

Contents

| | Page |
|--|-------------|
| Abstract | (3) |
| Acknowledgement | (5) |
| Contents | (6) |
| List of Illustrations | (9) |
| List of Tables | (14) |
| Abbreviations and Symbols | (15) |
| Chapter | |
| I Introduction | 1 |
| 1. Rationale for development of ester of NSAIDs | 1 |
| 2. Absorption across intestinal membrane | 6 |
| 2.1 Mechanisms of intestinal membrane permeation | 6 |
| 2.1.1 Passive Transcellular Transport | 7 |
| 2.1.2 Passive Paracellular Transport | 8 |
| 2.1.3 Carrier mediated transport and efflux | 8 |
| 2.2 Factors affecting gastrointestinal absorption | 10 |
| 2.2.1 Physicochemical parameters: pKa and lipophilicity | 11 |
| 2.2.2 Physiological parameters | 14 |
| 2.3 Cell culture model used in assessing intestinal absorption | 15 |
| 2.4 Drug Efflux Transporters as an absorption barrier | 17 |
| 3. Functional activity assay for drug efflux transporters | 21 |
| 3.1 ATPase assay | 22 |
| | (6) |

Contents (Continued)

| | Page |
|---|-------------|
| 3.2 Calcein AM efflux inhibition assay | 22 |
| Objectives of the research | 24 |
| II Experimentals | 25 |
| Research outlines | 25 |
| 1. Materials | 26 |
| 1.1 Esters of mefenamic acid | 26 |
| 1.2 Chemicals | 26 |
| 1.3 Chemicals and reagents used for cell cultures | 26 |
| 2. Methods | 27 |
| 2.1 Solubility determination | 27 |
| 2.2 Chemical Stability | 28 |
| 2.3 Enzymatic Stability | 28 |
| 2.3.1 Caco-2 homogenate preparation | 30 |
| 2.3.2 Rat liver homogenate preparation | 30 |
| 2.3.3 Human plasma | 31 |
| 2.3.4 Protein content determination | 31 |
| 2.4 Cell culture | 31 |
| 2.4.1 Preparation of culture medium | 32 |
| 2.5 Sample Analysis | 33 |
| 2.5.1 HPLC systems | 33 |

Contents (Continued)

| | Page |
|--|-------------|
| 2.5.2 Sample preparation | 34 |
| 2.6 Transepithelial transport studies of mefenamic ester prodrugs across Caco-2 monolayer | 35 |
| 2.7 Data Analysis | 37 |
| 2.8 Efflux inhibition studies | 38 |
| 2.9 Calcein AM inhibition assay | 40 |
| 2.10 Colorimetric MTT toxicity assay | 40 |
| | |
| III Results and Discussions | 42 |
| 1. Solubility | 42 |
| 2. Chemical Stability | 44 |
| 3. Enzymatic Stability | 45 |
| 4. Transport study | 49 |
| 4.1 Efflux inhibition study | 52 |
| 4.2 Calcein AM inhibition assay | 55 |
| | |
| IV Conclusions | 61 |
| | |
| Bibliography | 63 |
| | |
| Appendix | 88 |
| | |
| Vitae | 115 |

List of Illustrations

| Figure | | Page |
|--------|---|------|
| 1 | Arachidonic acid metabolism | 3 |
| 2 | Chemical structures of esters of mefenamic acid used in this study | 5 |
| 3 | Transport pathways across intestinal epithelium | 7 |
| 4 | Schematic representation of drug transport from a solid dosage form into systemic circulation. | 11 |
| 5 | Relationship between log D at pH 7.4 and the logarithm of apparent permeability across Caco-2 cell monolayers, (A) and fraction absorbed in human, (B). | 13 |
| 6 | Relationship between the absorbed fraction of structurally diverse sets of orally administered drugs and permeability coefficients obtained in cell monolayers. | 13 |
| 7 | Asymmetric distribution of ABC transporters in polarized intestinal absorptive cells | 19 |
| 8 | Chemical structures of calcein AM (A) and calcein (B) | 23 |
| 9 | Chemical structures of verapamil and indomethacin used in efflux inhibition studies | 39 |
| 10 | Degradation profile of 1 in human plasma | 47 |
| 11 | Degradation profile of 2 in human plasma and Caco-2 homogenate with and without protease inhibitor, PMSF. | 48 |

List of Illustrations (Continued)

| Figure | | Page |
|--------|--|------|
| 12 | Cytotoxicity of phenylmethyl sulfonyl fluoride (PMSF) to Caco-2 monolayer using MTT assay. | 50 |
| 13 | Bidirectional transport across Caco-2 monolayers of four ester derivatives of mefenamic acid. | 52 |
| 14 | Inhibition effects on calcein efflux in Caco-2 cells | 57 |
| 15 | Effect of inhibitors on calcein accumulation in Caco-2 cells, presented as percentage of the maximum response obtained from verapamil. | 58 |
| 16 | Hydrolysis of profiles of the esters <u>2</u> , <u>3</u> , and <u>6</u> in phosphate buffer pH 7.4 at 37°C | 92 |
| 17 | Hydrolysis of <u>1</u> in human plasma and phosphate buffer pH 7.4 at 37°C | 92 |
| 18 | Hydrolysis of <u>2</u> in human plasma and phosphate buffer pH 7.4 at 37°C | 93 |
| 19 | Hydrolysis of <u>3</u> in human plasma and phosphate buffer pH 7.4 at 37°C | 93 |
| 20 | Hydrolysis of <u>4</u> in human plasma and phosphate buffer pH 7.4 at 37°C | 94 |
| 21 | Hydrolysis of <u>5</u> in human plasma and phosphate buffer pH 7.4 at 37°C | 94 |
| 22 | Hydrolysis of <u>6</u> in human plasma and phosphate buffer pH 7.4 at 37°C | 95 |

List of Illustrations (Continued)

| Figure | | Page |
|---------------|--|-------------|
| 23 | Hydrolysis of <u>7</u> in human plasma and phosphate buffer pH 7.4 at 37°C | 95 |
| 24 | Degradation profiles of <u>1</u> in human plasma (pH 7.4, 37°C) | 96 |
| 25 | Degradation profiles of <u>1</u> in Caco-2 homogenate (pH 7.4, 37°C) | 96 |
| 26 | Degradation profiles of <u>1</u> in rat liver homogenate (pH 7.4, 37°C) | 97 |
| 27 | Degradation profiles of <u>2</u> in human plasma (pH 7.4, 37°C) | 97 |
| 28 | Degradation profiles of <u>2</u> in Caco-2 homogenate (pH 7.4, 37°C) | 98 |
| 29 | Degradation profiles of <u>2</u> in rat liver homogenate (pH 7.4, 37°C) | 98 |
| 30 | Degradation profiles of <u>3</u> in human plasma (pH 7.4, 37°C) | 99 |
| 31 | Degradation profiles of <u>3</u> in Caco-2 homogenate (pH 7.4, 37°C) | 99 |
| 32 | Degradation profiles of <u>3</u> in rat liver homogenate (pH 7.4, 37°C) | 100 |
| 33 | Degradation profiles of <u>4</u> in human plasma (pH 7.4, 37°C) | 100 |
| 34 | Degradation profiles of <u>4</u> in Caco-2 homogenate (pH 7.4, 37°C) | 101 |
| 35 | Degradation profiles of <u>4</u> in rat liver homogenate (pH 7.4, 37°C) | 101 |
| 36 | Degradation profiles of <u>5</u> in human plasma (pH 7.4, 37°C) | 102 |

List of Illustrations (Continued)

| Figure | | Page |
|---------------|---|-------------|
| 37 | Degradation profiles of <u>5</u> in Caco-2 homogenate (pH 7.4, 37°C) | 102 |
| 38 | Degradation profiles of <u>5</u> in rat liver homogenate (pH 7.4, 37°C) | 103 |
| 39 | Degradation profile of <u>6</u> in human plasma (pH 7.4, 37°C) | 103 |
| 40 | Degradation profile of <u>6</u> in Caco-2 homogenate (pH 7.4, 37°C) | 104 |
| 41 | Time course of <u>5</u> in human plasma, Caco-2 homogenate, and rat liver homogenate (pH 7.4, 37°C) | 104 |
| 42 | Representative chromatograms of degradation of <u>1</u> in rat liver homogenate. | 105 |
| 43 | Representative chromatograms of transport of <u>1</u> across Caco-2 monolayer. | 106 |
| 44 | Representative chromatograms of BL to AP transport of <u>3</u> across Caco-2 monolayer. | 107 |
| 45 | Representative chromatograms of transport of <u>3</u> with 100 µM indomethacin | 108 |
| 46 | Representative chromatograms of transport of <u>3</u> with 100 µM verapamil | 110 |
| 47 | Representative chromatograms of hydrolysis of <u>4</u> in Caco-2 homogenate | 112 |
| 48 | Representative chromatograms of transport of <u>4</u> with 100 µM indomethacin | 113 |

List of Illustrations (Continued)

| Figure | | Page |
|--------|--|------|
| 49 | Representative chromatograms of transport of <u>4</u> with 100 μ M verapamil | 114 |

List of Tables

| Table | | Page |
|--------------|---|-------------|
| 1 | Most commonly used cell culture models for estimating intestinal transcellular flux | 16 |
| 2 | ABC Transporters that confer multidrug resistance | 20 |
| 3 | Mobile phase used for HPLC analysis and retention time of each compound. | 34 |
| 4 | Solubility of esters of Mefenamic acid | 43 |
| 5 | Apparent half-lives of mefenamic acid esters <u>1-7</u> in 0.05M buffer solution at pH 2.0, 5.0, and 7.4 at 37°C | 45 |
| 6 | Apparent half-lives of mefenamic acid esters <u>1-7</u> in various biological media | 46 |
| 7 | Bidirectional apparent permeability coefficients of esters of mefenamic acid across Caco-2 monolayer and the efflux ratio | 51 |
| 8 | Effects of verapamil and indomethacin on apparent permeability of <u>3</u> and <u>4</u> across Caco-2 monolayer | 53 |
| 9 | Solubility of mefenamic esters <u>2</u> and <u>3</u> in various solvents | 89 |
| 10 | Rate constants and squared correlation coefficients of hydrolysis of mefenamic acid esters in buffers at varying pH's | 90 |
| 11 | Rate constants and squared correlation coefficients of hydrolysis of mefenamic acid esters in biological media | 91 |

Abbreviations and Symbols

| | |
|----------------|---|
| ABC | Adenosine Triphosphate Binding Cassettes |
| AP | Apical |
| BL | Basolateral |
| BCRP | Breast Cancer Resistance Proteins |
| cm/s | Centimeter per second |
| DMSO | Dimethyl Sulfoxide |
| ER | Efflux Ratio |
| F | Fluorescence intensity |
| GI | Gastrointestine |
| HBSS | Hank's Balanced Salt Solution |
| HPLC | High Performance Liquid Chromatography |
| HEPES | <i>N</i> -hydroxyethylpiperazine- <i>N'</i> -2-ethane sulfonate |
| h | hour |
| KOH | Potassium hydroxide |
| MES | 2-(<i>N</i> -Morpholino) ethane sulfonic acid |
| MRP | Multidrug resistance proteins |
| min | minute |
| nm | nanometer |
| NSAIDs | Non-steroidal anti-inflammatory drugs |
| P_{app} | Apparent permeability coefficient |
| $P_{app, A-B}$ | Apparent permeability coefficient, in apical to basolateral |
| $P_{app, B-A}$ | Apparent permeability coefficient, in basolateral to apical |
| Pgp | P-glycoprotein |
| PBS | Phosphate Buffer Saline |
| PMSF | Phenylmethyl sulfonyl fluoride |
| μg | microgram |
| μL | microliter |
| μM | micromolar |
| rpm | Round per minute |
| TER | Transepithelial electrical resistance |

Abbreviations and Symbols (Continued)

v/v Volume by volume