skin surface. The European Medicines Evaluation Agency and Food and Drug Administration is, however, concerned that damaging the complex structure of the skin can enhance environmental contamination from bacteria (Barry, 2001:115).

2.17.3.5 Intraject

This device which was developed from the vaccine gun, inserts liquids through the skin without using needles (Barry, 2001:115).

2.17.4 Drug delivery vehicles

2.17.4.1 Liposomes

These colloidal particles form concentric bimolecular layers that entrap drugs and deliver them to the skin. Liposomes usually facilitate a local drug effect with drug molecules concentrated in the stratum corneum or the upper skin layers (Barry, 2001:116).

2.17.4.2 Transferosomes

These vesicles are characterized by being ultra deformable to such an extent that they could squeeze through pores in the stratum corneum when driven by a hydration gradient. Data for transferosomes indicated that 50% of the dosage of a peptide or protein drug penetrated the skin *in vivo* within 30 minutes (Barry, 2001:116).

2.17.4.3 Ethosomes

Ethosomes are liposomes high in ethanol content. They can penetrate the skin and enhance compound delivery both to deep skin strata and systemically. This is because the ethanol fluidises both ethosomal lipids and the lipid bilayers of the stratum corneum. The soft malleable vesicles then penetrate the disorganised lipid bilayers of the stratum corneum (Barry, 2001:116).

2.17.4.4 Niosomes

Vesicles formed from nonionic surfactants are known as niosomes (Barry, 2001:116). They were actively promoted by the cosmetic industry in the 1970's. In modern times the focus has, however, shifted to the potential of these carries in the transport of medicating active ingredients. These

carriers are able to effectively encapsulate hydrophilic compounds and carry them in the same manner as phospholipid carries would (Uchegbu & Vyas, 1998:34).

2.18 Possible solutions to the problem of transdermal delivery of diclofenac

During this study preparations containing the various diclofenac salts will be formulated. Pheroid™ technology was implemented as a method to enhance transdermal diffusion.

2.18.1 Advantages of Pheroid™ incorporation

Research has shown that formulations containing Emzaloid™ particles (a carrier similar to Pheroid™) showed an increase in fluidity of the membranes and intercellular lipids. This is caused by the anaesthetic action of nitrous oxide in the formulation, and transport therefore, occurs in close association with the membranes. Another factor involves the possibility that the Emzaloid™ particles may be able to squeeze themselves through intercellular regions of the stratum corneum as intact structures, and by this mechanism, deliver encapsulated drugs to the dermis (Saunders *et al.,* 1999:105).

Although Emzaloid™ particles can be considered to fall into the same general category as liposomes, niosomes or sibmicron emulsions, they differ both in constitution and preparation techniques, and are regarded as unique (Saunders *et al.,* 1999:105).

Effective skin penetration enhancers show distinct properties. Amongst others they should be pharmacologically inert, non-toxic, non-irritating and should be compatible within a wide range of dosage forms. Another factor that favours the use of Emzaloid™ particles is that the essential fatty-acids in the formulation most likely play a role in replenishing a lipid deficiency in the skin by incorporating phospholipids and maintaining the integrity of the epidermal permeability barrier. The oleic acid in the Emzaloid™ formulation may also play a role in enhancing transdermal penetration by temporarily disrupting the packed structure of the intercellular lipids because of the incorporation of its kinked structure (Saunders *et al.,* 1999:105).

Other advantages of Emzaloid™ include a faster onset of action using a lower concentration of the drug in topical formulation and rejuvenation of skin lipids without irritation. Futhermore, it has been

speculated that the apparent ability of emzaloids to penetrate tissue as an intact structure might overcome the major limitation pertaining to the use of liposomes (Saunders *et al.,* 1999:105).

2.19 Summary

Pain is a major medical problem which affects a wide variety of individuals. Back pain is a major medical condition which affects most individuals at some or other stage in their lives and it is a very costly condition to treat. Pain is also associated with an array of traumatic events, research by Carstenson *et al.,* (2008:1), showed that people involved in a car accident will suffer from mental and physical pain for up to a year after the accident has occurred. In the professional sports arena 5 – 16% of an athlete's career will be spent recovering from a painful condition.

Topical diclofenac salts have been chosen as a group to be used in formulations that might enhance the absorption and the systemic availability of the active ingredient. Due to diclofenac's unfavourable physicochemical characteristics the formulation of the salt is of the utmost importance.

2.20 References

BANKER, G.S. & RHODES, C.T. 2002. Modern Pharmaceutics. New York. Marcel Dekker. 235p.

BARRY, B.W. 1983. Dermatological Formulations: Percutanous Absorption. New York. Marcel Dekker. 18.

BARRY, W.B. 2001. Novel mechanisms and devices to enable successful transdermal delivery. *European Journal of Pharmaceutical Science*. 14: 101-114.

CARSTENSON, T.B.W., FROSTHOLM, L., OERNBOEL, E., KONSTED, A., KASCH, H., JENSEN, T.S. & FINK, P. 2008. Post-trauma ratings of pre-collision pain and psychological distress predict poor outcome following acute whiplash trauma: A 12-month follow-up study. *Pain,* 139(2).

CASHMAN. N.J. 1996. The mechanisms of action of NSAIDs in analgesia. Drugs. 52:513-231.

ENCARTA ENCYCLOPEDIA DELUXE EDITION. 2004. Anatomy of the skin. Microsoft Corporation.

FELDMANN, R.J. & MAIBACH, H.I. 1970. Absorption of some organic compounds through the skin in man. *Journal of Investigative Dermatology*. 54:399-404.

FINI, A., FAZIO, G., GONZALES-RODRIQUEZ, M., CAVALLARI, C., PASSERINI, N. & RODRIQUEZ, L. 1999. Formation of ion-pairs in aqueous solutions of diclofenac salts. *International Journal of Pharmaceutics*. 187(5):163-173.

FIORUCCI, S. 2001. NO-releasing NSAIDs are capitase inhibitors. *Trends in Immunology*. 22(5):232-235.

GRANT, M.A. 1998. Pain control with EMDR, an information processing approach. Denver. Mentor Books. 194p.

HADGRAFT, J. 2004. Skin deep. *European Journal of Pharmaceutics and Biopharmaceutics.* 58(2):291-299.

HARDMAN, J.G., LIMBIRD, L.E. & GILMAN, A.G. 2001. Goodman & Gilman's THE PHARMACOLOGICAL BASIS OF THERAPEUTICS. New York : McGraw-Hill. 2148p.

Pain. 2009. International association for the study of pain. [http://www.iasp-pain.org]. Date of use 7 Desember 2009.

KENAWI, I.M. 2006. DFT analysis of diclofenac activity and cation type influence on the theoretical parameters of some diclofenac complexes. *Journal of Molecular Structure.* 761(3):151-157.

LIEBESKIND, J.C. 1998. History of Pain Collection. Symposium held at the Louise M. Darling Biomedical Library. UCLA.

MAMBERG, A.B., YAKSH, T.L. 1992. Hyperalgesia mediated by spinal glutamate or substance P receptor blocked by spinal cyclooxygenase inhibition. *Science.* 257:5074.

MELDRUM, M.L. 2003. Medical Biology: History of Pain Management. *Journal of American Medicine.* Assoc. 290:2470.

O'CONNOR, K.M. & CORRIGAN, 0. 2001. Comparison of the physicochemical properties of the N-(2-hydroxyethyl) pyrrolidine, diethylamie and sodium salt forms of diclofenac. *International Journal of Pharmaceutics.* 222(2):281-293.

SAUNDERS, C.J., DAVIS, H.J., COETZEE, L., BOTHA, S., KRUGER, A.E. & GROBLER, A. 1999. A Novel Skin Penetration Enhancer: Evaluation By Membrane Diffusion And Confocal Microscopy. *Journal of Pharmaceutical Science.* 2(3): 99-107.

TANOJO, H. & MAIBACH, H.I. 2000. Percutaneous absorption. *Current Problems in Dermatology* 13(3): 137-140 [www.sciencedirect.com] [Date of use 5 June 2008]

UCHEGBU, I.F. & VYAS, S.P. 1998. Non-ionic surfactant based vesicles (niosomes) in drug delivery. *International Journal of Pharmaceutics.* 172(15):33-70.

WOOLF. C.J. 1995. Somatic pain-phatogenesis and prevention. *British Journal of Anesthesia.* 75:169-176.

WESTER, R.C., MAIBACK, H.I., BUCKS, D.A.W. & AUFRERE, M.B. 1984. In vivo percutaneous absorption of paraquat from hand, leg and forearm of humans. *Journal of Toxicology and Environmental Health.* 14:759-762. [Sited in medline] [Date of use 5 June 2008]

WIDMAIER, E.P., RAFF, H. & STRANG, K.T. 2004. Vander, Sherman, & Luciano's HUMAN PHYSIOLOGY THE MECHANISMS OF BODY FUNCTION. New York : McGraw-Hill. 825p.

WILLIAMS, A.C. 2003. Transdermal and topical drug delivery. London, Pharm. Press, 222p.

WILLIAMS, A.C. & BARRY, B.W. 2003. Penetration enhancers. *Advanced Drug Delivery Reviews.* 56(5):603-618.

CHAPTER 3: ARTICLE FOR PUBLICATION IN THE INTERNATIONAL JOURNAL OF PHARMACEUTICS

In vitro release and transdermal diffusion of diclofenac in gel preparations

Heidi Steyn, Jan L. du Preez*, Joe Viljoen, Minja Gerber and Jeanetta du Plessis

Unit for Drug Research and Development, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa

* Corresponding author. Tel.: +2718 299 2236; Fax: +2718 299 2225. E-mail address: JanDuPreez@nwu.ac.za (JL du Preez)

Abstract

Muscular pain is a condition which influences most people some or other time during their lives. The most commonly used drugs to reduce inflammation and pain are the non-steroidal anti-inflammatory drugs (NSAIDs). The main side effect of oral NSAID usage manifests itself in the form of peptic ulcers. The transdermal route was chosen as a method to overcome the harmful effects of diclofenac on the stomach mucosa. Physicochemical determinations were done on each one of the salts to predict possible skin diffusion. Various preparations containing diclofenac salts were then produced by implementing Pheroid™ technology. The main focus of the study was to develop Pheroid™ and non-Pheroid™ preparations containing diclofenac diethylamine, diclofenac hydroxyethylpyrrolidine and diclofenac sodium and to compare them with each other, as well as with formulations available on the market. The preparations produced were then tested to determine efficacy and stability. Products were then compared by means of human skin Franz cell diffusion to determine systemic availability and efficacy; as the deep muscle tissue region is the main target area. Determination of various stability parameters were investigated over the duration of the study. Parameters investigated included assay, aesthetics, mass variation, pH variation, uniformity of content and viscosity.

Keywords: Transdermal delivery, Stability testing, Pheroid™, Diclofenac salts

1. Introduction

Research has shown that formulations containing Emzaloid™ (a carrier system now known as Pheroid™) showed an increase in fluidity of the membranes and intercellular lipids. This is caused by the anaesthetic action of nitrous oxide in the formulation, and transport therefore, occurs in close association with the membranes. Another factor involves the possibility that the Pheroid™ particles may be able to squeeze themselves through intercellular regions of the stratum corneum as intact structures, and by this mechanism, deliver encapsulated drugs to the dermis (Saunders et al., 1999). Incorporating the salts into Pheroid™ vesicles can contribute to the enhancement of the systemic availability of each one of the diclofenac salts. During this study relevant physicochemical characteristics of each one of the salts were evaluated. The various diclofenac salts, namely diclofenac diethylamine (Voltaren® emulgel), diclofenac hydroxyethylpyrrolidine (Panamor® gel) and diclofenac sodium (Arthruderm® lotion), were evaluated during this study and are available on the South African market. These salts were then formulated as Pheroid™ and non-Pheroid™ preparations and accelerated stability determinations were done to determine if preparations complied to standards as set by the Medicines Control Council (MCC) of South Africa (MCC, 2009). The preparations were evaluated to determine if the products reached the site of action (the deep muscle tissue) by performing *in vitro* Franz cell skin diffusion studies. Tape stripping studies were done to determine the amounts of active ingredients contained within the epidermal and dermal layers. Data obtained from the various Pheroid™, non-Pheroid™ and marketed products were compared to determine which showed optimum efficacy.

2. Materials and Methods

2.1. Reagents and raw materials

Diclofenac diethylamine, diclofenac hydroxyethylpyrrolidine and diclofenac sodium were all obtained from Amoli organics (India). Water used was HPLC (high performance liquid chromatography) grade deionised with a Milli-Q academic purification system (Millipore, Milford, USA). Methanol AR grade (Merck, South Africa) was used for sample preparation. Acetonitrile Lichrosolv (Merck, South Africa), methanol Lichrosolv (Merck, South Africa) and octane-sulphonic acid were used in HPLC mobile phase preparation. Potassium dihydrogen orthophosphate (Merck, South Africa) and potassium hydroxide (Merck, South -Africa) were both used in the preparation of phosphate buffer solution pH 7.4 (PBS) for solubility determinations. n-Octanol (Fluka) was used in the determination of log D (octanol-buffer partition coefficient). Materials used for the preparation of non-Pheroid™ gel were carbopol 934 PNF (Noveon), ethanol (Saarchem South Africa), polyethylene glycol (PEG 400) (Saarchem South Africa) and triethanolamine (Saarchem South Africa). Market products used in membrane and skin diffusion flux determination included Arthruderm® lotion (Meyer Zall laboratories), Panamor® gel (Aspen Pharmacare) and Voltaren® gel (Novartis).

2.2. Methods

2.2.1. Preparation of gel formulations containing different diclofenac salts

2.2.1.1. Pheroid™ gel preparation

The following ingredients were measured by using a Metler Toledo balance (USA): diclofenac salt (1.0 g), methyl paraben (0.4 g), BHA (butylated hydroxyanisole) (0.02 g), BHT (butylated hydroxytoluene) (0.2 g) and PEG (polyethylene glycol) 400 (6 g). PEG 400 was heated gently to enhance the solubility of the diclofenac salts, methyl paraben and antioxidants. The diclofenac salt, methyl paraben, BHA and BHT were then dissolved into the PEG 400, stirring constantly with a glass stirrer. The Pheroid™ portion was prepared separately; as soon as it had cooled down the Pheroid™ portion was added to the PEG 400 mixture. The preparation was left to cool down to room temperature while stirring. After the preparation has sufficiently cooled down the content was spooned into plastic containers. Pheroid™ preparations were produced containing the same percentage of active ingredient

as the non-Pheroid™ preparations. The Pheroid™ containing portion was produced in the formulations facility at the North-West University. Due to the confidential nature of the Pheroid™ formulation the ingredients contained within the Pheroid™ portion cannot be stipulated. Anti-oxidants (BHA and BHT) were added to enhance the stability and integrity of the formulations.

2.2.1.2. Non-Pheroid™ gel preparation

An amount of 1.0 g of the diclofenac salt was measured by using a Metler Toledo balance (USA). Thereafter PEG 400 (10 ml) and ethanol (15 ml) were measured. The diclofenac salt was stirred into a mixture of the PEG 400 and ethanol using a glass stirrer. Carbopol (0.4 g) and water (10 ml) were mixed using a Wise stir mixer (Korea) at 800 rpm until a gel consistency was obtained. The PEG 400 mixture was added to the carbopol mixture and the mixing speed was slowly increased to 1200 rpm. An amount of 1.3 ml triethanolamine was added to the mixture as a neutralizing agent. The preparation was left to stir for 15 minutes. After the preparation had reached proper gel consistency it was removed from the stirrer and spooned into plastic containers.

2.2.2. Stability determinations

Stability determinations were done at 25 °C/60% RH (relative humidity), 30 °C/60% RH and 40 °C/75% RH (MCC, 2009). Preparations were placed in Labcon stability ovens for accelerated stability over a period of six months.

2.2.2.1. High performance liquid chromatography method for stability testing (Assay)

Determinations were done using an Agilent gradient system, Luna C18-2 column, 150 x 4.6 mm, 5 µm, (Phenomenex, Torrance, CA). The flow rate was set at 1 ml/min and the detection wavelength was set at 280 nm. A mobile phase of 0.005 M octane-sulphonic acid (pH 3.5) and methanol (Lichrosolv) was injected in gradient elution. The gradient elution was started at 50% for the first minute, followed by a linear increase to 100% methanol after 8 minutes. The composition remained at 100% methanol until 20 minutes had elapsed. A total run time of 25 minutes was used for each sample run. Retention times for the ingredients varied from ± 5 minutes for methyl paraben, ± 9 minutes for BHA, ± 10 minutes for diclofenac

sodium, diclofenac diethylamine and diclofenac hydroxyethylpyrrolidine to ± 12 minutes for BHT and ± 18 minutes for vitamin E.

2.2.2.1.1. Sample preparation for assay determination

Samples for chromatographic assay determinations were prepared according to the following method. A 5 ml syringe was fitted with a 15 cm piece of silicone tubing by sliding the tubing over the nozzle of the syringe. An amount of the various preparations was then spooned into the empty syringes. Approximately 2g of each sample were deposited into a 50 ml volumetric flask, which was then filled to volume with analytical grade methanol. The volumetric flask was first shaken manually and then placed on a Transonic 540 (Germany) sonicating bath. Another 5 ml syringe was taken and fitted with a 0.45 µm pre-filter. The syringe was filled with 5 ml of the solution containing methanol and the dissolved preparation, it was then emptied into vials and analysed by HPLC.

2.2.2.1.2. Standard solution preparation

Amounts of approximately 40 mg diclofenac salt, 8 mg methyl paraben, 4 mg BHT, 0.4 mg BHA and 4 mg vitamin E were weighed. All of the ingredients were dissolved in methanol (50 ml) and sonicated for approximately 10 minutes. The dissolved solution was then transferred into vials and analysed by means of HPLC in triplicate.

2.2.2.2. Aesthetics

Photos were taken in direct sunlight using the super macro function on a FUJI S710 (Japan) camera. These photos were then compared over one hundred and eighty days to determine colour changes and consistency of the preparations.

2.2.2.3. *Confocal laser scanning microscopy*

Determinations were done using a confocal laser scanning microscope (CLSM) Nikon D-eclipse C1 (Japan) si utilising a violet diode laser 400-450 nm, a He/Ne laser – 543 nm and a Argon ion later 457-514 nm. The samples for determination were prepared by weighing 0.1 g of the preparation in an eppendorf vial and adding 5 µl nile red and 1 ml water. A vortex mixer was used to mix the contents of the eppendorf vial. A 20 µl sample was then placed on a sample glass and covered with a cover glass.

2.2.2.4. Mass variation

The mass of the formulations were determined at day zero, thirty, sixty, ninety and one hundred and eighty using a Shimadzu (Japan) balance. Preparations were weighed off in the plastic containers. The obtained values were evaluated to determine if a loss in mass has occurred over the duration of one hundred and eighty days.

2.2.2.5. pH variation

A Metler Toledo pH meter was used in the monthly determinations. Before initiating pH determinations the pH meter was calibrated at pH values of 4.00, 7.01 and 10.00.

2.2.2.6. Viscosity

A Brookfield viscosity meter (USA) was used in the determinations of viscosity. Using the spindle size D at 0.3 rpm. The viscosity of the preparations (gels) was evaluated by taking measurements at 10 second intervals. For each viscosity determination 32 measurements were taken and the average viscosity was determined using these values. A Wingather 32 software program was utilized to interpret the flow character of each formulation.

2.2.3. Physicochemical properties

2.2.3.1. High performance liquid chromatography method for the determination of physical properties and skin diffusion

The system used was similar to the system used in assay. Isocratic elution with a mobile phase of acetonitrile:octane-sulphonic acid (50:50) at pH 3.5 was used. The flow rate was set at 1 ml/min and the detection wavelength was set at 280 nm. The diclofenac salts all showed similar retention times of ± 9 minutes.

2.2.3.1.1. Standard preparation

The standard solution was prepared by dissolving 10 mg of each of the diclofenac salts in 100 ml methanol.

2.2.3.2. Aqueous solubility determinations

A shaker bath was preheated to 32 °C. Standard preparations were prepared and injected for chromatographic determinations. Three test tubes were filled with 10 ml PBS with a pH of 7.4 (British Pharmacopoeia, 2007). The test tubes were then placed in a shaker bath and the shaker mechanism was activated. Spoonfuls of the active ingredient were added to the three test tubes at hourly intervals over a period of 24 h to ensure constant saturation of the

solution. An oven was preheated to 32 °C an hour before the test tubes were to be removed from the shaker bath. Three 5 ml plastic syringes and three 0.45 µm filters were placed in an oven preheated to 32 °C, half an hour before removing the test tubes from the shaker bath. The test tubes were removed one at a time leaving the remaining test tubes in a stationary position in the shaker bath, maintaining the temperature at 32 °C. The clear solution was then transferred from the test tubes to the syringes with pre-filters. The solution was then filtered and diluted to a factor relevant to the character of the active ingredient. Finally the solutions were transferred into separate vials and analysed by HPLC in duplicate.

2.2.3.3. Octanol-buffer partition coefficient determinations

At the outset a shaker bath was preheated to 25 °C. The sample solutions were prepared by measuring off 5 mg of the active ingredient. An amount of 10 ml methanol (sample grade) was added to the active ingredient in a 50 ml volumetric flask and filled up to volume with PBS (pH 7.4). Thereafter 5 ml of the aforementioned solution was transferred into a 50 ml volumetric flask and filled up to volume using PBS (pH 7.4). Amounts of 3 ml were then pipetted into three test tubes; 3 ml of n-octanol were added to each tube and placed in the shaker bath at 25 °C for 45 minutes. The test tubes were then removed and centrifuged at 4000 rpm for 30 minutes. A Pasteur pipette was used to remove the PBS phase from the test tubes and it was injected into the HPLC system in duplicate with an injection volume of 10 µl. Subsequently, 1 ml of the octanol phase was removed and diluted with 5 ml of methanol and analysed by HPLC in duplicate with an injection volume of 10 µl (Leo et al., 1971).

2.2.4. Diffusion determinations

2.2.4.1. Human skin samples

Human Caucasian skin samples were obtained from cosmetic surgery abdominoplastic surgeries. Consent was obtained from the surgeons and the patients before any skin samples were removed from the facilities. The transdermal laboratory is an approved facility according to the Human Tissue Act (Reference 04D08).

2.2.4.2. Method of skin diffusion

Initially silicon tubing was cut into eleven, 10 cm pieces. Eleven 5 ml syringes were then removed and fitted with 18 gauge needles. The silicone tubing pieces were slid over the tips of all the needles. Ten Franz cells were used and the top half (donor phase) and the bottom half (receptor phase) were covered with a thin layer of Dow Corning vacuum grease (USA). A magnetic stirrer was placed in the bottom of the receptor phase of each Franz cell. A skin sample 15 mm in diameter (diffusional area of ± 1.075 cm^2) was mounted on top of a piece of Whatman® (USA) filter paper and placed on each of the receptor compartments. The donor compartment was then placed on top of the skin and clamped together using a horseshoe clamp. Syringes were used to fill the receptor compartments with 2 ml PBS (pH 7.4). The preparation to be evaluated was then placed into the donor phase in 1 ml amounts. Pieces of Parafilm® (USA) were placed over the donor phase and then sealed off. The Franz cells were placed into a Franz cell stand upon a Variomac magnetic stirrer (Germany) at 750 rpm and it was ensured that the Franz cells align with the indicated circles. The water bath was closed next and samples of PBS (pH 7.4) were withdrawn at intervals of 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0 hours. After 2 ml samples were extracted at the prescribed intervals, the receptor phase was filled up with PBS (pH 7.4, 37 °C) to mimic sink conditions and analysed by HPLC. After the diffusion process was completed tape stripping was performed.

2.2.5. Tape stripping method

After 12 hours of diffusion, the diffusion cells were carefully dismantled and the skin was pinned on a piece of Parafilm® which was stapled to a solid surface and numbered according to the numbers of each Franz cell. The imprints of the flanges from the diffusion cells clearly marked the exposed diffusion area which had an area of about 1.075 cm^2. The skin was then dried by dabbing it with pieces of tissue paper. The first tape strip was discarded and the following 15 strips were placed in a vial containing 5 ml of PBS at pH 7.4. It is seen as normal practice to discard the first strip as it is considered to be part of the cleaning procedure and it may be contaminated with drug from the formulation. After 15 tape strips the skin showed the glistening of the viable epidermis and this indicated that the tape stripping process was completed. Excess skin was trimmed away from the flange imprints and the remaining dermis and epidermis were placed in separate vials containing 2 ml of

PBS at pH 7.4 (Pellet et al., 1997). The samples were then refrigerated overnight at a temperature of about 8 °C.

2.2.3.4.3. Statistical determinations

The Kruskal-Wallis test was implemented as a non-parametric (distribution free) test, it is used to compare three or more groups of sample data. The Kruskal-Wallis test is used when assumptions of ANOVA are not met. ANOVA is a statistical data analysis technique that is used when there are more than two independent variable groups. In ANOVA, it is assumed that each group has a normal distribution. The Kruskal-Wallis test does not make any assumption about the distribution, thus it is a distribution free test. If normality assumptions are met, then the Kruskal-Wallis test is not as powerful as ANOVA. If the null-hypothesis, being the hypothesis that the samples originate from the same population, is rejected ($p<0.05$), then the conclusion is that there is a statistically significant difference between at least two of the subgroups (SAS institute Inc., 2005). The Kruskal-Wallis test was implemented to evaluate multiple comparisons of p-values using an independent (grouping) variable method (Steyn et al., 1994).

3. Results and discussion

3.1. Stability testing

3.1.1. Assay

The chromatographic recovery values obtained were compared to original values obtained and the following changes in ingredient content were observed. The changes observed after ninety days within formulations subjected to 25 °C were listed according to ingredients contained within the formulations. Changes observed in the recovery of diclofenac diethylamine in Pheroid™ gel **(1)** at 25 °C, are as follows: 11% increase for BHA, 23% increase for BHT, 20% decrease for diclofenac, 22% increase for methyl paraben and 30% increase for vitamin E. The following changes were observed in the recovery of **(1)** at 30 °C: BHA a 12% increase, BHT a 2% decrease, diclofenac a 31% decrease, methyl paraben a 5% decrease and vitamin E a 32% decrease. The following changes were observed in the recovery of **(1)** at 40 □C: BHA a 2% increase, BHT a 65% increase, diclofenac a 32% decrease, methyl paraben a 19% decrease and vitamin E a 9% decrease. Changes observed in recovery for diclofenac diethylamine non-Pheroid™ gel **(2)** at 25 °C, are as follows: 44% decrease for diclofenac and 11% decrease for methyl paraben. The following changes in **(2)** were observed at 30 °C: diclofenac a 30% increase and methyl paraben a 12% increase. The following changes were observed in the recovery of **(2)** at 40 °C: diclofenac a 46% decrease and methyl paraben a 31% decrease. Changes observed in the recovery of diclofenac hydroxyethylpyrrolidine Pheroid™ gel **(3)** at 25 °C, are as follows: 15% increase for BHA, 4% decrease for BHT, 23% decrease for diclofenac, 19% decrease for methyl paraben and 35% increase for vitamin E. The following changes in **(3)** were observed at 30 °C: BHA a 17% decrease, BHT a 3% decrease, diclofenac a 31% increase, methyl paraben a 29% increase and vitamin E a 24% increase. The following changes in **(3)** were observed at 40 °C: BHA a 0.7% increase, BHT a 68% increase, diclofenac a 28% decrease, methyl paraben a 17% increase and vitamin E a 13% increase. Changes observed in the recovery diclofenac hydroxyethylpyrrolidine non-Pheroid™ gel **(4)** at 25 °C, are as follows: 26% decrease for diclofenac and 37% increase for methyl paraben. The following changes were observed in the recovery of **(4)** at 30 °C: diclofenac a 3% decrease and methyl paraben

a 52% increase. The following changes were observed in the recovery of **(4)** at 40 °C: diclofenac a 38% decrease and methyl paraben a 6% increase. Changes observed in the recovery of diclofenac sodium Pheroid™ gel **(5)** at 25 °C, are as follows: 6% increase for BHA, 22% increase for BHT, 25% decrease for diclofenac, 17% decrease for methyl paraben and 12% decrease for vitamin E. The following changes were observed in the recovery of **(5)** at 30 °C: BHA a 27% decrease, BHT an 8% decrease, diclofenac a 49% decrease, methyl paraben a 15% decrease and vitamin E a 37% increase. The following changes were observed in the recovery of **(5)** at 40 °C: BHA a 3% decrease, BHT a 22% increase, diclofenac a 31% decrease, methyl paraben a 17% increase and vitamin E a 21% decrease. Changes observed in the recovery of diclofenac sodium non-Pheroid™ gel **(6)** at 25 °C, are as follows: 36% decrease for diclofenac and 31% decrease for methyl paraben. The following changes were observed in the recovery of **(6)** at 30 °C: diclofenac a 30% decrease and methyl paraben a 44% increase. The following changes were observed in the recovery of **(6)** at 40 °C: diclofenac a 35% decrease and methyl paraben a 36% increase. None of the evaluated preparations showed stability at accelerated stability conditions. Increases in ingredient concentrations might be attributed to water loss from within formulation and the use of different HPLC columns as the months progressed.

3.1.2 pH Variation

All of the Pheroid™ preparations containing diclofenac salts showed a substantial decrease in pH over the term of six months from approximately. Non-Pheroid™ preparations showed only a minimal decrease in average pH value.

3.1.3. Viscosity variation

Pheroid™ and non-Pheroid™ preparation viscosity data showed no substantial decrease in viscosity over the term of six months at a stability temperature of 25 °C. At higher accelerated stability temperatures (30 and 40 °C) the non-Pheroid™ preparations would potentially show higher viscosity values due to loss in water mass. Pheroid™ preparations maintained acceptable viscosity as the water mass within the gel preparations were contained within the Pheroid™ portion of the preparation and as a result the water content did not evaporate.

3.1.4. Aesthetics

Changes in colour were evaluated over the course of 180 days and the following changes were noted. **(1)** changed in colour from a white gel to a light yellow gel. **(2)** was initially colourless, depicted an initial colour of a a clear transparent gel. Over the term of stability testing the preparation changed to an orange transparent gel. **(3)** was initially a gel white in colour which changed to a mustard-yellow colour. **(4)** was initially a colourless transparent gel which changed in colour to an orange transparent gel. **(5)** a gel, initially displayed a white colour which changed to a dark mustard colour. **(6)** a transparent gel, displayed an orange colour initially of an orange see-through gel which changed to a darker coloured orange transparent gel.

3.1.5. Confocal laser scanning microscopy

The particle size were observed for 6 months and it was determined that the particle size remained the same over a period of 6 months. The preparations displayed no formation of crystals and remained homogenous over the period of accelerated stability testing.

3.1.6. Mass variation

The following changes in mass, of the diclofenac containing preparations, were noted over the period of 180 days. **(1)** displayed a 7% decrease (25 °C), a 9% decrease (30 °C) and a 36% decrease (40 °C). **(2)** displayed a 14% decrease (25 °C), a 27% decrease (30 °C) and a 36% decrease (40 °C). **(3)** displayed a 4% decrease (25 °C), a 17% decrease (30 °C) and a 3% decrease (40 °C). **(4)** displayed a 10% decrease (25 °C), a 37% decrease (30 °C) and a 40% decrease (40 °C). **(5)** displayed a 4% decrease (25 °C), a 10% decrease (30 °C) and a 20% decrease (40 °C). **(6)** displayed a 7% decrease (25 °C), a 4% decrease (30 °C) and a 59% decrease (40 °C). Comparison of non-Pheroid™ and Pheroid™ formulations highlighted that the non-Pheroid™ formulations displayed a bigger degree of loss in mass than the Pheroid™ formulations. It was clear that Pheroid™ formulations remained more stable even at higher stability temperatures than the non-Pheroid™ formulations. The mass instabilities as depicted by the mass variation data obtained for non-Pheroid™ preparations can be contributed to a high degree of water mass loss especially at higher accelerated stability temperatures.

3.2. Physicochemical properties

3.2.1. Aqueous solubility and Log D determinations

Solubility determinations were done at 32 °C and pH 7.4 using PBS as the solvent. The following aqueous solubility and log D values were obtained, respectively: 4.3 mg/ml and 1.6 (diclofenac diethylamine), 7.1 mg/ml and 1.9 (diclofenac hydroxyethylpyrrolidine) and 6.2 mg/ml and 1.7 (diclofenac sodium). According to aqueous solubility data obtained for the diclofenac salts the compounds will be able to diffuse through the skin as the solubility values are higher than 1 mg/ml (Naik et al., 2000). For drugs to effectively move through the skin the solubility in oil and water should be balanced (Barry, 2001) and the octanol-water partitioning coefficient (log P) should have a value of 1-3 (Hadgraft, 2004). The aqueous solubility data, as well as the log D values obtained suggested that diclofenac hydroxyethylpyrrolidine has the potential to reach the highest concentration within the dermis (water soluble environment of skin), followed by diclofenac sodium and then diclofenac diethylamine.

3.3. Skin diffusion

3.3.1. *In vitro* permeation studies

No flux values were obtained for skin diffusion studies after experiments were done on either the diclofenac formulations (containing non-Pheroid™ and Pheroid™) or on the South African market products, over the range of all of the diclofenac salts evaluated. This is due to the lack of penetration through the layers of the skin. Therefore the target site (deep muscle tissue region) was not penetrated.

3.3.2. Tape stripping data

(1) and **(2)** had the largest amount of diclofenac salt within the epidermis, both with a concentration of 2.93 µg/ml. These values were only slightly higher than the values obtained from the retail product Voltaren® (2.89 µg/ml). The diclofenac dermis concentration of **(1)** (7.45 µg/ml) and **(2)** (7.40 µg/ml) is roughly the same and the concentration of the retail product Voltaren®, (7.27 µg/ml) was slightly lower. Of all the other formulations and patented products it was found that **(1)** and **(2)** had the largest concentration obtained within the dermis. The lowest concentration contained within the epidermis was **(3)** (0.41 µg/ml) and

(4) (0.43 µg/ml). The retail product Panamor® (0.64 µg/ml) showed a higher concentration in the epidermis than the aforementioned formulations containing the same diclofenac salt. The lowest concentration detected within the dermis was 1.04 µg/ml in the case of **(5)** and it also had a lower concentration than the retail product Panamor® (1.60 µg/ml). After comparing the Pheroid™ with the non-Pheroid™ preparations, it was observed that in the case of the Pheroid™ preparations higher levels of diclofenac were detected in both the epidermis and dermis, although **(4)** had a slightly higher concentration in the epidermis than **(3)**. When comparing the Pheroid™ and non-Pheroid™ formulations with the retail product, all the formulations had better concentration values in both the epidermis and dermis than the retail product. This was not true for **(6)** where there was a lower concentration in the epidermis and in the case of **(3)** and **(4)** there was a lower concentration in both the epidermis and dermis. The containment within the specific areas of the skin cannot be clearly explained by inspection of the solubility and log D values of the respective salts. According to the log D values and the aqueous solubility values obtained, these salts should move through the skin effectively (Hadgraft, 2004). These salts, however, do not reach sufficient concentration at the site of action, the deep skin tissue. After closer consideration of diclofenac's other physical properties it became clear that diclofenac salts have molecular masses (between 200 and 500) that result in slow transdermal diffusion of these salts (Goosen et al., 1998). Diclofenac has the tendency to form more than two hydrogen bonds; this factor also results in negligible transdermal flux values (Pugh *et al.*, 1996). Another contributing factor is the bonding of diclofenac to the stratum corneum. This could result in insufficient levels of active ingredient at the target of action (Fini *et al.*, 1995).

Figure 1: The tape stripping epidermis and the dermis concentration values (µg/ml) for **(a)** diclofenac diethylamine, **(b)** diclofenac hydroxyethylpyrrolidine and **(c)** diclofenac sodium; obtained after skin diffusion studies.

3.3.3. Statistical analysis of data

Statistical analysis of the p-values obtained from tape stripping values illustrates the statistical differences as contained in Table 1 and Table 2. No statistical significant differences were depicted by the p-values obtained for epidermis to epidermis concentration

comparisons. The following statistical significant differences were observed by comparing the p-values obtained for dermis to dermis concentrations (µg/ml) between formulations: **(1)** and **(3)**, **(1)** and **(4)**, **(1)** and **(5)**, **(1)** and Panamor®, **(2)** and **(3)**, **(2)** and **(4)**, **(2)** and **(5)**, **(2)** and Arthruderm®, **(2)** and Panamor®, **(3)** and Voltaren®, **(4)** and **(6)**, **(4)** and Arthruderm®, **(4)** and Voltaren®, **(5)** and Panamor®.

Table 1: p-Values depicting statistical significant variance between the epidermal values obtained

Table 2: p- Values depicting statistical significant variance between the dermal values obtained

4. Conclusions

The goal of this study was to evaluate the differences in efficacy of three structurally different diclofenac salts formulated as a Pheroid™ and non-Pheroid™ preparation. These formulations were then subjected to accelerated stability conditions for the duration of one hundred and eighty days. Stability determination indicated uniformity of content of the active ingredient concentrations in the case of diclofenac diethylamine. Diclofenac hydroxyethylpyrrolidine did not comply with standards as set by the ICH (International conference on Harmonisation) (2005). Both of these preparations showed values which varied with more than 5% from the initial standard dosage of 100%. The diclofenac sodium preparations, however, remained more stable over the duration of the accelerated stability determinations. The receptor phase showed that skin diffusion did not occur and no flux values for any of the preparations (Pheroid™, non-Pheroid™ and retail products) were attained. According to data obtained from skin diffusion studies none of the active ingredient reached the target site of action, namely deep muscle tissue. The epidermis and dermis values obtained for the preparations did not conclusively show a correlation with the physicochemical data obtained (Hadgraft, 2004).

The most significant concentration of active ingredient contained within the epidermis and dermis (the site closest to the site of action) was seen in the determinations done with preparation **(1)** and **(2)**. The outcome of this evaluation could be summarised by highlighting that no specific diclofenac salt or preparation evaluated was more successful in reaching substantial active ingredient content at the target site, the systemic circulation or deep muscle tissue.

Acknowledgments

The authors would like to thank Dr Jan du Plessis for assistance in the representation of statistical data and the National Research foundation for providing the funds necessary to undertake this project. The authors would also take pleasure in thanking all of the physicians, Dr. A Lamont (Carstenhoff clinic, Midrand), Dr L Gordon (The Glynnwood, Benoni) and Dr DA Hoffman (Sunward Park hospital, Boksburg), who participated by providing human skin samples.

References

Ah, Y., Choi, J., Choi, Y., Ki, H., Bae, J., 2009. A novel transdermal patch incorporating meloxicam: In vitro in vivo characterization. Int. J. Pharm, 385: 12-19.

Barry, W.B. 2001. Novel mechanisms and devices to enable successful transdermal delivery. J. Phar. Pharm. Sci, 14: 101-114. J. Phar. Pharm. Sci., 14, 101-114.

British Pharmacopoeia, 2007. Volume IV, Appendix ID, p. A140.

Fini, A., Fazio, G., Hervas, M.F., Holdago, M.A., Rabasco, A.M., 1995. Factors governing the dissolution of diclofenac salts. J. Phar. Pharm. Sci, 4, 231-238.

Goosen, C., Du Plessis, J., Muller, J., Janse van Rensburg, D.J. 1998. Correlation between physicochemical characteristics, pharmacokinetics properties and transdermal absorption of NSAID's. Int. J. Pharm, 163, 203-209.

Hadgraft, J., 2004. Skin deep. Int. J. Pharm, 58, 291-299

ICH: International conference on harmonization of technical requirements for registration of pharmaceuticals for human use. 2005. Validation of analytical procedures: Text and methodology, Q2(R1).

Leo, A., Hansch, C., Elkins, D., 1971. Chem. Rev. 71, 525-555.

Medicines Control Council, 2009. Stability. Registration of medicine, Dep. Health, 24 p. www.mccza.com/documents/2.05%20Stability%23Jun09%20v4.doc Date of access: 18 March 2009.

Naik, A., Kalia, Y.N., Guy, R.H., 2000. Transdermal drug delivery: overcoming the skin's barrier function. Pharmaceut. Sci. Tech. **Today.**, 3, 318-325.

Pellet, M.A., Roberts, M.S., Hadgraft, J., 1997. Supersaturated solutions evaluated with an *in vitro* stratum corneum tape stripping technique. Int. J. Pharm., 151, 91-98.

Pugh, W.J., Roberts, M.S., Hadgraft, J., 1996. Epidermal permeability – penetrant structure relationships: The effect of hydrogen bonding interactions and molecular size on diffusion across the stratum corneum. Int. J. Pharm., 138, 149-165.

SAS Institute Inc., 2005. SAS institute Inc., SAS OnlineDoc®, version 9.1, Cary, NC.

Saunders, C.J., Davis, H.J., Coetzee, L., Botha, S., Kruger, A.E., Grobler, A., 1999. A Novel Skin Penetration Enhancer: Evaluation by membrane diffusion and confocal microscopy. J. Phar. Pharm. Sci., 2, 99-107.

Steyn, A.G.W, Smit, C.F., Du Toit, S.C.H., Strasheim, C., 1994. Modern statistics in practice, J.L. van Schaik, Pretoria, South Africa, pp. 604-606.

United States Pharmacopoeia (USP), 2005. Physical Tests / (905) Uniformity of Dosage Units, 28th ed., The United States Pharmacopeial Convention. pp. 2503-2510.

FIGURE LEGENDS:

Figure 1: The tape stripping epidermis and the dermis concentration values (µg/ml) for **(a)** diclofenac diethylamine, **(b)** diclofenac hydroxyethylpyrrolidine and **(c)** diclofenac sodium; obtained after skin diffusion studies

Table 1: p-Values depicting statistical significant variance between the epidermal values obtained.

	(1)	(2)	Voltaren®	(3)	(4)	Panamor®	(5)	(6)	Arthruderm®
(1)		0.710	0.928	4.383	5.383	5.880	1.807	3.085	3.034
(2)	0.710		1.639	5.092	6.094	6.569	2.512	3.796	3.744
Voltaren®	0.928	1.639		3.453	4.455	4.930	0.873	2.156	2.106
(3)	5.383	6.094	4.455		1.000	0.475	3.582	2.298	2.349
(4)	4.382	5.092	3.455	1.001		1.476	2.580	1.296	1.340
Panamor®	5.883	6.569	4.490	0.457	1.476		4.057	2.773	2.845
(5)	1.801	2.512	0.873	2.580	3.582	4.057		1.238	0.051
(6)	3.085	3.796	0.873	1.296	2.289	2.773	1.283		1.232
Arthruderm®	3.085	3.744	2.156	1.348	2.349	2.854	1.232	0.051	

Table 2: p-Values depicting statistical significant variance between the dermal values obtained.

	(1)	(2)	Voltaren®	(3)	(4)	Panamor®	(5)	(6)	Arthruderm®
(1)		1.000	1.000	0.000*	0.000*	0.000*	0.004*	1.000	0.112
(2)	1.000		1.000	0.000*	0.000*	0.000*	0.003*	1.000	0.088
Voltaren®	1.000	1.000		0.000*	0.013*	0.011*	0.368	1.000	1.000
(3)	0.000*	0.000*	0.000*		1.000	1.000	1.000	0.252	0.022*
(4)	0.000*	0.000*	0.013*	1.000			0.368	0.006*	0.592
Panamor®	0.000*	0.000*	0.011*	1.000	1.000		0.035*	1.000	1.000
(5)	0.003*	0.004*	0.368	0.006*	0.252	0.035*		1.000	1.000
(6)	1.000	1.000	1.000	0.022*	1.000	0.529	1.000		1.000
Arthruderm®	0.080	0.122	1.000	0.368	1.000	1.000	1.000	1.000	

*indicates statistical significant variance

FIGURES:

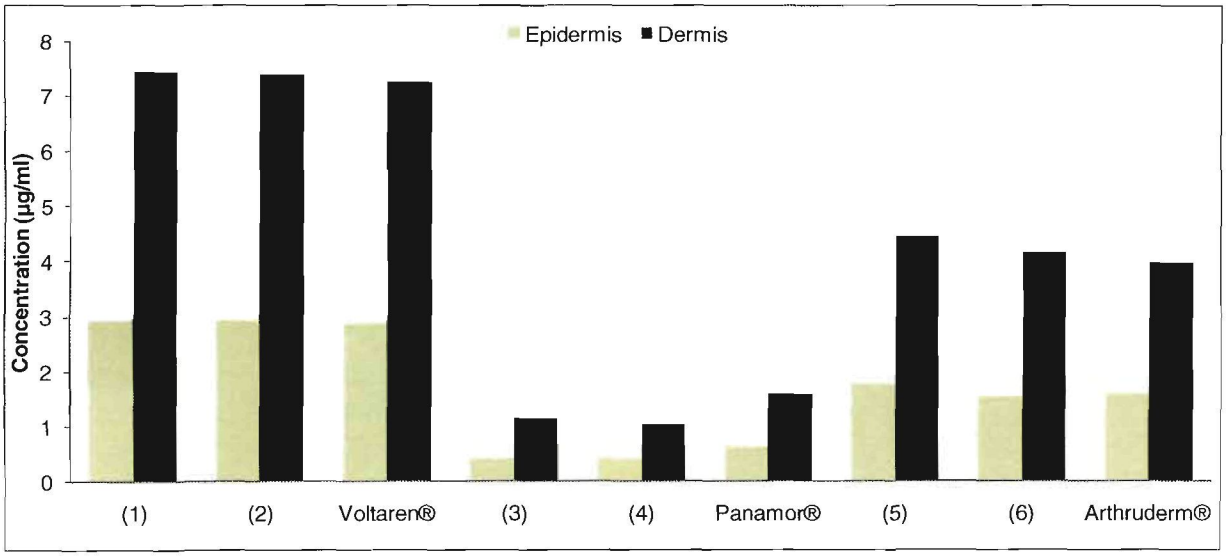

Figure 1: The tape stripping epidermis and the dermis concentration values (µg/ml) for **(a)** diclofenac diethylamine, **(b)** diclofenac hydroxyethylpyrrolidine and **(c)** diclofenac sodium; obtained after skin diffusion studies

Chapter 4

Final conclusions and future perspectives

4.1 Study conclusions

Muscular pain disorders affect a large amount of people worldwide every year. In the Netherlands alone the treatment cost of lower back pain in the workforce has amounted to US $ 4.6 billion (Van Tulder *et al*, 1995:233). Muscle pain in the back muscles can also result in severe tension headaches by sensitising neurons in the central nervous system (Olesen, 1991:125). After evaluation of the literature it became apparent that pain is a condition which is very complex in nature. Pain treatment can only be effective if the condition is seen and understood as a multi facetted condition. The treatment should be psychological, physical and preventative in nature.

The skin was chosen as a route for administration of non-steroidal anti-inflammatory drugs for the treatment of pain in the deep muscle tissue areas. The skin, however, forms a very effective barrier against the penetration of chemicals from the outside environment the stratum corneum. The stratum corneum is a bio membrane unique in structure and consistency (Charalambopoulou *et al.*, 2004:603).

Various diclofenac salts were evaluated to determine if a specialised carrier system (Pheroid™) could be utilised to enhance the transdermal diffusion of these compounds. All the diclofenac salts have a few characteristics which result in insufficient transdermal fluxes. The diclofenac salts investigated all have molecular masses of between 200 and 500 which causes slow transdermal permeation (Goosen *et al*, 1998:206). The melting points of the diclofenac salts are also very high, resulting in ineffective transdermal diffusion (Ah *et al*, 2009:12). Formation of hydrogen bonds also plays a role in the determination of subcutaneous permeation. Molecules that show the potential to form more than two hydrogen bonds may bind to the stratum corneum and therefore not diffuse through the skin (Pugh *et al*, 1996:164). Diclofenac salts tend to form multiple hydrogen bonds when exposed to an aqueous medium (for example the environment within a gel medium). This formation of hydrogen bonds might result in stratum corneum bonding (Fini *et al*, 1995:237).

Diclofenac salts were formulated as Pheroid™ and non-Pheroid™ preparations and the transdermal diffusion were evaluated by means of Franz cell diffusion studies. Membrane diffusion studies were also done as a comparative study to determine which formulation showed the best release of active ingredient from the formulation. Transdermal and membrane diffusion studies were done on Pheroid™, non-Pheroid™ and commercial

products containing diclofenac diethylamine, diclofenac hydroxyethylpyrrolidine and diclofenac sodium. Results obtained indicated that diclofenac diethylamine showed the highest levels of containment within the epidermal and dermal layers, there was, however, no indication of transdermal flux. Membrane diffusion studies highlighted a diclofenac sodium formulation as the formulation releasing the largest amount of active ingredient from the preparation. The study highlighted that none of the preparations evaluated showed sufficient transdermal flux to reach the target of action: the deep muscle tissue.

This study has shown that the following possibilities exist for the future study of the transdermal delivery of diclofenac salts. The various diclofenac salts can be formulated together with different penetration enhancers. The transdermal diffusion of these preparations can be evaluated to determine which enhancers produce the most effective flux. The formulations can also be adjusted to determine which preservative and antioxidant combination would result in the most effective and stable preparation. After the successful formulation of a product which shows efficacy and stability, clinical studies can be initiated.

Natural alternatives for the treatment of muscular inflammation can also be explored. In order to find topical alternatives for the treatment of pain and inflammation research done on the anti-inflammatory properties of emu oil may be investigated. Emu oil formulated in a topical muscle gel has shown efficacy in the relief of inflammation in muscular areas (Lopez *et al.*, 1999:1). Research has also found that emu oil can be utilised in the treatment of chronic pain conditions when formulated together with a penetration enhancer. These preparations act by mediating a local immune regulation response. These oil formulations depicted stability against accelerated stability conditions and are able to withstand high temperatures. Possible problems in formulation include unpredictable potency of commercial samples and irregular distribution of actives within preparations (Whitehouse *et al.*, 1998:2).

References

AH, Y., CHOI, J., CHOI, Y., KI, H. & BAE, J. 2009. A novel transdermal patch incorporating meloxicam: In vitro in vivo characterization. *International Journal of Pharmaceutics.* 385:12-19.

CHARALAMBOPOULOU, G.C., STEROITIS, T.A., HAUSS, T., STUBOS, A.K. & KANELLOPOULOS, N.K. 2004. Structural alterations of fully hydrated human stratum corneum. *Physica.* 350:603-606.

FINI, A., FAZIO, G., HERVAS, M.F., HOLGADO, M.A. & RABASCO, A.M. Factors

Governing the dissolution of diclofenac salts. *European Journal of Pharmaceutical Sciences.* 4:231-238.

GOOSEN, C., DU PLESSIS, J, MULLER, D.J. & JANSE VAN RENSBURG, L.F. 1998. Correlation between physicochemical characteristics, pharmacokinetic properties and transdermal absorption of NSAID's. International Journal of Pharmaceutics, 163(2):203-209.

LOPEZ, A., SIMS, D.E., ABLETT, R.F., SKINNER, R.E., LEGER, L.W., LARIVIERE, C.M., JAMIESON, L.A. and MARTINEX, B.J. 1999. Effect of emu oil on auricular inflammation induced with croton oil in mice. *American Journal of Veterinary research.* 60(12):1-2.

OLESEN, J. 1991. Clinical and pathophysiological observations in migraine and tension-type headache explained by integration of vascular, supraspinal and myofascial inputs. *Pain.* 46:125-132.

PUGH, W.J., ROBERTS, M.S. & HADGRAFT, J. 1996. Epidermal permeability – penetrant structure relationships: 3. The effect of hydrogen bonding interactions and molecular size on diffusion across the stratum corneum. *International Journal of Pharmaceutics.* 138:149-165.

VAN TULDER, M.W., KOES, B.W. & BOUTER, L.M. 1995. A cost-of-illness study of back pain in the Netherlands. *Pain.* 62:233-240.

WHITEHOUSE, M.W., TURNER, A.G., DAVIS, C.K.C. & ROBERTS, M.S. 1998. Emu oil(s): A source of non-toxic transdermal anti-inflammatory agents in aboriginal medicine. *Inflammopharmacology.* 6(1):1-8.

ANNEXURE A

Method development and validation for the chromatographic determination of diclofenac salts

A.1 Introduction

A liquid chromatographic method was developed and validated to quantify the active ingredient concentration obtained in skin diffusion and membrane diffusion samples during the study.

A.2 HPLC (High performance liquid chromatography) method validation for diclofenac

A.2.1 Methods and Reagents

Analytical instrument	An Agilent 1100 series HPLC (Agilent Technologies, Palo Alto, CA) equipped with a gradient pump, auto sampler, UV detector and Chemstation Rev. A.08.03 data acquisition and analysis software or equivalent were used during the analysis of diclofenac salt concentrations.
Column	A Luna C18-2 column, 150 x 4.6 mm, 5 µm, 100 Å pores, 17.8% carbon load, end capped, Phenomenex, Torrance, CA was used.
Mobile phase	The mobile phase consisted of acetonitrile/water (50/50), pH adjusted to 3.5 using phosphoric acid, isocratic elution.

Flow rate	1.0 ml/min
Injection volume	10 µl
Detection	UV at 280 nm
Retention time	Approximately 9 minutes for Diclofenac Sodium, Diclofenac Diethylamine, and Diclofenac Hydroxyethyl pyrrolidine.
Stop time	12 minutes
Solvents	Methanol (10% during standard preparation) and deionised water.

A.2.2 Standard preparation

Approximately 10 mg of each of the diclofenac salts were individually and accurately weighed. The salts were dissolved in 100 ml deionised water. A further 5 ml was diluted to 50 ml. The last step was performed 2 times. The standard solution was then transferred into auto sampler vials and analysed using the high performance liquid chromatography system.

A.3.1 Validation of test procedure and acceptance criteria

A.3.1.1 Linearity

Linearity is defined as the ability of an analytical procedure to produce results that is directly proportional to the concentration of active substances in a sample within a given range. Linear regression analysis complies when it yields a regression coefficient of (R^2) ≥ 0.99 (Hong *et al.*, 2000:366-367). Table A1 lists the peak area values for diclofenac sample in methanol and water.

Table A1: Peak area values of diclofenac.

Linearity			
µg/ml	**Peak Area**		**Mean**
0.2	55.4	55.2	55.3
0.3	82.4	82.5	82.5
0.4	111.3	110.7	111.0
0.5	138.0	138.1	138.1
1.0	231.4	230.0	230.7
2.0	459.9	462.6	461.3
3.0	690.8	691.3	691.1
4.0	925.8	921.0	923.4
5.0	1156.4	1146.8	1151.6
10.0	2292.1	2291.0	2291.6
20.0	4580.9	4587.0	4584.0
30.0	6864.8	6863.3	6864.1
R^2	**0.999988**	Lower 95%	Upper 95%
Intercept	-9.14607	-10.4629	-7.82926
Slope	6.195034	6.179629	6.210439

Figure A1 indicates the linear regression curve for diclofenac sodium.

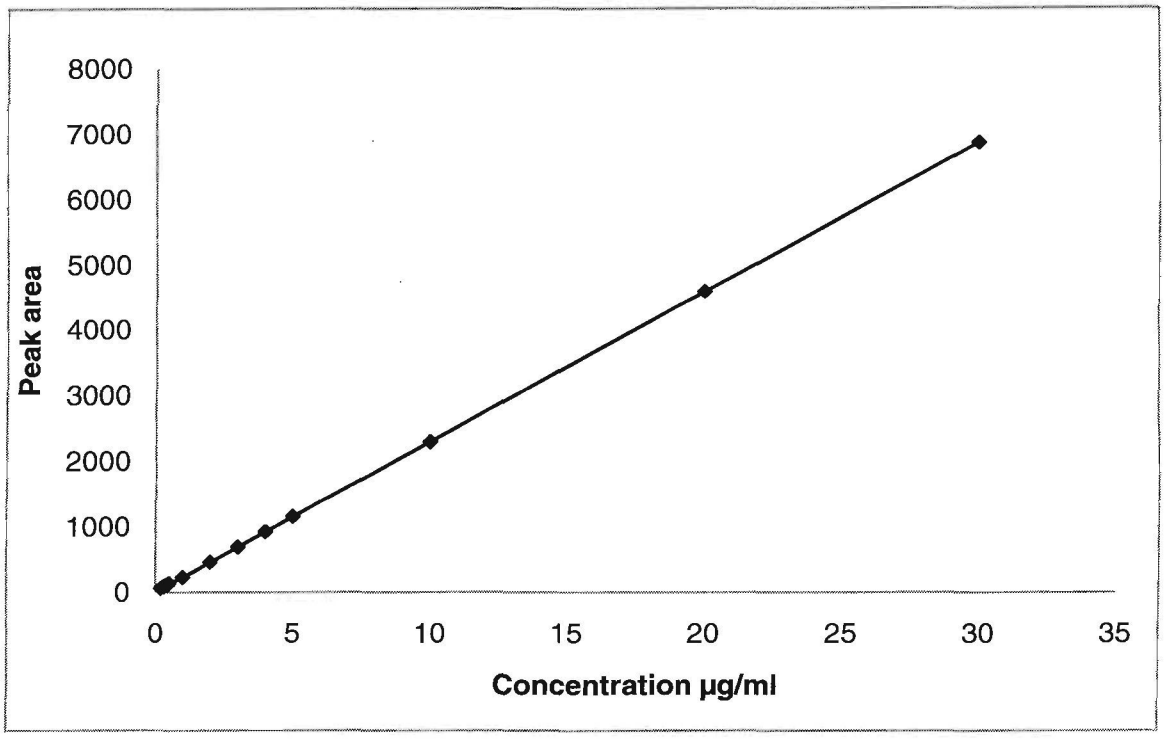

Figure A1: Linear regression curve of diclofenac.

A.3.1.2 Accuracy

Accuracy is the indication of the closeness of the experimental value to the true value. During this stage of method development the percentage active substances recovered by assay is measured and compared to a standard preparation. It should be established across the specified range of the analytical procedure. Acceptance criteria for accuracy are recovery values of 98 to102% (Hong *et al.*, 2000:336-367). Table A2 lists the accuracy values for diclofenac samples in methanol and water.

Table A2: Table indicating accuracy values of diclofenac samples in methanol and water.

Accuracy:					
Conc.spiked					**Recovery**
µg/ml	**Area ***	**Area ***	**Mean**	**µg/ml**	**%**
8.2	498.1	497.3	498	81.8	102.0
8.2	499.2	498.4	499	82.0	102.2
8.2	494.2	495.4	495	81.3	101.4
10.2	621.5	623.2	622	101.9	101.8
10.2	621.5	619.8	621	101.7	101.5
10.2	620.6	622.2	621	101.8	101.6
12.2	741.4	742.4	742	121.2	100.9
12.2	743.6	744.1	744	121.5	101.1
12.2	748.5	746.1	747	122.1	101.6
				Mean	**101.6**
				SD **	**0.4**
				% RSD ***	**0.4**

Area * refers to peak area

SD ** refers to standard deviation % RSD *** refers to relative standard deviation

A.3.1.3 Precision

The closeness of data values to each other for a number of measurements under the same analytical conditions is called precision. Precision refers to the distribution of test results around their average, and is expressed as the percent relative standard deviation for a statistical significant number of samples (Hong *et al.*, 2000:336-367). Table A3 lists the precision values of a diclofenac salt sample in methanol and water.

Table A3: Precision values of diclofenac sample in methanol and water.

Precision						
Mass (g)	**Area**	**Area**	**Mean**	**Conc.µg/ml**	**mg/5 ml**	**%**
8.490	462.4	463.0	462.7	75.5	52.0	103.9
9.040	492.2	491.9	492.1	80.3	51.9	103.8
8.560	457.6	457.6	457.6	74.7	51.0	101.9
10.750	584.1	583.9	584.0	95.3	51.8	101.6
10.540	572.7	572.1	572.4	93.4	51.8	101.6
10.730	580.0	580.2	580.1	94.7	51.5	103.1
12.400	672.4	671.8	672.1	109.7	51.7	103.4
12.4	670.6	670.8	670.7	109.5	51.5	103.1
12.7	685.8	686.4	686.1	112.0	51.3	107.2
				Mean	**51.6**	**103.2**
				SD	**0.29**	**0.58**
				RSD %	**0.56**	**0.56**

A.3.1.4 Inter-day precision

Inter-day precision are the results of the HPLC method operating over a short time interval under the same conditions; also termed intra-assay precision. Injector performance and analysis of samples are evaluated during this procedure. The acceptance for inter-day precision is a RSD ≤ 5% (Hong *et al.*, 2000:336-367). Table A4 lists the inter-day precision values of a diclofenac salt sample in methanol and water on day 2.

A.3.1.4.1 Day 2 inter-day precision

Table A4: Inter day precision values of a diclofenac sample in methanol and water on day 2.

Inter day precision						
Mass (g)	**Area**	**Area**	**Mean**	**Conc.**	**mg/5 ml**	**%**
10.7	5.82.2	583	579	95.1	51.6	103.2
10.6	568.8	570	573	93.0	51.5	102.9
10.7	575.8	576	579	94.1	51.3	102.5
			Mean		**51.4**	**102.8**
			SD		**0.13**	**0.27**
			RSD %		**0.26**	**0.26**

A.3.1.4.2 Day 3 inter-day precision

Table A5 lists the inter-day precision values of a diclofenac sample in methanol and water on day 3.

Table A5: Inter-day precision values of a diclofenac sample in methanol and water on day 3.

Inter day precision						
Mass (g)	**Area**	**Area**	**Mean**	**Conc.**	**mg/5 ml**	**%**
10.7	576.4	580.8	579	94.5	51.7	103.3
10.6	569.7	575.3	573	93.5	51.3	102.7
10.7	576.9	580.9	579	94.5	51.4	102.9
			Mean		**51.4**	**102.9**
			SD		**0.13**	**0.26**
			RSD %		**0.25**	**0.25**

A.3.1.5 Ruggedness

Samples should be analysed within the period before degradation of 2% can occur. If degradation does occur, precaution should be taken to compensate for the loss. During this validation procedure the sample stability is evaluated over a period of 12 hours. Acceptance criteria are RSD values ≤ 2 % (Hong *et al.*, 2000:366-367). Table A6 lists ruggedness values obtained over a time span of 12 hours.

Table A6: Table indicating ruggedness values of a diclofenac sample in methanol and water.

Time	Peak area	Sample
0	670.7	100.0
1	670.2	99.9
2	671.2	100.1
3	671.6	100.1
4	671.2	100.1
5	671.4	100.1
6	673	100.3
7	671.3	100.1
8	670.9	100.0
9	671.5	100.1
10	670.4	100.0
11	669.5	99.8
12	671.2	100.1
Mean	**671.1**	**100.1**
SD	**0.80**	**0.12**
RSD %	**0.12**	**0.12**

A.4 HPLC (High performance liquid chromatography) method validation for Pheroid™ and non-Pheroid™gel preparations containing diclofenac

A.4.1 Methods and Reagents

Analytical instrument	An HP1100 series HPLC, equipped with a gradient pump, auto sampler, UV detector and Chemstation Rev. A.08.03 data acquisition and analysis software or equivalent were used during the study.
Column	A Luna C18-2 column, 150 x 4.6 mm, 5 µm, 100 Å pores, 17.8% carbon load, end capped, Phenomenex, Torrance, CA was used.
Flow rate	1.0 ml/min
Injection volume	20 µl
Detection	UV at 280 nm
Retention time	The retention time for diclofenac diethylamine, diclofenac hydroxyethylpyrrolidine and diclofenac sodium was approximately ± 10 minutes. BHA elutes at ± 9 minutes, BHT at ± 12 minutes. Methyl paraben eluted at ± 5 minutes and vitamin E at ± 20 minutes.
Stop time	25 minutes

Solvents	Methanol sample grade

The mobile phase for chromatographic determinations consisted of orthophosphoric acid **(A)** 1 g in 1000 ml water, pH adjusted to 3.5 with phosphoric acid and Lichrosolve grade methanol **(B)**. During the chromatographic determinations the mobile phases were injected in different percentages, table A7 indicates the ratios of orthophosphoric acid to lichrosolve methanol injected per timed sample run.

Table A7: Gradient table of mobile phase ingredient ratios.

Orthophosphoric acid (A)	**Methanol (B)**	**Duration (minutes)**
50	50	1
0	100	8
0	100	20
50	50	20.1

Standard preparation

Approximately 2 grams of each one of the non-Pheroid™ and Pheroid™ preparations that contain diclofenac sodium, diclofenac diethylamine, and diclofenac hydroxyethylpyrrolidine were weighed off and dissolved in 50 ml methanol in a 50 ml volumetric flask.

A.4.2 Validation of test procedure and acceptance criteria

A.4.2.1 Linearity

The ability of an analytical procedure to produce results that is directly proportional to the concentration of active substances in a sample within a given range is called the linearity (Hong *et al.*, 2000:366-367). Tables A8 to A12 list the linearity values of BHA, BHT, diclofenac, methyl paraben and vitamin E. Corresponding Figures A2 to A6 depict the linearity curves for BHA, BHT, diclofenac, methyl paraben and vitamin E.

A.4.2.1.1 Linearity of BHA

Table A8: Linearity values of BHA in preparation sample.

Linearity			
µg/ml	**Area**		**Mean**
6.0	141.1	127.0	134.0
7.0	191.2	191.5	191.4
8.0	217.1	229.4	223.3
9.0	332.1	305.9	319.0

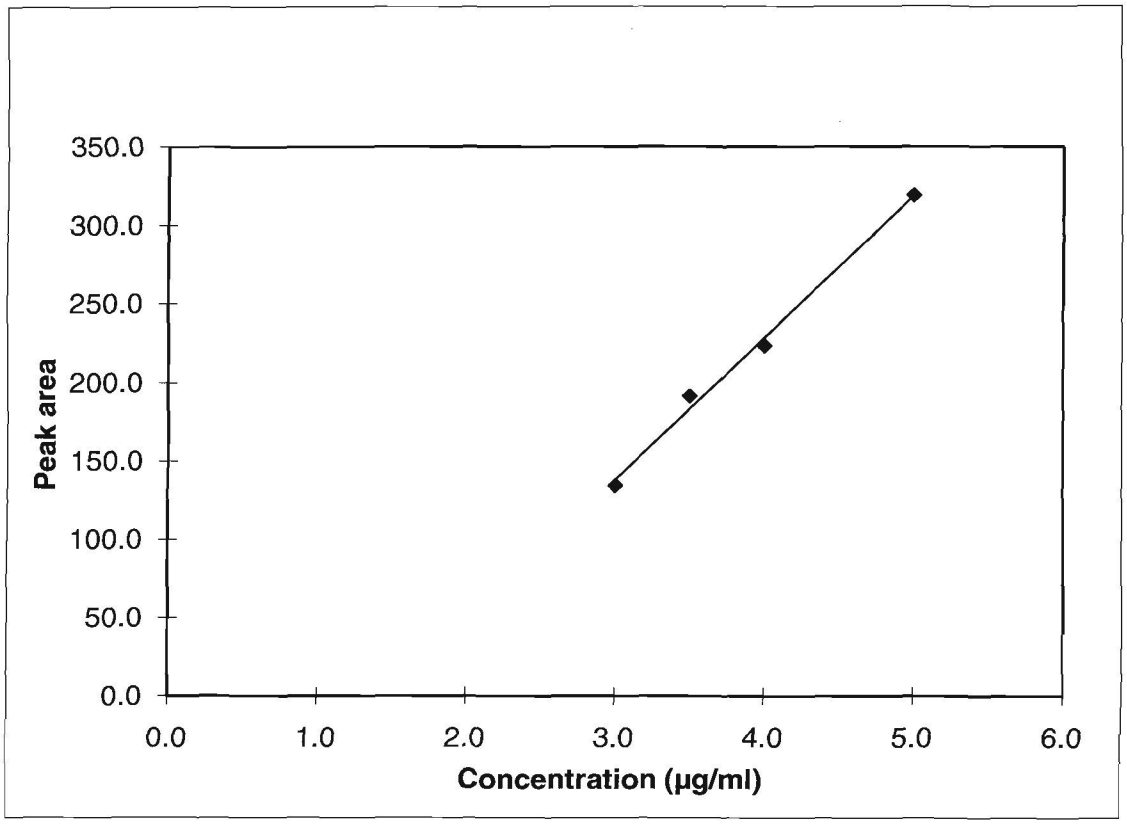

Figure A2: Curve indicating linearity of BHA.

A.4.2.1.2 Linearity of BHT

Table A9: Linearity values of BHT in preparation sample.

Linearity			
µg/ml	Area		Mean
30	2127.0	2144.0	2135.5
40	2319.0	2320.0	2319.5
50	2984.0	2806.0	2895.0
60	3383.0	3435.0	3409.0

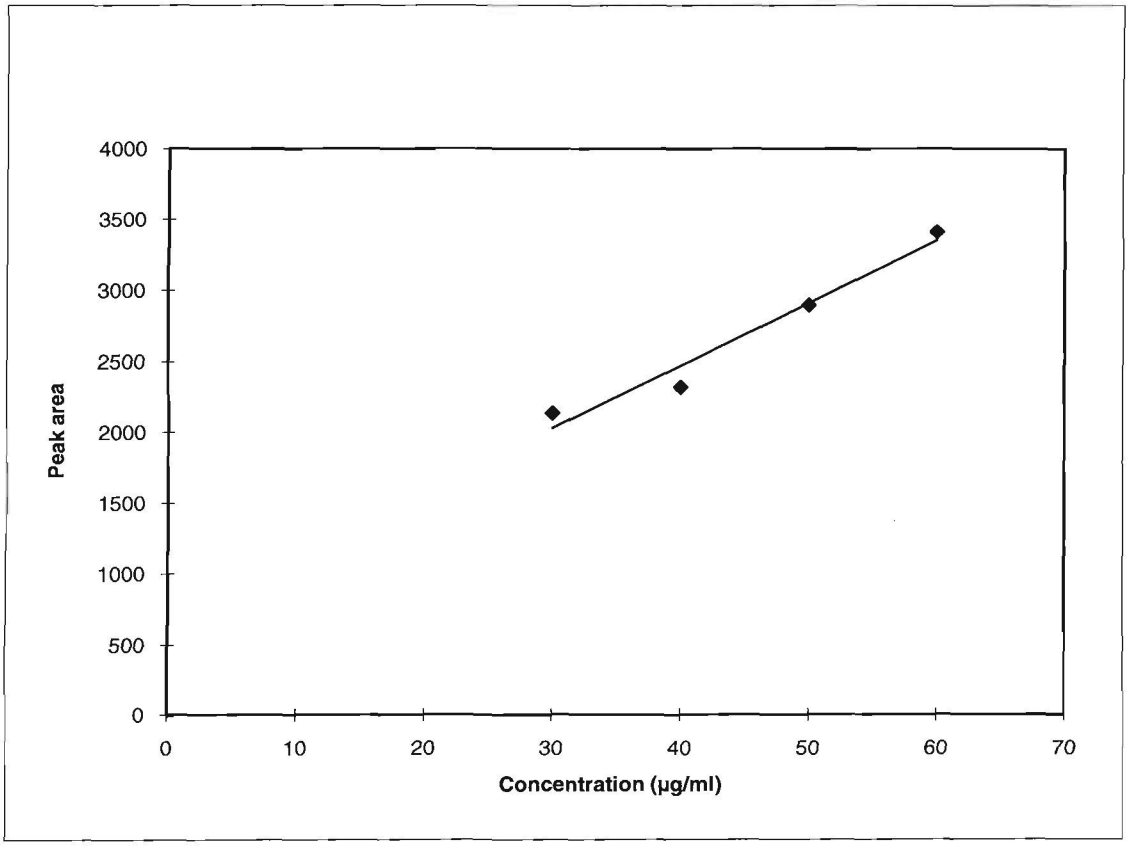

Figure A3: Curve indicating linearity of BHT.

A.4.2.1.3 Linearity of diclofenac

Table A10: Linearity values of diclofenac salt in preparation sample.

Linearity			
µg/ml	**Area**		**Mean**
280	15333.8	15319.6	15326.7
320	20362.0	20651.0	20506.5
360	21007.0	21863.0	21435.0
400	23543.0	23419.0	23481.0

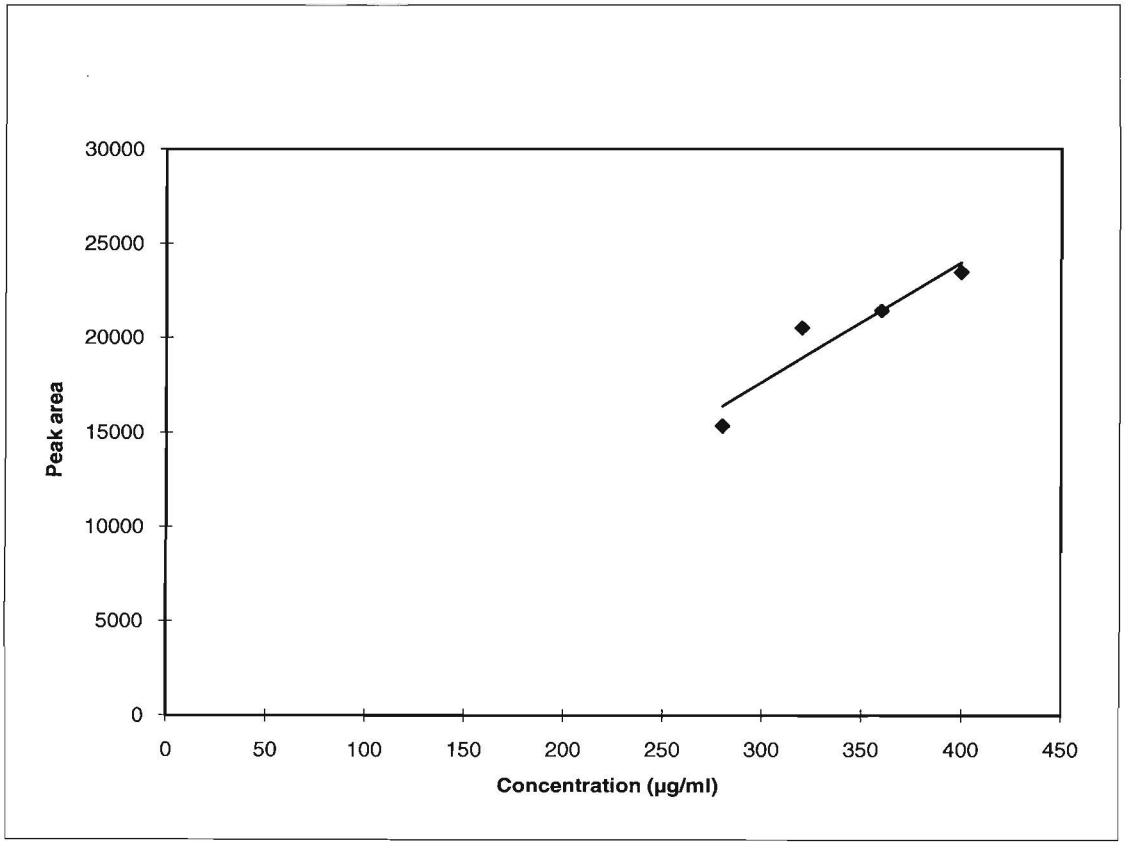

Figure A4: Curve indicating linearity of diclofenac.

A.4.2.1.4 Linearity of methyl paraben

Table A11: Linearity values of methyl paraben in preparation sample.

Linearity			
µg/ml	Area		Mean
100.0	1907.0	1903.0	1905.0
160.0	2556.0	2543.0	2549.5
220.0	3178.0	3121.0	3149.5
280.0	3765.0	3759.0	3762.0

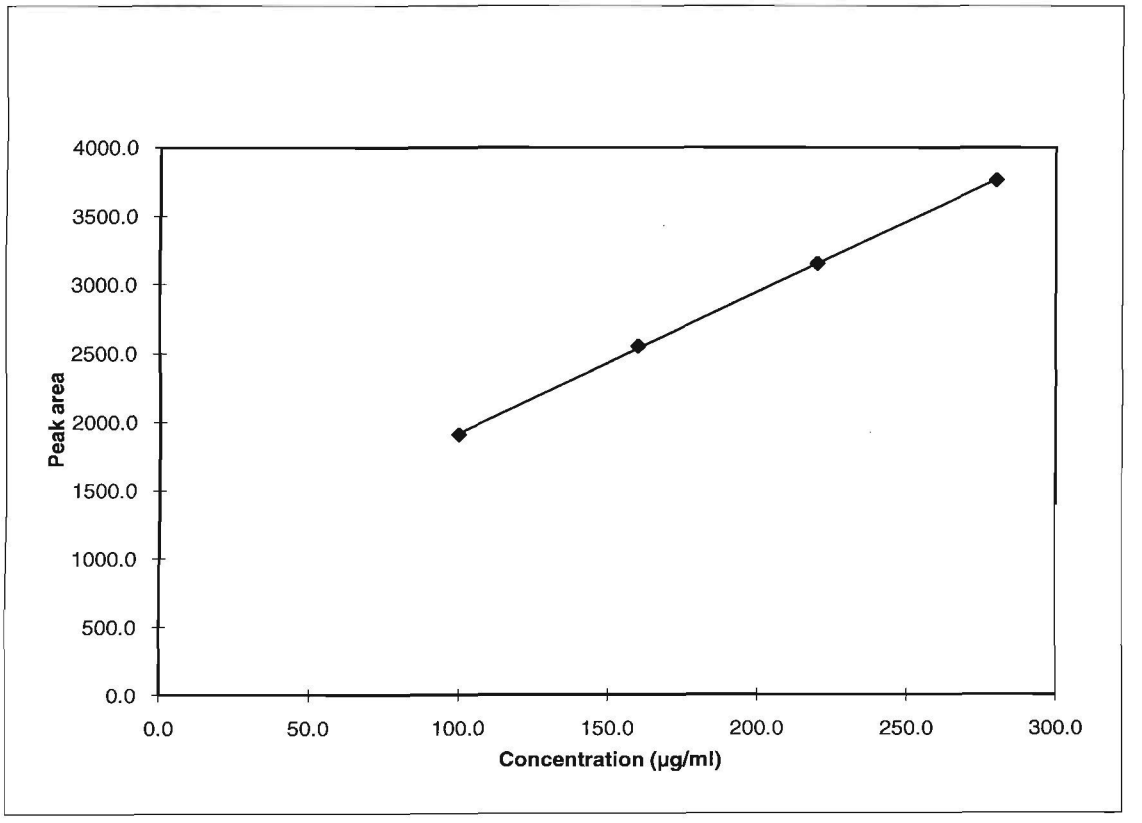

Figure A5: Curve indicating linearity of methyl paraben.

A.4.2.1.5 Linearity of vitamin E

Table A12: Linearity values of vitamin E in preparation sample.

Linearity			Mg/100ml
µg/ml	Area		Mean
40.0	1833.0	1842.0	1837.5
100.0	2112.0	2119.0	2115.5
160.0	2556.0	2576.0	2566.0
220.0	2985.0	2979.0	2982.0

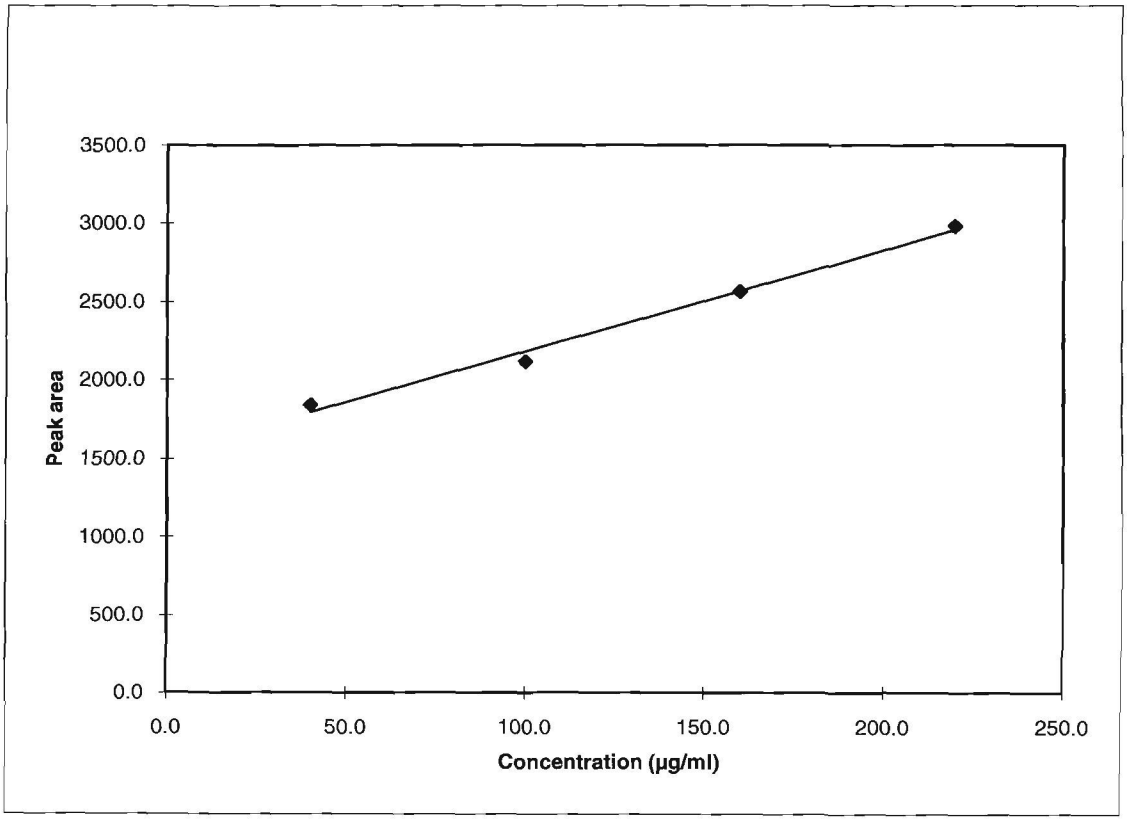

Figure A6: Curve indicating linearity of vitamin E.

A.4.2.2 Accuracy

Is the indication of the closeness, of the experimental value to the true value. During this stage of method development the percentage active substances recovered by assay is measured and compared to a standard preparation concentration. It should be established across the specified range of the analytical procedure (Hong *et al.*, 2000:336-367). The following tables A13 to A17 list the accuracy values of BHA, BHT, diclofenac, methyl paraben and vitamin E.

A.4.2.2.1 Accuracy of BHA

Table A13: Accuracy values of BHA in the preparation sample.

Accuracy:					
Conc.spiked				**Recovery**	
µg/ml	**Area**	**Area**	**Mean**	**µg/ml**	**%**
6.4	188.7	184.3	187	31.6	98.7
6.4	191.3	189.6	190	32.2	100.7
6.4	194.5	193.5	194	32.8	102.5
8.0	251.4	247	249	41.7	104.3
8.0	243.1	236.9	240	40.2	100.5
8.0	237.7	241.3	240	40.1	100.3
9.6	281.3	285.4	283	47.2	98.4
9.6	294	288.6	291	48.5	101.0
9.6	321.2	243	282	47.0	97.9
				Mean	100.5
				SD	1.9
				% RSD	1.9

A.4.2.2.2 Accuracy of BHT

Table A14: Accuracy values of BHT in the preparation sample.

Accuracy:					
Conc.spiked				**Recovery**	
µg/ml	**Area**	**Area**	**Mean**	**µg/ml**	**%**
64	2013.0	2011.1	2012	326.3	102.0
64	2010.9	2014	2012	326.3	102.0
64	2008.6	2014.7	2012	326.2	101.9
80	2443	2435	2439	395.2	98.8
80	2452	2466	2459	398.4	99.6
80	2459	2453	2456	397.9	99.5
96	2988	2978	2983	483.0	100.6
96	2994	2954	2974	481.5	100.3
96	3136	3106	3121	505.3	105.3
				Mean	101.1
				SD	1.8
				% RSD	1.8

A.4.2.2.3 Accuracy of diclofenac

Table A15: Accuracy values of diclofenac in the preparation sample.

Accuracy:					
Conc.spiked				**Recovery**	
µg/ml	**Area**	**Area**	**Mean**	**µg/ml**	**%**
320.0	20032.0	20054.0	20043	3236.8	101.2
320.0	20031	20041	20036	3235.7	101.1
320.0	20015	20029	20022	3233.4	101.0
400.0	24532	24544	24538	3962.4	99.1
400.0	24560	24533	24547	3963.8	99.1
400.0	24541	24583	24562	3966.3	99.2
480.0	29655	29641	29648	4787.2	99.7
480.0	29642	29652	29647	4787.1	99.7
480.0	29665	29671	29668	4790.5	99.8
				Mean	100.0
				SD	0.8
				% RSD	0.8

A.4.2.2.4 Accuracy of methyl paraben

Table A16: Accuracy values of methyl paraben in the preparation sample.

Accuracy:					
Conc.spiked				**Recovery**	
µg/ml	**Area**	**Area**	**Mean**	**µg/ml**	**%**
128	2013.0	2012.0	2013	326.3	102.0
128	2006	2009	2008	325.5	101.7
128	2001	1998	2000	324.2	101.3
160	2505	2501	2503	405.5	101.4
160	2498	2497	2498	404.6	101.2
160	2487	2492	2490	403.3	100.8
192	2998	3001	3000	485.7	101.2
192	2988	2991	2990	484.0	100.8
192	2983	2996	2990	484.0	100.8
				Mean	101.3
				SD	0.4
				% RSD	0.4

A.4.2.2.5 Accuracy of vitamin E

Table A17: Accuracy values of vitamin E in the preparation sample.

Accuracy:					
Conc. spiked				**Recovery**	
µg/ml	**Area**	**Area**	**Mean**	**µg/ml**	**%**
64	1976.0	1966.0	1971	319.6	99.9
64	1978	1981	1980	321.0	100.3
64	1982	1980	1981	321.2	100.4
80	2535	2532	2534	410.4	102.6
80	2530	2528	2529	409.7	102.4
80	2540	2438	2489	403.2	100.8
96	2877	2897	2887	467.5	97.4
96	2899	2902	2901	469.7	97.8
96	2903	2905	2904	470.2	98.0
				Mean	100.0
				SD	1.8
				% RSD	1.8

A.4.2.2.5 Accuracy of vitamin E

Precision is defined as the closeness of data values to each other for a number of measurements under the same analytical conditions. It refers to the distribution of test results around their average. And is expressed as the percent relative standard deviation for a statistically significant number of samples (Hong *et al.*, 2000:336-367). Tables A18 to A22 list the precision values obtained for BHA, BHT, diclofenac, methyl paraben and vitamin E.

A.4.2.3.1 Precision of BHA

Table A18: Precision values of BHA.

Precision					
Mass (g)	**Area**	**Area**	**Mean**	**Conc.µg/ml**	**%**
0.012	166.6	167.8	167.2	27.3	99.0
0.012	171.3	171.8	171.6	28.0	100.0
0.012	169.8	172.4	171.1	27.9	100.7
0.02	214.5	211.8	213.2	34.8	99.1
0.02	205.0	207.0	206.0	33.6	97.7
0.02	212.0	195.0	203.5	33.2	96.8
0.028	255.4	261.0	258.2	42.2	102.1
0.028	259.3	251.0	255.2	41.7	101.1
0.028	267.5	240.0	253.8	41.4	100.8
				Mean	99.72
				SD	1.62
				RSD %	1.63

A.4.2.3.2 Precision of BHT

Table A19: Precision values of BHT.

Precision					
Mass (g)	**Area**	**Area**	**Mean**	**Conc.µg/ml**	**%**
0.12	2011	2013	167.2	27.3	99.0
0.12	2031	2025	171.6	28.0	100.0
0.12	2019	2016	171.1	27.9	100.7
0.2	2455	2458	213.2	34.8	99.1
0.2	2449	2451	206.0	33.6	97.7
0.2	2453	2456	203.5	33.2	96.8
0.28	2996	2993	258.2	42.2	102.1
0.28	2305	2309	255.2	41.7	101.1
0.28	2997	2993	253.8	41.4	100.8
				Mean	99.72
				SD	1.62
				RSD %	1.63

A.4.2.3.3 Precision of diclofenac

Table A20: Precision values of diclofenac.

Precision					
Mass (g)	**Area**	**Area**	**Mean**	**Conc.µg/ml**	**%**
0.22	16852.0	16886.0	16869.0	2753.9	100.5
0.27	16853.0	16889.0	16871.0	2754.2	100.5
0.24	17391.0	17415.0	17403.0	2841.1	103.7
1.10	20746.0	21200.0	20973.0	3423.9	100.0
1.02	20720.0	20179.0	20449.5	3338.4	97.5
1.01	20754.0	20608.0	20681.0	3376.2	98.6
1.82	24886.0	24864.0	24875.0	4060.9	98.8
1.81	24876.0	24859.0	24867.5	4059.7	98.8
1.80	24890.0	24875.0	24882.5	4062.1	98.8
				Mean	99.69
				SD	1.70
				RSD %	1.70

A.4.2.3.4 Precision of methyl paraben

Table A21: Precision values of methyl paraben.

Precision					
Mass (g)	**Area**	**Area**	**Mean**	**Conc.µg/ml**	**%**
0.3	2011.0	2016.0	2013.5	525.5	102.3
0.3	2013.0	2014.0	2013.5	525.5	102.3
0.3	2007.0	2012.0	2009.5	524.4	102.1
0.4	2556.0	2558.0	2557.0	667.3	97.4
0.4	2554.0	2552.0	2553.0	666.3	97.3
0.4	2561.0	2559.0	2560.0	668.1	97.5
0.48	3154.0	3156.0	3155.0	823.4	96.2
0.48	3103.0	3104.0	3103.5	809.9	94.6
0.48	3144.0	3139.0	3141.5	819.8	95.8
				Mean	98.38
				SD	2.85
				RSD %	2.90

A.4.2.3.5 Precision of vitamin E

Table A22: Precision values of vitamin E.

Precision					
Mass (g)	**Area**	**Area**	**Mean**	**Conc.µg/ml**	**%**
0.16	1965.0	1943.0	1954.0	255.0	99.3
0.16	1987.0	1985.0	1986.0	259.1	100.9
0.16	1956.0	1959.0	1957.5	255.4	99.4
0.20	2543.0	2544.0	2543.5	331.9	96.9
0.22	2550.0	2551.0	2550.5	332.8	97.2
0.21	2557.0	2559.0	2558.0	333.8	97.5
0.28	3211.0	3215.0	3213.0	419.2	97.9
0.28	3244.0	3266.0	3255.0	424.7	99.2
0.28	3261.0	3251.0	3256.0	424.9	99.2
				Mean	98.62
				SD	1.24
				RSD %	1.26

A.4.2.4 Inter-day precision

Inter-day precision is termed as the results of the HPLC method operating over a short time interval under the same conditions; also termed intra-assay precision. Injector performance and analysis of samples are evaluated during this procedure (Hong *et al.,* 2000:336-367). Tables A23 and A24 list the interday precision values for BHA on day 2 and 3. Tables A25 and A26 list the interday precision values for BHT on day 2 and 3. Tables A27 and A28 list the interday precision values for diclofenac on day 2 and 3. Tables A29 and A30 list the interday precision values for methyl paraben on day 2 and 3. And tables A31 and A32 list the interday precision values for vitamin E on day 2 and 3.

A.4.2.4.1 Day 2 inter day precision of BHA

Table A23: Inter-day precision of BHA day 2.

Inter day precision					
Mass (g)	**Area**	**Area**	**Mean**	**Conc.**	**%**
0.012	161.3	164.8	163	26.6	97.2
0.012	169.9	170.1	170	27.8	101.3
0.012	177.6	160	169	27.6	100.6
			Mean		99.68
			SD		1.81
			RSD %		1.81

A.4.2.4.2 Day 3 inter-day precision BHA

Table A24: Inter-day precision of BHA on day 3.

Inter-day precision					
Mass (g)	**Area**	**Area**	**Mean**	**Conc.**	**%**
0.012	166.4	163.6	165	26.9	98.3
0.012	172.4	168.3	170	27.8	101.5
0.012	171.2	172.2	172	28.0	102.3
			Mean		100.71
			SD		1.72
			RSD %		1.71

A.4.2.4.3 Day 2 inter-day precision of BHT

Table A25: Inter-day precision values of BHT on day 2.

Inter-day precision					
Mass (g)	**Area**	**Area**	**Mean**	**Conc.**	**%**
0.2	1765	1766	1766	88.2	103.0
0.2	1769	1754	1762	88.0	97.3
0.2	1782	1785	1784	89.1	97.1
			Mean		99.15
			SD		2.74
			RSD %		2.77

A.4.2.4.4 Day 3 inter-day precision BHT

Table A26: Inter-day precision values of BHT on day 3.

Inter-day precision					
Mass (g)	**Area**	**Area**	**Mean**	**Conc.**	**%**
0.2	1788	1786	1787	89.3	97.6
0.2	1782	1789	1786	89.2	98.0
0.2	1893	1792	1843	92.1	100.2
			Mean		98.62
			SD		1.13
			RSD %		1.15

A.4.2.4 5 Day 2 inter-day precision of diclofenac

Table A27: Inter-day precision of diclofenac on day 2.

Inter-day precision					
Mass (g)	**Area**	**Area**	**Mean**	**Conc.**	**%**
0.22	16843	16855	16849	2750.6	100.4
0.22	16554	16887	16721	2729.7	99.6
0.22	16598	16587	16593	2708.8	98.9
			Mean		99.63
			SD		0.62
			RSD %		0.63

A.4.2.4.6 Day 3 inter-day precision of diclofenac

Table A28: Inter-day precision of diclofenac on day 3.

Inter-day precision					
Mass (g)	**Area**	**Area**	**Mean**	**Conc.**	**%**
0.22	16557	16543	16550	2701.8	98.6
0.22	16598	16578	16588	2708.0	98.8
0.22	16547	16521	16534	2699.2	98.5
			Mean		98.66
			SD		0.13
			RSD %		0.14

A.4.2.4.7 Day 2 inter-day precision of methyl paraben

Table A29: Inter-day precision of methyl paraben on day 2.

Inter-day precision					
Mass (g)	**Area**	**Area**	**Mean**	**Conc.**	**%**
0.3	2014	2010	2012	525.1	102.2
0.3	2011	2009	2010	524.5	102.1
0.3	2007	2003	2005	523.2	101.9
			Mean		102.06
			SD		0.15
			RSD %		0.15

A.4.2.4.8 Day 3 inter-day precision methyl paraben

Table A30: Inter-day precision of methyl paraben on day 3.

Inter-day precision					
Mass (g)	**Area**	**Area**	**Mean**	**Conc.**	**%**
0.3	2011	2004	2008	523.9	102.0
0.3	2016	2012	2014	525.6	102.3
0.3	2008	2006	2007	523.8	102.0
			Mean		102.09
			SD		0.16
			RSD %		0.16

A.4.2.4.9 Day 2 inter-day precision of vitamin E

Table A31: Inter-day precision of vitamin E on day 2.

Inter-day precision					
Mass (g)	**Area**	**Area**	**Mean**	**Conc.**	**%**
0.16	1955	1934	1945	253.7	98.8
0.16	1976	1978	1977	258.0	100.4
0.16	1987	1988	1988	259.3	101.0
			Mean		100.06
			SD		0.93
			RSD %		0.93

A.4.2.4 9 Day 3 inter-day precision of vitamin E

Table A32: Inter-day precision of vitamin E on day 3.

Inter-day precision					
Mass (g)	**Area**	**Area**	**Mean**	**Conc.**	**%**
0.16	1965	1966	1966	256.5	99.9
0.16	1967	1977	1972	257.3	100.2
0.16	1966	1965	1966	256.5	99.9
			Mean		99.96
			SD		0.16
			RSD %		0.16

A.4.2.5 Ruggedness

Samples should be analysed within the period before degradation of 2% can occur in order to determine the ruggedness of the samples. If degradation does occur, precaution should be taken to compensate for the loss. During this validation procedure the sample stability is evaluated over a period of 12 hours. Tables A33 to A37 list the ruggedness values obtained for BHA, BHT, diclofenac, methyl paraben and vitamin E (Hong *et al.*, 2000:366-367).

A.4.2.5.1 Ruggedness of BHA

Table A33: Ruggedness of BHA.

Time (hr)	Peak area	Stability
0	457	100.0
1	455.6	99.7
2	449	98.2
3	458.6	100.4
4	427	93.4
5	449	98.2
6	454	99.3
7	449	98.2
8	451	99.6
9	455	99.6
10	458	100.2
11	451.8	98.9
12	451	98.7
Mean	451.2	98.8
SD	7.73	1.71
RSD %	1.71	1.73

A.4.2.5.2 Ruggedness of BHT

Table A34: Ruggedness of BHT.

Time (hr)	Peak area	Stability
0	3059	100.0
1	3059	100.0
2	3021	98.8
3	2937	96.0
4	3085	100.8
5	3091	101.0
6	3074	100.5
7	3084	100.8
8	3070	100.4
9	3120	102.0
10	3066	100.2
11	3066	100.2
12	3074	100.5
Mean	3062.0	100.1
SD	41.99	1.37
RSD %	1.37	1.37

A.4.2.5.3 Ruggedness of diclofenac

Table A35: Ruggedness of diclofenac.

Time (hr)	Peak area	Stability
0	18650	100.0
1	18639	99.9
2	18650	100.0
3	18716	100.4
4	18744	100.5
5	18658	100.0
6	18575	99.6
7	18637	99.9
8	18577	99.6
9	18752	100.5
10	18576	99.6
11	18477	99.1
12	18642	100.0
Mean	18637.9	99.9
SD	72.58	0.39
RSD %	0.39	0.39

A.4.2.5.4 Ruggedness of methyl paraben

Table A36: Ruggedness of methyl paraben.

Time (hr)	Peak area	Stability
0	6409	100.0
1	6536	102.0
2	6693	104.4
3	6739	105.1
4	6622	103.3
5	6643	103.7
6	6621	103.3
7	6408	100.0
8	6731	105.0
9	6513	101.6
10	6415	100.1
11	6503	101.5
12	6788	105.9
Mean	6586.2	102.8
SD	126.92	1.98
RSD %	1.93	1.93

A.4.2.5.5 Ruggedness of vitamin E

Table A37: Ruggedness of vitamin E.

Time (hr)	Peak area	Stability
0	2976	100.0
1	2962	99.5
2	2917	98.0
3	2932	98.5
4	2903	97.5
5	2709	91.0
6	2746	92.3
7	3026	101.7
8	2919	98.1
9	2928	98.4
10	2949	99.1
11	2972	99.9
12	2908	97.7
Mean	2911.3	97.8
SD	85.10	2.86
RSD %	2.92	2.92

A.5 Conclusion

The methods developed for the evaluation of active ingredients and adjuvant content within samples and preparations complied to all of the standards as set by the ICH (International conference on harmonisastion, 2005). These methods could thus be implemented in order to determine the amount of active ingredient flux contained within the Franz cell receptor phase and the amount of active and adjuvant ingredients within formulated preparations.

A.6 References

HONG, D.D., SHAH, M. 2000. Development and validation of HPLC Stability Indicating Assays. (In CARSTENSEN, J.T., RHODES, C.T. 2000. Drug stability: Principles and practices. 3rd ed. Marcel Dekker Inc. New York. P329-384.)

ICH: International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use. 2005. Validation of analytical procedures: Text and methodology Q2(R1)

ANNEXURE B

Method for the determination of solubility and Log D in phosphate buffer

B.1 *Introduction*

Prior to initiation of any transdermal study, relevant physicochemical properties were determined. The solubility and the Log D value are important parameters to evaluate as these characteristics influence the permeation of solutes in transdermal studies. The purpose of this study was to determine the solubility and Log D values of diclofenac diethylamine, diclofenac hydroxyethylpyrrolidine and diclofenac sodium.

B.2 Preparation of phosphate buffer pH 7.4 (PBS)

2.72 Grams of KH_2PO_4 were dissolved in 1 liter of distilled water. A solution of KOH was prepared to adjust the pH to a higher value, this solution was prepared by dissolving 0.56 grams of KOH in 20 ml of distilled water. To lower the pH value, H_3PO_4 was used. The pH of the solution was adjusted to 7.4 in accordance to the prescribed method (BP electronic reference., 2007:A140).

B.3 Method for the determination of the solubility of diclofenac in PBS

A shaker bath was preheated to 32 °C to simulate the temperatures at the area of topical application, namely the surface of the skin. Test tubes were then filled with 10 ml of PBS in triplicate. The test tubes were placed in the shaker bath and the shaker mechanism was activated. Spoonfuls of the active ingredient were added to the three test tubes at hourly intervals over a period of 24 hours to ensure constant saturation of the solution. An oven was preheated to 32 °C an hour before the test tubes were due to be removed from the shaker bath for HPLC determination. Three 5 ml plastic syringes and three 0.45 µm filters were placed in an oven preheated to 32 °C half an hour before removing the test tubes from the shaker bath. The test tubes were removed one at a time leaving the remaining test tubes in a stationary position in the shaker bath while maintaining the temperature at 32 °C. The clear solution was then converted from the test tubes to the syringes fitted with pre-filters, after which the solution was filtered and diluted to a factor relevant to the character of the

active ingredient. The solutions were then transferred into separate vials. Chromatographic determinations were done in duplicate.

B.4 Method for determining octanol-buffer partitioning coefficient (log D)

Phosphate buffer system was prepared according to the method as described in section B.2 (BP electronic reference., 2007:A140).

B.4.1 Saturation of n-octanol and PBS

24 Hours before the commencement of the determination n-octanol should be saturated with PBS and vice versa according to the method as described in section B.4.2.

B.4.2 Method for saturation of octanol and PBS

- 200 ml of n-octanol and 200 ml of PBS (pH 7.4) were measured off respectively.
- The contents of each flask were then emptied into a single pear shaped titration flask.
- The contents were then mixed by gently tilting the pear shaped flask.
- The pear shaped flask was left to saturate in a ventilation unit.

B.4.3 Method for determining log D values

A shaker bath was preheated to 25 °C. Standard solutions were prepared with active ingredient (diclofenac salt) concentrations of 100, 10, 1, and 0.1 µg/ml. The solutions were transferred into different auto sampler vials and injected for chromatographic determination in duplicate. After the standard solution preparation, the sample for analysis was prepared. Sample solutions were prepared by measuring 5 mg of the active ingredient. An amount of 10 ml methanol was added to the active ingredient in a volumetric flask. The flask was filled to volume using a solution of PBS (pH 7.4). 5 ml of the above mentioned solution was transferred into a 50 ml volumetric flask and filled to volume using PBS (pH 7.4). Amounts of 3 ml were pipetted into three test tubes and placed in the shaker bath at 25 □C for 10 minutes. After 25 minutes, 3 ml of the solutions obtained from the shaker bath were pipetted into test tubes. 3 ml Octanol was added to each of the test tubes containing test solution. The test tubes where placed into a shaker bath for an additional 45 minutes. After 45

minutes the test tubes were removed and centrifuged at 4000 rpm for 30 minutes. Standard solutions were injected for chromatographic determination in duplicate with an injection volume of 10 µl before chromatographic determination of the analytical samples. A Pasteur pipette was used to remove the PBS phase from the test tubes and it was directly transferred into auto sampler vials. The octanol phase was removed and dissolved with 5 ml of sample grade methanol. Samples were then transferred into auto sampler vials for chromatographic determination.

B.5 Results

Solubility determinations were done at 32 □C and pH 7.4 using PBS (phosphate buffer system) as the solvent. Table B1 lists the solubility and log D values obtained for diclofenac diethylamine, diclofenac hydroxyethylpyrrolidine and diclofenac sodium.

Table B1: Solubility and log D values of diclofenac diethylamine, diclofenac hydroxyethylpyrrolidine and diclofenac sodium.

Diclofenac salt	**Solubility (µg/ml)**	**Log D value**
Diclofenac diethylamine	4.3	1.6
Diclofenac hydroxyethylpyrrolidine	7.1	1.9
Diclofenac sodium	6.2	1.7

B.6 Conclusion

The values obtained from the solubility and log D determinations were used in the chromatographic method development and in the prediction of possible skin diffusion

outcomes. Values obtained after solubility determinations were implemented when developing a chromatographic method for flux determinations of membrane and skin diffusion studies. The log D values obtained could be used to predict and interpret skin diffusion flux values.

B.7 References

B. Pharmacopoeia, 2007. Volume IV, Appendix ID, p. A140

ANNEXURE C

Formulation of Pheroid™ and non-Pheroid™ formulations containing diclofenac salts

C.1 Introduction

The objective of this study was to formulate Pheroid™ and non-Pheroid™ products containing diclofenac diethylamine, diclofenac hydroxyethylpyrrolidine and diclofenac sodium. These formulations were used to determine release of active ingredient and active ingredient stability within formulations.

C.2 Methods and materials

C.2.1 Ingredients of Pheroid™ preparations containing a diclofenac salt

Pheroid™ preparations were produced containing the same percentage of active ingredient as the non-Pheroid™ preparations. Pheroid™ preparations were produced in the formulation facility at the North-West university. Due to the confidential nature of the Pheroid™ formulation the entrapment procedure of the Pheroid™ portion cannot be stipulated. Anti oxidants were added to enhance the stability and integrity of the formulations. Table C1 indicates all the preparation ingredients.

Table C1: Amounts of ingredients in Pheroid™ formulations containing a diclofenac salt.

Ingredient	Amount in grams (g)
Diclofenac salt	1 g
Carbopol	1 g
Methyl paraben	0.4 g
Polyethylene glycol 400 (PEG 400)	6 g
Pheroid™ portion	91.38 g
Butylated hydroxyanisole (BHA) (anti oxidant)	0.02 g
Butylated hydroxytoluene (BHT) (anti oxidant)	0.2 g
Total	100 g

C.2.2 Method for the preparation of Pheroid™ gel containing diclofenac salts

Appropriate amounts of each ingredient were measured using a Mettler Toledo balance (Greifensee, Switzerland). The diclofenac salt, methyl paraben and antioxidants were dissolved in heated PEG 400 (± 60 □C) and constantly stirred with a glass stirrer. The Pheroid™ portion was prepared separately. As soon as the Pheroid™ portion was sufficiently cooled, it was added to the PEG 400 mixture. The preparation was left to stir as it cooled down to room temperature. After the preparation had been sufficiently cooled, the content was spooned into plastic containers.

C.3.1 Ingredients of non-Pheroid™ preparations containing a diclofenac salt

Table C2 indicates the preparation ingredients of a diclofenac containing non-Pheroid™ gel.

Table C2: Amounts of ingredients in a diclofenac containing non-Pheroid™ gel preparation.

Ingredient	Amount in grams (g)
Diclofenac salt	1.0 g
Methyl paraben	0.4 g
Carbopol	1.0 g
Triethanolamine	1.3 g
Ethanol	15.0 g
Propylene glycol	10.5 g
Water	71.2 g
Total weight	100 g

C.3.2 Method for the preparation of non-pheroid™ gel containing diclofenac salts

Appropriate amounts of each ingredient was measured using a Mettler Toledo balance (Greifensee, Switzerland). Carbopol and water were mixed using a wise stir mixer (korea) at 800 rpm until a gel consistency was obtained. The propylene glycol was added to the carbopol mixture and the mixing speed was slowly increased to 1200 rpm. 1.3 ml Triethanolamine (Saarchem, South-Africa) was added to the mixture as a neutralising agent. The preparation was left to stir for 15 minutes. After the preparation reached proper gel consistency, it was removed from the stirrer and spooned into plastic containers.

C.4 Summary

Preparations of Pheroid™ and non-Pheroid™ nature were obtained. The non-Pheroid™ preparations all showed a transparent color the Pheroid™ preparations obtained were all of a clear white color.

Suitable formulations of all the different diclofenac salts were manufactured. Non- Pheroid™ formulations were transparent whereas Pheroid™ formulations were a translucent white.

ANNEXURE D

Transdermal and membrane diffusion studies of preparations containing diclofenac salts

D.1. Introduction

Franz cell diffusion studies were conducted in order to determine the concentration of active ingredient absorbed through human skin. Human skin samples obtained from abdominoplasty plastic surgery procedures, were uses in the diffusion studies. Human skin samples were obtained from plastic surgeons after consultation with the surgeon and consent of the patient. Skin diffusion studies were completed and tape stripping was done on the skin samples in order to determine the amounts of active ingredient present in the stratum corneum of the excised skin after the application of formulated non-Pheroid™ and Pheroid™ preparations. This study was to determine to the amount of active ingredient absorbed through the skin, as well as concentrations contained within the epidermis and dermis (Pellet *et al.,* 1997:92). Fluxes obtained from Franz cell diffusion studies were compared in order to evaluate which preparation was able to produce more significant concentrations at the site of action, namely the deep tissue muscle. Membrane studies were done as a comparative study between formulated preparations and retail products containing diclofenac salts. These studies were done to determine and highlight the strengths and weaknesses of each one of the evaluated preparations and products.

D.2. Membrane diffusion studies

The method for membrane diffusion studies will be explained step by step in the following sections.

D.2.1.1. Standard solution preparation

Standard solutions with concentrations of 100, 10, 1 and 0.1 µg/ml were prepared and injected into the chromatograph to obtain a calibration curve.

D.2.1.2. Membrane diffusion preparation

Silicon tubing was cut into eleven 10 cm pieces. Eleven 5 ml syringes were fitted with 18 gauge needles. The silicone tubing pieces were slid over the tips of each of the needles. Ten Franz cells with a surface area of 1.075 cm^2 were prepared for diffusion by covering the top half (donor phase) and the bottom half (receptor phase) with a thin layer of vacuum grease (Dow Corning, Midland, MI, USA). A magnetic stirrer was placed in the bottom of the receptor phase of each Franz cell. A cellulose acetate membrane was placed on each of the receptor phases taking care to place it with its rough side up. The donor phase was placed on top of the receptor phase and clamped together using a horseshoe clamp. Syringes were used to fill each of the receptor phases in the Franz cells with 2 ml PBS. Amounts of 1 ml of each preparation were placed into the donor phase. A volumetric flask filled with PBS was placed in a separate water bath (Grant Instruments Ltd, Cambridge, UK) at 37 □C. Another water bath was preheated to a temperature of 32 □C this water bath would be used for the diffusion study.

D.2.1.3 Membrane diffusion

The Franz cell container containing the cells was placed into a preheated water bath at 32 □C on top of a magnetic stirrer (Variomag, Daytona Beach, FL, USA). The Franz cell container was placed on top of the magnetic stirrer ensuring that the circles align with the stirrer which was set to 750 rpm. The water bath was closed and samples of PBS were withdrawn at intervals of 0.5, 1, 2, 4, 6 and 8 hours and refilled up with fresh PBS (37 □C).

D.3 Results

D.3.1 Results of membrane diffusion studies

The following graphical representations (figure D1, D2, D4, D5, D6, D8, D9 and D10) illustrate the median flux values (µg/cm.h^{-1}) of the respective Pheroid™, non-Pheroid™ and retail products. Figure D3, D7 and D11 shows box plot representations. The box plot figures show the statistical comparisons between the average and the median flux values. For the purpose of this study either the average or median value can be used as these values do not differ significantly.

Figure D1 illustrates a graphical representation of diclofenac diethylamine Pheroid™ gel's cumulative amounts diffused.

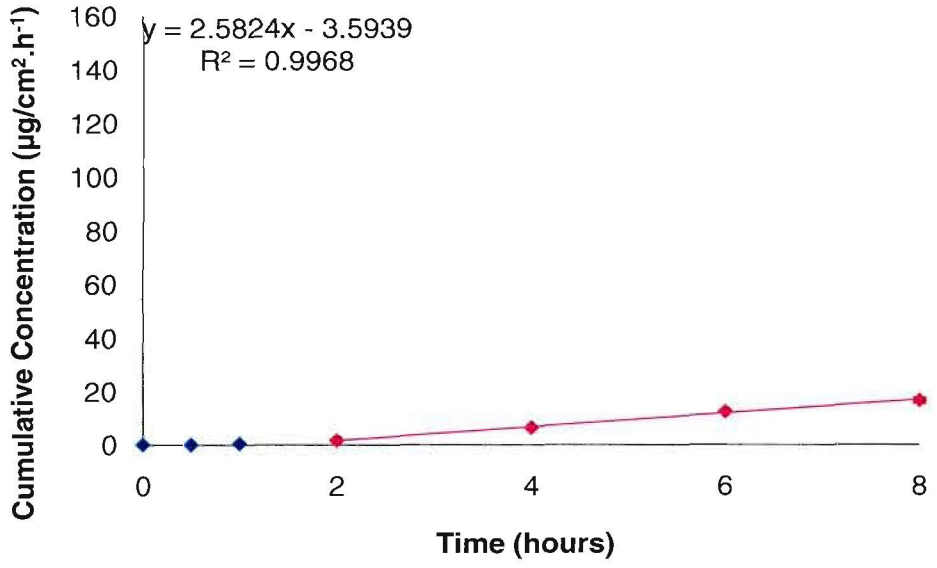

Figure D1: Average membrane cumulative concentration amounts of diclofenac diethylamine Pheroid™ gel.

An average flux of 2.58 ± 1.63 (µg/ml.h $^{-1}$) and median flux of 1.94 ± 1.46 (µg/ml.h $^{-1}$) was obtained.

Figure D2 illustrates a graphical representation of diclofenac diethylamine non-Pheroid™ gel's cumulative amounts diffused.

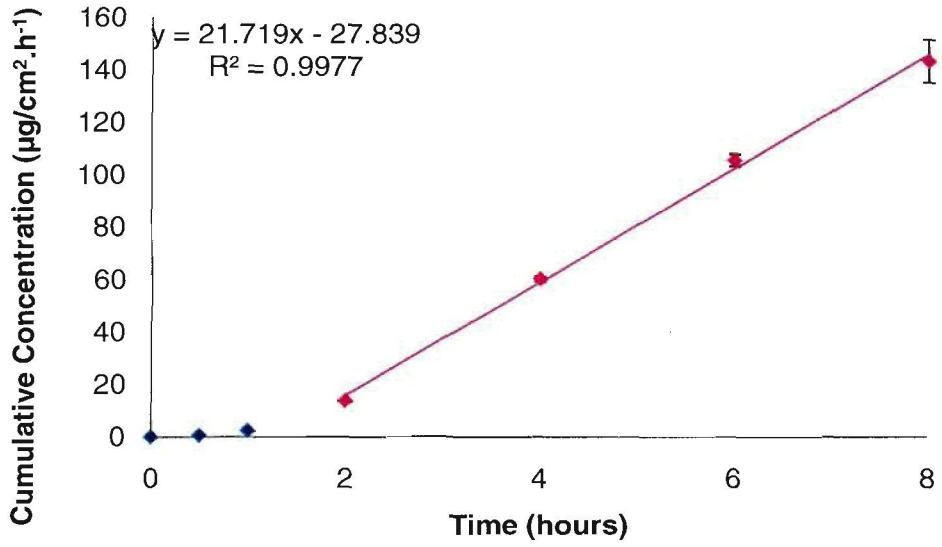

Figure D2: Average membrane cumulative amounts diffused of diclofenac diethylamine non-Pheroid™ gel.

An average flux of 21.71 ± 4.19 (µg/ml.h $^{-1}$) and median flux of 21.47 ± 4.09 (µg/ml.h $^{-1}$) was obtained.

Voltaren® emulgel (the market product containing diclofenac diethylamine) showed no release. The possibility exists that the method used in the determinations and the receptor phase of methanol/water did not accommodate the flux of Voltaren®. The value obtained, therefore, does not suggest that Voltaren® is an inferior formulation.

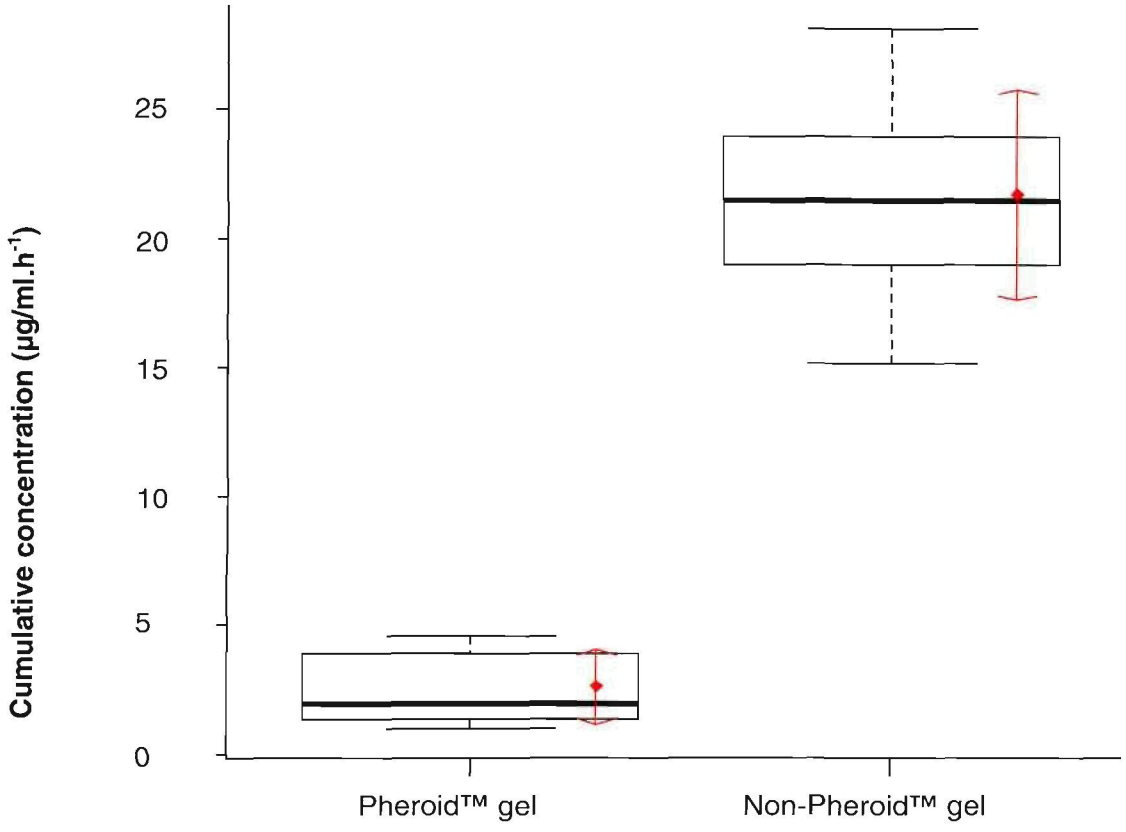

Figure D3: Box plots of the Pheroid™ and non-Pheroid™ preparations containing diclofenac diethylamine show black lines to illustrate median flux and red lines to illustrate the average flux values.

Figure D4 illustrates a graphical representation of diclofenac hydroxyethylpyrrolidine Pheroid™ gel's cumulative amounts diffused.

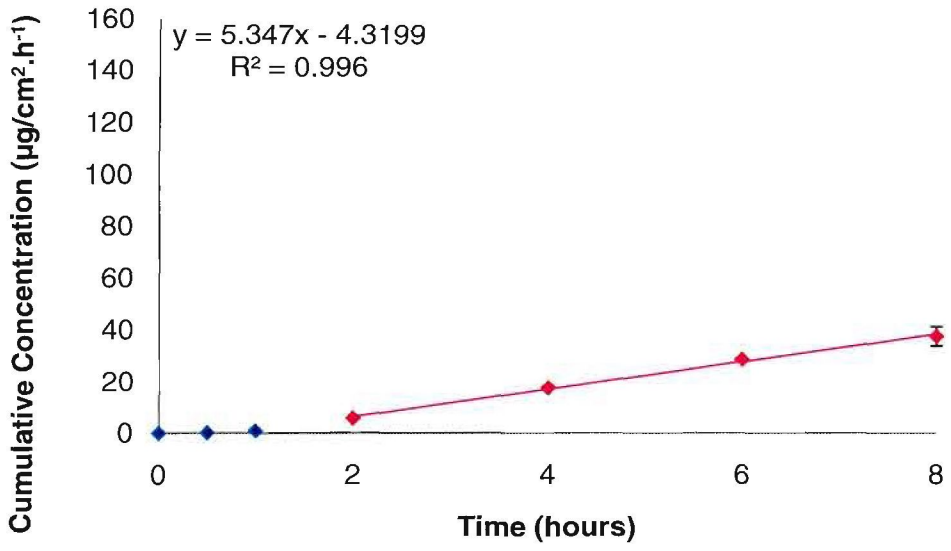

Figure D4: Cumulative amounts diffused of diclofenac hydroxyethylpyrrolidine Pheroid™ gel.

An average flux of 5.35 ± 1.33 (µg/ml.h $^{-1}$) and median flux of 5.74 ± 1.07 (µg/ml.h $^{-1}$) was obtained.

Figure D5 illustrates a graphic representation of diclofenac hydroxyethylpyrrolidine non-Pheroid™ gel's cumulative amounts diffused.

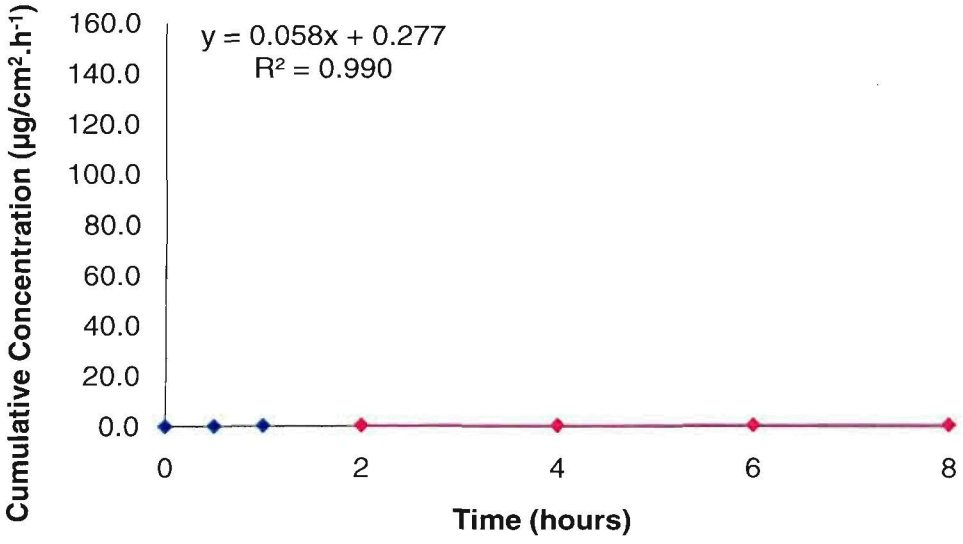

Figure D5: Cumulative amounts diffused of diclofenac hydroxyethyl non-Pheroid™ gel.

An average flux of 0.05 ± 0.40 (µg/ml.h $^{-1}$) and median flux of 0.06 ± 0.01(µg/ml.h $^{-1}$) was obtained.

Figure D6 illustrates a graphical representation of Panamor® gel's cumulative amounts diffused.

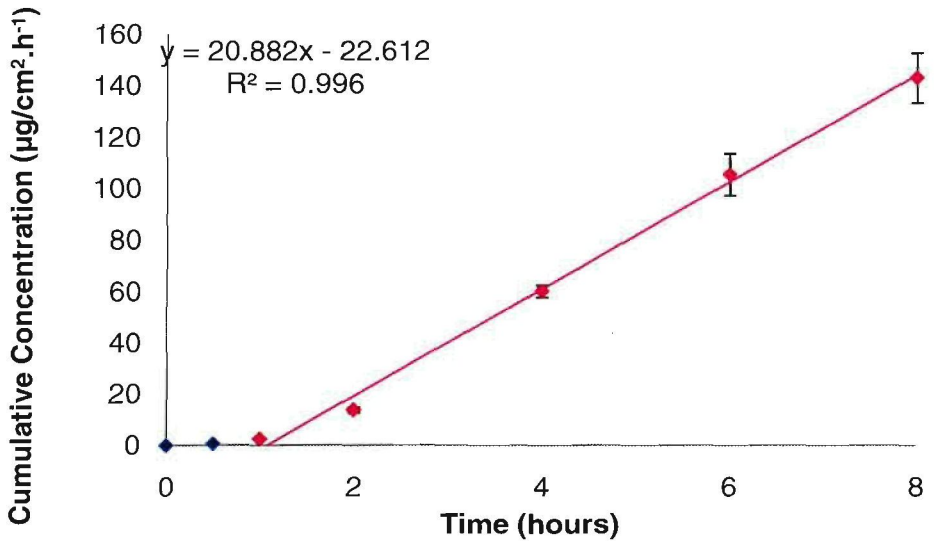

Figure D6: Cumulative amounts diffused of Panamor® gel.

An average flux of 21.71 ± 4.22 (µg/ml.h $^{-1}$) and median flux of 22.57 ± 3.91 (µg/ml.h $^{-1}$) was obtained.

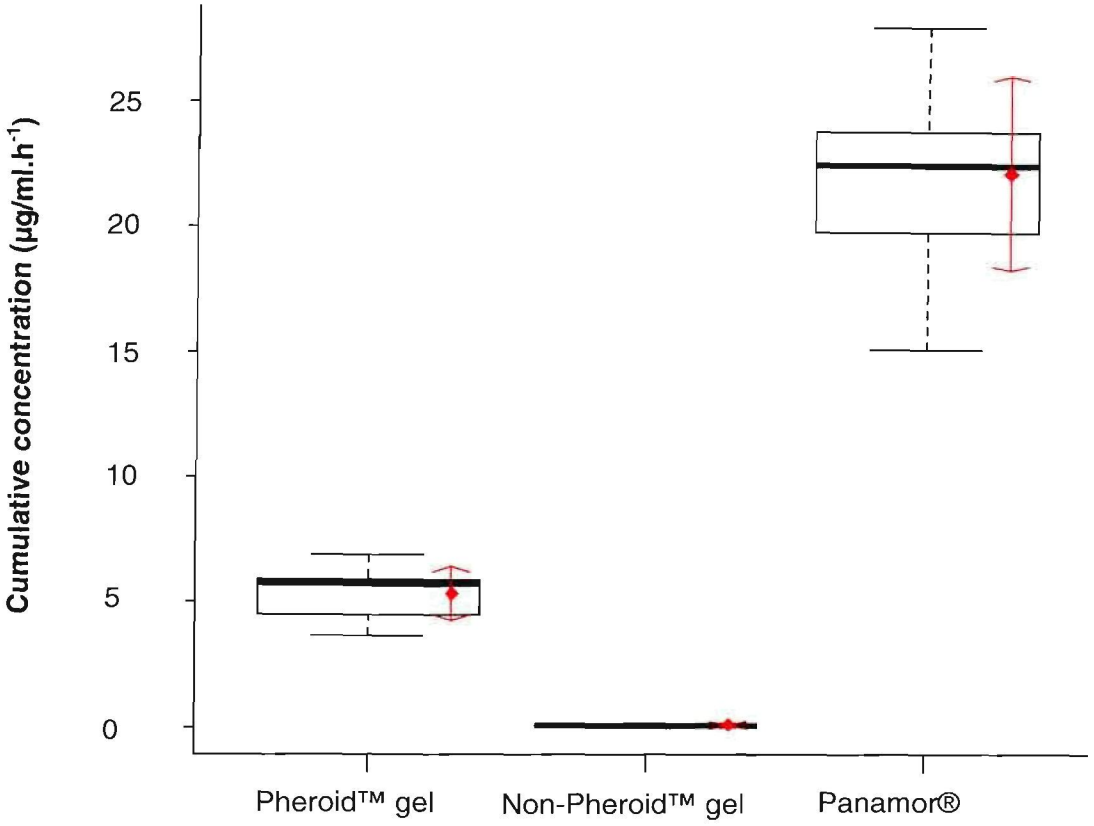

Figure D7: Box plots of the Pheroid™, non-Pheroid™ and retail product, containing diclofenac hydroxyethylpyrrolidine show black lines to illustrate median flux and red lines to illustrate the average flux values.

Figure D8 illustrates a graphical representation of diclofenac sodium Pheroid™ gel's cumulative amounts diffused.

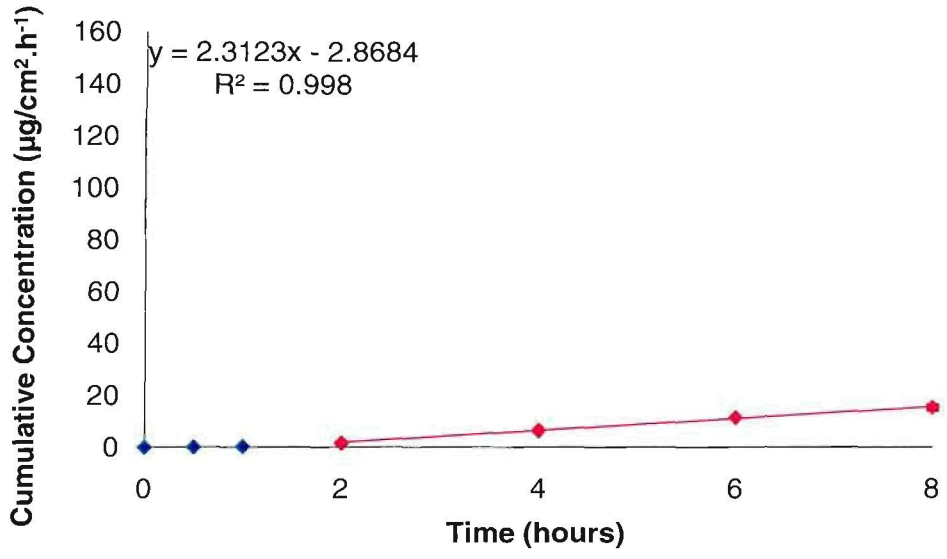

Figure D8: Cumulative amounts diffused of diclofenac sodium Pheroid™ gel.

An average flux of 2.31 ± 1.02 (µg/ml.h $^{-1}$) and median flux of 2.32 ± 0.66 (µg/ml.h $^{-1}$) was obtained.

Figure D9 illustrates a graphical representation of diclofenac sodium non-Pheroid™ gel's cumulative amounts diffused.

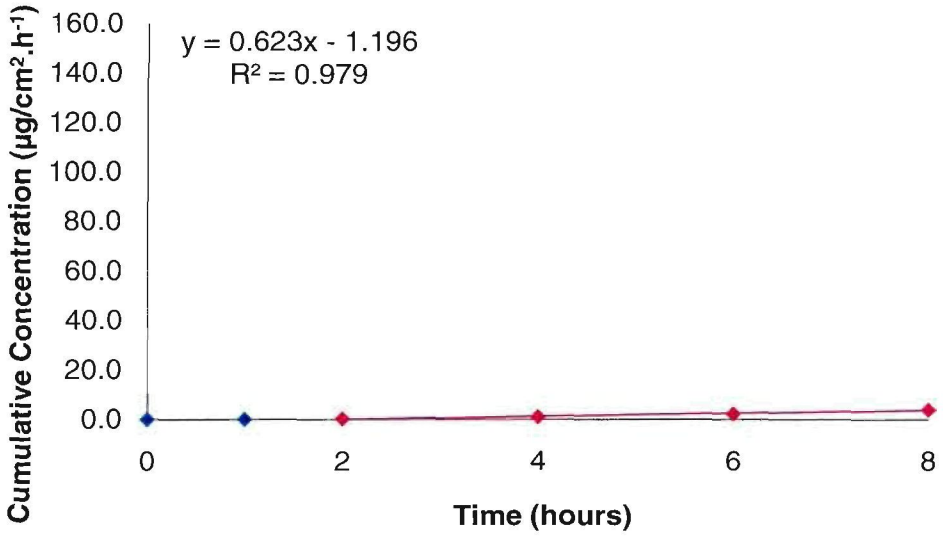

Figure D9: Cumulative amounts diffused of diclofenac sodium non-Pheroid™ gel.

An average flux of 0.63 ± 0.34 (µg/ml.h $^{-1}$) and median flux of 0.95 ± 0.26 (µg/ml.h $^{-1}$) was obtained.

Figure D10 illustrates a graphical representation of Arthruderm® lotion's cumulative amounts diffused.

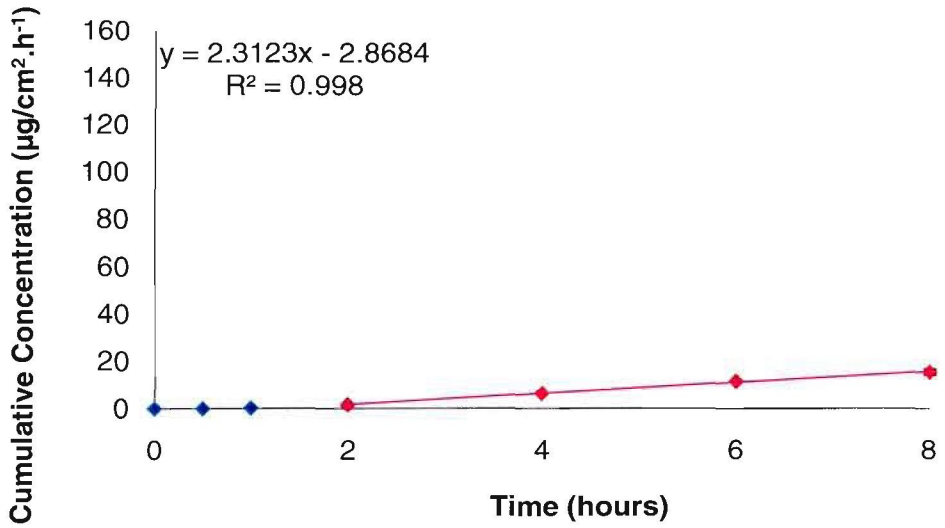

Figure D10: Cumulative amounts diffused of Arthruderm® lotion.

An average flux of 29.49 ± 15.76 (µg/ml.h $^{-1}$) and median flux of 28.36 ± 13.18 (µg/ml.h $^{-1}$) was obtained.

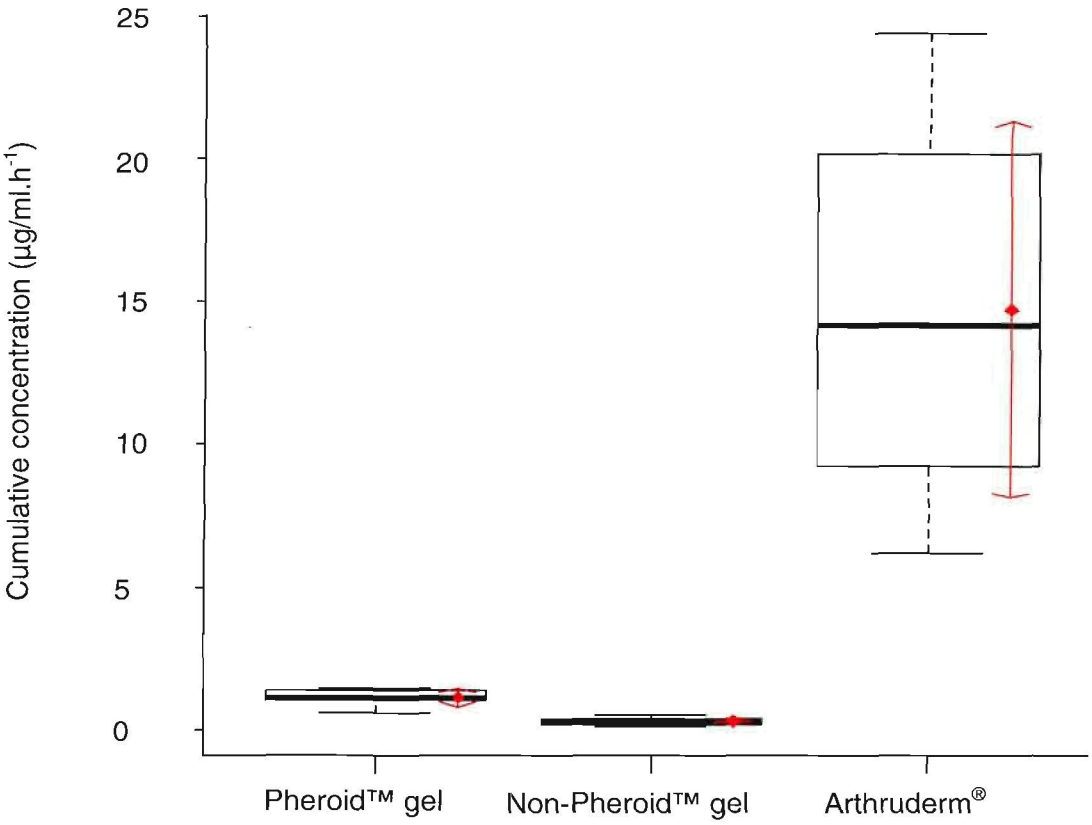

Figure D11: Box plots of the Pheroid™, non-Pheroid™ and retail product, containing diclofenac sodium show black lines to illustrate median flux and red lines to illustrate the average flux values.

D.3.2. Membrane diffusion discussion

The box-plots obtained from membrane diffusion study flux data proved that the standard deviation between the average and median flux values was not considerable. Thus any one of the two sets of values can be used. Tablea D1 lists the average flux values obtained for diclofenac diethylamine Pheroid™ gel **(1)**, diclofenac diethylamine non-Pheroid™ gel **(2)**, Voltaren® emulgel **(3)**, diclofenac hydroxyethylpyrrolidine Pheroid™ gel **(4)**, diclofenac hydroxyethylpyrrolidine non-Pheroid™ gel **(5)**, Panamor® gel **(6)**, diclofenac sodium Pheroid™ gel **(7)**, diclofenac sodium non-Pheroid™ gel **(8)** and Arthruderm® lotion **(9)**.

Table D1: Average and median flux values of preparations containing various diclofenac salts.

Dosage form	Average flux ($\mu g/ml.h^{-1}$)	Median flux ($\mu g/ml.h^{-1}$)
(1)	2.58 ± 1.63	1.94 ± 1.46
(2)	21.71 ± 4	21.47 ± 4.09
(3)	0	0
(4)	5.35 ± 1.33	5.47 ± 1.07
(5)	0.05 ± 0.04	0.06 ± 0.01
(6)	21.7 ± 4.22	22.57 ± 3.91
(7)	2.31 ± 1.02	2.32 ± 0.66
(8)	0.63 ± 0.34	0.95 ± 0.26
(9)	29.49 ± 15.76	28.36 ± 0.26

The data obtained shows that Arthruderm® showed the highest average and median flux values. Data obtained indicates that Arthruderm®, product containing diclofenac sodium, can be distinguished as the formulation that showed the best potential to release the active ingredient from the formulation. The non-Pheroid diclofenac diethylamine gel is the only other formulation that came close to the Arthruderm. All of the other formulations had less than 20% of the flux.

D.4 Skin diffusion

D.4.1 Method for skin diffusion studies

D.4.1.1 Standard solution preparation

Standard solutions with concentrations of 100, 10, 1 and 0.1 µg/ml were prepared and injected into the chromatograph to obtain a calibration curve.

D.4.1.2 Preparation for diffusion

Silicon tubing was cut into eleven 10 cm pieces. Eleven 5 ml syringes were fitted with 18 gauge needles. The silicone tubing pieces were slid over the tips of each of the needles. Ten Franz cells with a size of 1.075 cm^2 were taken and the top half (donor phase) and the bottom half (receptor phase) were covered with a thin layer of Dow Corning vacuum grease (USA). A magnetic stirrer was placed in the bottom of the receptor phase of each Franz cell. A sample of skin with a diameter of ±1 cm mounted on top of a piece of Whatman® filter paper with the same diameter was placed on each one of the receptor phases. The donor phase was placed on top of the receptor phase and clamped together using a horseshoe clamp. Syringes were used to extract PBS (phosphate buffer system) and each of the receptor phases of the 10 Franz cells were filled with 2 ml PBS.

1 ml of the preparation to be evaluated was placed into the donor phase. Pieces of self adhesive plastic film (Parafilm® M, Pechiney Plastic Packaging, Chicago, IL, USA) were placed over the top parts of the donor phase and the donor phase was sealed off from the external environment by placing a plastic cap over the Parafilm®. The Franz cells were placed into a Franz cell stand assigning a number to each Franz cell. A 100 ml volumetric flask filled with PBS was placed in a separate water bath (Grant USA) at 37 □C. Another

water bath was preheated to a temperature of 32 □C this water bath would be used for the diffusion study.

D.4.1.3 Diffusion

The Franz cell container containing the cells was placed into a preheated water bath at 32 □C on top of a magnetic stirrer (Variomag, Daytona Beach, FL, USA). The Franz cell container was placed on top of the magnetic stirrer ensuring that the circles align with the stirrer which was set to 750 rpm. The water bath was closed and samples of PBS were withdrawn at intervals of 0.5, 1, 2, 4, 6, 8, 10 and 12 hours. After 2 ml samples were withdrawn at the prescribed intervals, the receptor phase was filled up with PBS (37 □C).

D.4.1.4 Tape stripping

At the time interval of 12 hours after removal of the donor and receptor phases, the diffusion cells were carefully dismantled and the skin was pinned on a piece of Parafilm® which was stapled to a solid surface and numbered according to the numbers of each Franz cell. The imprints of the flanges from the diffusion cells clearly marked the exposed diffusional area of about 1.25 cm^2. The next step was to dab the skin dry using pieces of tissue paper. The first tape strip was discarded and the following 15 strips were placed in a vial containing 5 ml of PBS pH 7.4. It is seen as normal practice to discard the first strip as it is considered to be part of the cleaning procedure and it may be contaminated with drug from the formulation.

After 15 tape strips the stratum corneum showed the glistening of the viable epidermis and this indicated that the tape stripping process was completed. Excess skin was trimmed away from the flange imprints and the remaining dermis and epidermis were placed in separate vials containing 2 ml of PBS pH 7.4 (Pellett *et al.*, 1997:94). The samples were left overnight in a refrigerator at a temperature of about 8 □C. The PBS solutions were removed from the vials the following day and analysed using HPLC. The concentration values obtained depicts the amounts of active ingredient contained within the layers of the dermis and epidermis.

D 5.1 Results

D.5.1.1 Skin diffusion flux values

No flux values were obtained for any of the formulations including non-Pheroid™, Pheroid™ and products on the South African market. None of the evaluated formulations reached substantial amounts in the target area the systemic circulation and the deep tissue muscle. This indicated that either the drugs did not reach the systemic circulation or it was in amounts below the detection limit of the analytical method.

D.5.1.2 Tape stripping results

The following lists of values indicate the concentration of active ingredient found within the epidermal and dermal layers of the skin. Each preparation's values were listed individually. The following table D2 lists the concentration of active ingredient contained within the epidermis and dermis of various preparations containing diclofenac salts.

Table D2: Concentration of diclofenac in epidermis and dermis after application of various preparations containing diclofenac salts.

Dosage form	Epidermis (µg/ml) (n = 10)	Dermis (µg/ml) (n = 10)
(1)	2.93 ± 0.03	7.45 ± 0.01
(2)	2.93 ± 0.03	7.40 ± 0.12
(3)	2.89 ± 0.005	7.27 ± 0.01
(4)	0.41 ± 0.02	1.15 ± 0.03
(5)	0.43 ± 0.03	1.04 ± 0.07
(6)	0.64 ± 0.008	1.60 ± 0.004
(7)	1.79 ± 0.16	4.47 ± 1.49
(8)	1.56 ± 0.04	4.16 ± 0.61
(9)	1.59 ± 0.09	4.00 ± 0.12

D 6 Discussion

The purpose of membrane diffusion was to evaluate the potential of the preparations to release the drug for absorption through the skin. Membrane diffusion only indicates the amount of active ingredient which is possible to be released from a formulation, not the actual amount that crosses the skin. Skin diffusion gives an indication of the amount of the active ingredient that crossed the skin.

In this study no conclusive data was found that indicated a certain superior diclofenac salt or a certain superior formulation. Membrane studies were implemented as a method to differentiate between release rates, fluxes and possible weaknesses of each respective formulation. The diclofenac diethylamine salt contained within the originator formulation (Voltaren®) had the highest membrane diffusion rate. None of the formulations were able to get diclofenac through the skin. The diclofenac diethylamine Pheroid™ gel reached the highest concentration in both the dermis and epidermis, followed by the diclofenac diethylamine non-Pheroid™ and the originator's product, diclofenac diethylamine emulgel (Voltaren®). The formulations with the sodium salt attaned about half of this concentration, whereas the hydroxyethylpyrrolidine salts reached only about 15% of this concentration. This outcome is in sharp contrast with what one would expect from solubility and log D values, since the diethylamine salt is the least soluble of the three, and has a log D value between that of the other salts. Furthermore it can be concluded that membrane diffusion studies were of no scientific value as a predictive tool since it bore no resemblance to the outcome of skin penetration studies or accumulation of drug in the layers of the skin.

D.7 References

PELLET, M.A., ROBERTS, M.S. & HADGRAFT, J. 1997. Supersaturated solutions evaluated with an in vitro stratum corneum tape stripping technique. *International Journal of Pharmaceutics*, 151:91-98.

ANNEXURE E

Guide for Authors

Scope of the journal

The International Journal of Pharmaceutics publishes innovative papers, reviews, mini-reviews, rapid communications and notes dealing with physical, chemical, biological, microbiological and engineering studies related to the conception, design, production, characterisation and evaluation of drug delivery systems *in vitro* and *in vivo*. "Drug" is defined as any therapeutic or diagnostic entity, including oligonucleotides, gene constructs and radiopharmaceuticals.

Areas of particular interest include: pharmaceutical nanotechnology; physical pharmacy; polymer chemistry and physical chemistry as applied to pharmaceutics; excipient function and characterisation; biopharmaceutics; absorption mechanisms; membrane function and transport; novel routes and modes of delivery; responsive delivery systems, feedback and control mechanisms including biosensors; applications of cell and molecular biology to drug delivery; prodrug design; bioadhesion (carrier-ligand interactions); and biotechnology (protein and peptide formulation and delivery).

Note: In 2004, a new section was started on pharmaceutical nanotechnology. For more details, see Editorials in 279/1-2, 281/1, and 288/1.

Editorial Policy

The over-riding criteria for publication are originality, high scientific quality and interest to a multidisciplinary audience. Papers not sufficiently substantiated by experimental detail will not be published. Any technical queries will be referred back to the author, although the Editors reserve the right to make alterations in the text without altering the technical content. Manuscripts submitted under multiple authorship are reviewed on the assumption that all listed authors concur with the submission and that a copy of the final manuscript has been approved by all authors and tacitly or explicitly by the responsible authorities in the laboratories where the work was carried out. If accepted, the manuscript shall not be published elsewhere in the same form, in either the same or another language, without the consent of the Editors and Publisher.

Authors must state in a covering letter when submitting papers for publication the

novelty embodied in their work or in the approach taken in their research. Routine bioequivalence studies are unlikely to find favour. No paper will be published which does not disclose fully the nature of the formulation used or details of materials which are key to the performance of a product, drug or excipient. Work which is predictable in outcome, for example the inclusion of another drug in a cyclodextrin to yield enhanced dissolution, will not be published unless it provides new insight into fundamental principles.

Submission of Manuscripts

Authors are strongly encouraged to submit their manuscript electronically by using the Elsevier submission site at (⇨ http://www.elsevier.com/journals)

After registration, authors will be asked to upload their manuscript and associated artwork. Full instructions on how to use the online submission tool are available at the web address listed above.

If an author cannot submit their manuscript electronically, then for the initial submission of manuscripts for consideration, hardcopies are sufficient. The original plus two copies, complete with two sets of figures (including originals or duplicates of sufficient quality for clarity of reproduction) and tables, must be submitted in English. All data that would help referees to evaluate the paper should also be supplied. Manuscripts should be typewritten with double spacing and adequate margins on one side of the sheet only (not more than 26 lines per page). All pages should be numbered sequentially. Manuscripts should be sent to one of the following Editors-in-Chief according to the geographical origin of the author. Please include full contact information - corresponding author name, e-mail address, telephone and fax numbers, and full postal address.

After **final acceptance for publication**, your revised manuscript on disk together with two printed hard copies, should be submitted to the accepting editor. **It is important that the file on disk and the printout are identical.** Both will then be forwarded by the editor to Elsevier. In-depth guidelines for submitting artwork/illustrations can be found at: ⇨ http://www.elsevier.com/artworkinstructions.

When the paper is to be published as a Rapid Communication, this should be clearly indicated to the Editor-in-Chief.

Europe, Africa, Near East

Prof A.T. Florence, The School of Pharmacy, University of London, 29-39 Brunswick Square, London WCIN 1AX, UK, Fax: +44 20 7837 5092; E-mail: ijp@pharmacy.ac.uk

The Americas, Australia and New Zealand

Professor D.J. Burgess, University of Connecticut, School of Pharmacy, Room 432, 69 North Eagleville Road, Unit 3092, CT 06269-3092, Storrs, USA, Fax.: +1 860 486-2076; E-mail: d.burgess@uconn.edu

Japan and Far East

Prof. T. Sonobe, Department of Pharmaceutical Engineering, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Shizuoka-shi 422-8526, Japan. Fax: +81-54-264-5614; E-mail: sonobe@gakushikai.jp

Manuscript Types

(1) **Full Length Manuscripts**

The arrangement of full length papers should accord with the following:

(a) *Title*

The full title should not exceed 85 characters including spaces between words.

(b) *List of Authors*

Initial(s) (one given name may be used) followed by the surname of author(s) together with their affiliations. When the work has been carried out at more than one address, the affiliation of each author should be clearly indicated using superscript, lower-case letters. The author to whom correspondence should be directed must be indicated with an asterisk.

(c) *Affiliation(s)* Name(s) and address(es) of the establishment(s) where the work was done, designated by superscript, lower-case letters where appropriate.

(d) *Abstract*

An Abstract not exceeding 200 words (a single paragraph) should be provided typed on a

separate sheet.

(e) *Keywords*

A maximum of 6 keywords or short phrases suitable for indexing should be supplied. If possible keywords should be selected from Index Medicus or Excerpta Medica Index. Authors may also wish to refer to the Subject Index published in *International Journal of Pharmaceutics*, for example, Vol. 287/1-2, pp. 205-219.

(f) *Corresponding Author*

The author to whom correspondence should be directed should be designated with an asterisk (do not include the address unless different from that indicated by the author's affiliation). Telephone, fax and e-mail address of the corresponding author must be provided.

(g) *Text*

The text should be divided into main sections, such as the following: 1. Introduction. 2. Materials and methods. 3. Results. 4. Discussion. Acknowledgements. References, figure legends, tables and figures. These sections must be numbered consecutively as indicated. Subdivisions of a section should also be numbered within that section, for example, 2.1. Materials, 2.2. Relative humidity measurement, 2.3. Sample preparation, etc.

(h) *Nomenclature*

Standard nomenclature should be used throughout; unfamiliar or new terms and arbitrary abbreviations should be defined when first used. Unnecessary or ambiguous abbreviations and symbols are to be avoided. Data should be expressed in SI units.

(i) *Figure Legends, Table Legends, Footnotes*

Figure legends, tables and footnotes should be typed on separate sheets, lines double spaced. Footnotes, to be numbered consecutively in superscript throughout the text, should be used as little as possible.

(j) *References*

See below for full details.

(2) **Rapid Communications**

(a) These articles should not exceed 1500 words or equivalent space.

(b) Figures should not be included otherwise delay in publication will be incurred.

(c) Do not subdivide the text into sections. An Abstract should be included as well as a full reference list.

(3) **Notes**

Should be prepared as described for full length manuscripts, except for the following:

(a) The maximum length should be 1500 words, including figures and tables.
(b) Do not subdivide the text into sections. An Abstract and reference list should be included.

(4) **Reviews and Mini-Reviews**
Suggestions for review articles will be considered by the Editors-in-Chief. "Mini-reviews" of a topic are especially welcome.

References

(a) *Text citation*
The Harvard system of citation must be used. References should be cited in the text within parentheses: where several citations are given within a single set of parentheses, they should be arranged in ascending order of year of publication; where more than one reference with the same year of publication is cited, they should be arranged in alphabetical order of the first authors' names. When referring to a work of more than two authors, the name of the first author should be given, followed by et al.

Examples of text citations:
(Gesztes et al., 1988; Chestnut et al., 1989; Legros et al., 1990; Mhando and Li Wan Po, 1990; Korsten et al., 1991; Langerman et al., 1991, 1992a,b; Masters et al., 1991; Bonhomme et al., 1992; Kolli et al., 1992).

(Shaw et al., 1978; Nakano and Arita 1990b; Nakano et al., 1990a,b; Bone et al., 1992)

(b) *Reference list*

All references cited in the text should be listed at the end of the paper (typed with double spacing) and assembled alphabetically. More than one paper from the same author(s) in the same year must be identified by the letters a b c, etc. placed after the year of publication.

References must consist of names and initials of all authors, year, title of paper, abbreviated title of periodical, and volume and first and last page numbers. 'Personal communication' and 'unpublished data' should be cited in the text only. Papers referred to as 'submitted for publication' must include the name of the journal to which submission has been made. Journal titles should be abbreviated according to the '*List of Serial Title Word Abbreviations*' (available from International Serials Data System, 20, rue Bachaumont, 75002 Paris, France. ISBN 2-904939-02-8).

Example of arrangement in the reference list:

Crowe, J.H., Crowe, L.M., Chapman, D., 1984a. Infrared spectroscopic studies on interactions of water and carbohydrates with a biological membrane. Arch Biochem. Biophys., 232, 400-407.

Crowe, J.H., Crowe, L.M., Hoekstra, F.A., 1989. Phase transitions and permeability changes in dry membranes during rehydration. J. Bioenerg. Biomembr., 21, 77-92.

Crowe, J.H., Crowe, L.M., Carpenter, J.F., Aurell Wistrom, C., 1987. Stabilization of dry phospholipid bilayers and proteins by sugars. Biochem. J., 242, 1-10.

Crowe, J.H., Crowe, L.M., Carpenter, J.F., Rudolph, A.S., Wistrom, C.A., Spargo, B.J., Anchordoguy, T.J., 1988. Interactions of sugars with membranes. Biochim. Biophys. Acta, 947, 367-384.

Crowe, L.M., Crowe, J.H., Womersley, C., Reid, D., Appel, L., Rudolph, A., 1986. Prevention of fusion and leakage in freeze-dried liposomes by carbohydrates. Biochim. Biophys. Acta, 861, 131-140.

Crowe, L.M., Mouradian, R., Crowe, J.H., Jackson, S.A., Womersley, C., 1984b. Effects of carbohydrates on membrane stability at low water activities. Biochim. Biophys. Acta, 769, 141-150.

Examples of presentation for various types of publication:

Langerman, L., Chaimsky, G., Golomb, E., Tverskoy, M., Kook, A.I., Benita, S., 1990. A rabbit model for evaluation of spinal anesthesia: chronic cannulation of the subarachnoid space. Anesth. Analg., 71, 529-535.

Timsina, M.P., Martin, G.P., Marriott, C., Ganderton, D., Yianneskis, M., 1994. Drug delivery to the respiratory tract using dry powder inhalers. Int. J. Pharm., 101, 1-13.

Gibaldi, M. and Perrier, D., 1982. Pharmacokinetics, 2nd Ed., Dekker, New York.

Deppeler, H.P., 1981. Hydrochlorothiazide. In: Florey, K. (Ed.), Analytical Profiles of Drug Substances, Vol. 10, Academic Press, New York, pp. 405-441.

US Pharmacopeia XXII, 1990. US Pharmacopeial Convention, Rockville, MD, pp. 1434-1435.

Mueller, L.G., 1988. Novel anti-inflammatory esters, pharmaceutical compositions and methods for reducing inflammation. UK Patent GB 2 204 869 A, 23 Nov.

Du Plessis, J., 1992. Topical liposomal delivery of biologically active peptides. Ph.D Thesis, Potchefstroom University for CHE, South Africa.

(c) *Use of Digital Object Identifier (DOI)*

The digital object identifier (DOI) may be used to cite and link to electronic documents. The DOI consists of a unique alpha-numeric character string which is assigned to a document by the publisher upon the initial electronic publication. The assigned DOI never changes. Therefore, it is an ideal medium for citing a document particularly "Articles in press" because they have not yet received their full bibliographic information.

The correct format for citing a DOI is shown as follows: doi:10.1016/j.ijpharm.2005.01.041

Articles in Special Issues: Please ensure that the words 'this issue' are added (in the list and text) to any references to other articles in this Special Issue.

Figures and Tables

Figures

Line drawings (including graphs) should be drawn in black ink on white paper or on tracing

paper with blue or faint grey rulings; graduation will not be reproduced. Lettering should be large enough to permit photographic reduction. If figures are not to be reduced, their format should not exceed 16 x 20 cm. Photographs (or half-tone illustrations) must be of good quality, submitted as black and white prints on glossy paper, and have as much contrast as possible. The magnification of micrographs should be indicated by a scale bar in the figure. Figures should be clearly marked on the reverse side with the number, orientation (top) and author's name; a soft pencil or a felt-tipped pen should be used for marking photographs. The illustrations should be numbered with Arabic numerals. The legends should be typed separately with double spacing.

Colour illustrations should be submitted as original photographs, high-quality computer prints or transparencies, close to the size expected in publication, or as 35 mm slides. Polaroid colour prints are *not* suitable. If, together with your accepted article, you submit usable colour figures then Elsevier will ensure, **at no additional charge**, that these figures will appear in colour on the web (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in colour in the printed version. For colour reproduction in print, you will receive information regarding the total cost from Elsevier after receipt of your accepted article. The 2008 price for color figures is EUR 285 for the first page and EUR 191 for subsequent pages. For further information on the preparation of electronic artwork, please see ⇉http://www.elsevier.com/artworkinstructions.

Please note: Because of technical complications which can arise by converting colour figures to 'grey scale' (for the printed version should you not opt for colour in print) please submit in addition usable black and white prints corresponding to all the colour illustrations.

Tables

All tables must be numbered consecutively (with Arabic numerals) and be cited in the text. Titles should be short but descriptive. Tables should be compiled on separate sheets, together with a legend and/or footnotes identified by superscripts a.b.c, etc. Do not use vertical lines and keep horizontal rules to a minimum.

DNA sequences and GenBank Accession numbers.

Many Elsevier journals cite "gene accession numbers" in their running text and footnotes. Gene accession numbers refer to genes or DNA sequences about which further information can be found in the databases at the National Center for Biotechnical Information (NCBI) at the National Library of Medicine.

Authors are encouraged to check accession numbers used very carefully. **An error in a letter or number can result in a dead link.** Note that in the final version of the ***electronic***

copy, the accession number text will be linked to the appropriate source in the NCBI databases enabling readers to go directly to that source from the article.

Copyright guidelines for authors

Upon acceptance of an article, authors will be asked to sign a 'Journal Publishing Agreement' (for more information on this and copyright see ⇉http://www.elsevier.com/copyright). Acceptance of the agreement will ensure the widest possible dissemination of information. An e-mail (or letter) will be sent to the corresponding author confirming receipt of the manuscript together with a 'Journal Publishing Agreement' form or a link to the online version of this agreement.
Subscribers may reproduce tables of contents or prepare lists of articles including abstracts for internal circulation within their institutions. Permission of the Publisher is required for resale or distribution outside the institution and for all other derivative works, including compilations and translations (please consult ⇉http://www.elsevier.com/permissions). If excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article. Elsevier has preprinted forms for use by authors in these cases: please consult ⇉ http://www.elsevier.com/permissions.

Authors' rights

As an author you (or your employer or institution) retain certain rights; for details you are referred to ⇉http://www.elsevier.com/authorsrights.

Proofs, Offprints and Page Charges

One set of page proofs in PDF format will be sent by e-mail to the corresponding author (if we do not have an e-mail address then paper proofs will be sent by post). Elsevier now sends PDF proofs which can be annotated; for this you will need to download Adobe Reader version 7 or higher available free from ⇉ http://www.adobe.com/products/acrobat/readstep2.html. Instructions on how to annotate PDF files will accompany the proofs. The exact system requirements are given at the Adobe site ⇉http://www.adobe.com/products/acrobat/acrrsystemreqs.html#70win.
If you do not wish to use the PDF annotations function, you may list the corrections (including replies to the Query Form) and return to Elsevier in an e-mail. Please list your corrections quoting line number. If, for any reason, this is not possible, then mark the corrections and any other comments (including replies to the Query Form) on a printout of your proof and

return by fax, or scan the pages and e-mail, or by post.
Please use this proof only for checking the typesetting, editing, completeness and correctness of the text, tables and figures. Significant changes to the article as accepted for publication will only be considered at this stage with permission from the Editor. We will do everything possible to get your article published quickly and accurately. Therefore, it is important to ensure that all of your corrections are sent back to us in one communication: please check carefully before replying, as inclusion of any subsequent corrections cannot be guaranteed. Proofreading is solely your responsibility. Note that Elsevier may proceed with the publication of your article if no response is received.
The corresponding author, at no cost, will be provided with a PDF file of the article via e-mail or, alternatively, 25 free paper offprints. The PDF file is a watermarked version of the published article and includes a cover sheet with the journal cover image and a disclaimer outlining the terms and conditions of use. Additional paper offprints can be ordered by the authors. An order form with prices will be sent to the corresponding author.

Page Charges

There are no page charges.

Language Services.

Authors who require information about language editing and copyediting services pre- and post-submission please visit ⊟↦http://www.elsevier.com/locate/languagepolishing or contact authorsupport@elsevier.com for more information. Please note Elsevier neither endorses nor takes responsibility for any products, goods or services offered by outside vendors through our services or in any advertising. For more information please refer to our Terms & Conditions ⊟↦http://www.elsevier.com/termsandconditions.

Funding body agreements and policies
Elsevier has established agreements and developed policies to allow authors who publish in Elsevier journals to comply with potential manuscript archiving requirements as specified as conditions of their grant awards. To learn more about existing agreements and policies please visit ⊟↦http://www.elsevier.com/fundingbodies.

Author enquiries

For enquiries relating to the submission of articles (including electronic submission where available) please visit this journal's homepage at ⊟↦http://www.elsevier.com/locate/ijpharm.

You can track accepted articles at ⊡→http://www.elsevier.com/trackarticle and set up e-mail alerts to inform you of when an article's status has changed. Also accessible from here is information on copyright, frequently asked questions and more.

Contact details for questions arising after acceptance of an article, especially those relating to proofs, are provided after registration of an article for publication.

No responsibility is assumed by the Publisher for any use or operation of any methods, products, instructions or ideas contained in the material herein. Because of the rapid advances made in the medical sciences, independent verification of diagnoses and drug dosages should be made.