COPYRIGHT© INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

CHROMATOGRAPHIC METHOD DEVELOPMENT FOR THE DETECTION OF MEFENAMIC ACID IN HUMAN PLASMA USING HPLC WITH UV-VIS DETECTOR

BY

HUDA JAMILAH

A thesis submitted in fulfilment of the requirement for the degree of Master of Pharmaceutical Chemistry

Kulliyyah of Pharmacy
International Islamic University Malaysia

DECEMBER 2015

ABSTRACT

Mefenamic acid (MA) [2-[(2,3-dimethylphenyl) amino]benzoic acid], is from group of nonsteroidal anti-inflammatory drugs (NSAIDs). Generic MA is widely used nowadays as it is expected to have same efficacy and strength as the brand drug. Bioequivalence study for generic formulation is prime requisite of generic drug registration. Bioequivalence study consists of clinical part and analytical part. Method development and subsequent validation of MA in human plasma using HPLC UV-vis detector for analytical part of bioequivalence study was the aim of this study. Two sample preparation technique was successfully developed and validated which were protein precipitation technique and liquid liquid extraction technique. Optimization of MA was performed with selecting several mobile phase, column, and selection of internal standard candidates. Acetonitrile and 2 % triethylamine buffer adjusted to pH 4.2 by using phosphoric acid with ratio (60:40 v/v) was selected as mobile phase. Agilent Zorbax Eclipse XDB C 18 column with 3.5 µm particle size (150 mm x 4.6 mm) was selected as the column for separation of MA compound. Diclofenac sodium was selected as internal standard. Retention time of MA and diclofenac sodium was found 5.4 and 3.8 minutes respectively. Protein precipitation technique was performed using 500 µl spiked plasma sample added with 100 µl of internal standard and 2 ml acetonitrile. For liquid liquid extraction technique relatively small volume of sample was used, 100 µl of spiked plasma with MA, was added with 100 µl diclofenac sodium and 100 µl 20 mM ammonium acetate buffer with pH 3.9. Dichloromethane was used as extracting solvent for MA and IS. Both of these sample preparation technique was validated according to CDER bioanalytical method validation guideline. Protein precipitation technique and liquid liquid extraction was validated for selectivity and showed no interference at retention time of MA and IS. Calibration plot was linear within the range of 250 – 5000 ng ml^{-1} with the coefficient of determination (r^2) above 0.990 for both technique. Limit of detection was 40 ng ml^{-1} whereas limit of quantification was 250 ng ml^{-1}. QC samples consists of LLOQ 250 ng ml^{-1}, LQC 400 ng ml^{-1}, MQC 2700 ng ml^{-1}, and HQC 4500 ng ml^{-1}. Accuracy and precision was performed for QC samples for intraday and interday. The range for intraday or within day accuracy was between 91.67 % to 110.84 %. The range for intraday and interday precision (CV) was between 1.45 % to 5.5 %. Recovery of MA was more than 80 %. Limit of detection for MA for liquid liquid extraction was 50 ng ml^{-1} whereas limit of quantification 250 ng ml^{-1}. The range of accuracy for intraday and interday accuracy was within 95.11 % to 106.56 % whereas the range for intraday and interday precision (CV) was 1.47 % to 6.45 %. Recovery of MA was found more than 90 % for this technique. For the stability study for both liquid liquid extraction technique and protein precipitation technique, MA was found stable, for short term, freeze up to three cycle and thaw and long term stability the content of MA about 4 month found about 90 % for all QC samples. Thus two different chromatographic technique were developed and validated for MA in plasma which were efficient, reliable, economic and also fulfill the green chemistry approach. Both the methods are suitable for bioequivalence study of MA.

ملخص البحث

يعتبر حمض الميفيناميك {٢-{(٢،٣ ثنائي ميتيل فينيل) امينو} حمض البنزويك} أحد الادوية من مجموعة مضادات الالتهاب غير الستيروئيدية NSAIDS. تم اختيار الطور المتحرك ليكون مزيجا بنسبة ٦٠ الى ٤٠ بالمئة حجم على التوالي من الاسيتونتريل و ٢% من وقاء ثلاثي ايتيل الامين بعامل حموضة ٤,٢ حيث تم ضبط ال pH بواسطة حمض الفوسور. أما العمود المستخدم في هذه الدراسة فكان Agilent Zorbax Eclipse XDB C 18 بقياس جزيئات ٣,٥ ميكرومتر وأبعاد ١٥٠ ملم طولا و٤,٦ ملم قطرا وتم اختيار ديكلوفيناك الصوديوم كمعاير داخلي. أظهرت النتائج أن وقت الاحتفاظ كان ٥,٤ دقيقة و٣,٨ دقيقة لحمض الميفيناميك وديكلوفيناك الصوديوم على التوالي. تم انجاز طريقة ترسيب البروتينات باستخدام ٥٠٠ ميكروليتر من البلازما مع ١٠٠ ميكروليتر من المعاير الداخلي و ٢ مل من الاسيتونيتريل. أما بالنسبة لطريقة الاستخلاص السائل فقد تم استخدام ١٠٠ ميكروليتر من البلازما المزودة بحمض الميفيناميك مع ١٠٠ ميكروليتر من المعاير الداخلي و١٠٠ ميكروليتر من وقاء اسيتات الامونيوم ذو الحموضة ٣,٩. تم استخدام ثنائي كلور الميتان كعامل استخلاص. تم تحقيق الطريقة التحليلية باستخدام طريقتي الاستخلاص طبقا لمعايير ال CDER. تم التحقق من انتقائية الطريقة حيث أظهرت النتائج عدم وجود تداخل بين قمة حمض الميفيناميك مع القمم الناتجة عن البلازما. تراوحت تراكيز المنحنى الخطي المعياري بين ٢٥٠ و٥٠٠٠ ميكروغرام/مل مع معامل تحديد قدره ٠,٩٩٠ لكلا الطريقتين. كان التركيز الادنى المكتشف ٤٠ نانوغرام / مل والتركيز الادنى المعاير ٢٥٠ نانوغرام / مل. تم تحديد ٤ تراكيز لمراقبة الجودة وهي LLOQ بتركيز ٢٥٠ نانوغرام /مل و LQC بتركيز ٤٠٠ نانوغرام/مل و MQC بتركيز ٢٧٠٠ نانوغرام/مل و HQC بتركيز ٤٥٠٠ نانوغرام/مل. تم التحقق من دقة واحكام الطريقة باستخدام عينات مراقبة الجودة في اليوم نفسه و بين ايام مختلفة. أظهرت النتايج ان الدقة تراوحت بين ٩١,٦٧% و ١١٠,٨٤% بينما تراوحت قيمة الاحكام CV% بين ١,٤٥% و ٥,٥%. كانت قيمة استرداد حمض الميفيناميك أكثر من ٩٠% لهذه الطريقة. أما بالنسبة لدراسة الثباتية فقد كان حمض الميفيناميك ثابتا طبقا لدراسة الثباتية قصيرة الامد والثباتية بعد التجميد والحل لثلاث مرات والثباتية طويلة الامد لحوالي ٤ اشهر حيث أعطت عينات مراقبة الجودة تركيزا اعلى من ٩٠%. في الختام تم تطوير وتحقيق طريقتين كروماتوغرافيتين لحمض الميفيناميك في البلازما وهاتين الطرق كن عاليتي الفعالية والموثوقية والاقتصادية و متماشية مع الكيمياء الأمنة. كلتا الطريقتين مناسبتين لدراسات التكافؤ الحيوي لحمض الميفيناميك.

ABSTRAK

Asid mefenamik (AM) ialah 2-[(2,3-dimetilphenil) amino]asid benzoik. Ia merupakan salah satu ubat daripada kumpulan ubat anti radang bukan steroid. Ubat AM generik yang dihasilkan mempunyai kesan yang sama dengan syarikat inovator yang mengeluarkannya. Bagi mendaftarkan ubat generik, kajian bioekuivalen perlu dijalankan. Kajian bioekuivalen terdiri daripada dua bahagian iaitu kajian klinikal dan bahagian analisis. Kaedah untuk mengesan ubat AM daripada plasma manusia menggunakan kromatografi cecair berprestasi tinggi (HPLC) dengan menggunakan alat pengesan yang dinamakan ultraungu telah dijalankan untuk menganalisa kajian bioekuivalen. Terdapat dua teknik penyediaan sampel daripada plasma manusia telah berjaya dihasilkan dan disahkan iaitu kaedah penyah protein dan juga teknik pengekstrakan cecair. Pengoptimuman AM dilakukan dengan pemilihan fasa bergerak, turus dan pemilihan standard dalaman terbaik. Asetonitril dan air yang mengandungi 2 % trietilamina telah diubahusai kepada pH 4.2 dengan nisbah 60:40 menjadi pilihan sebagai larutan fasa bergerak. Turus yang digunakan ialah turus 18 karbon berjenama Agilent Zorbax Eclipse XDB (150 mm x 4.6 mm, 3.5 μm d.d) untuk pengasingan AM. Diclofenac sodium telah dipilih sebagai standard dalaman. Masa yang dapat direkodkan untuk AM dan diclofenac sodium ialah pada 5.4 dan 3.8 minit. Kaedah penyahprotein dilaksanakan dengan hanya menggunakan 500 μl isipadu plasma yang mengandungi AM. Standard dalaman telah dimasukkan ke dalam plasma sebanyak 100 μl dan 2 ml asetonitril. Teknik pengekstrakan menggunakan cecair hanya memerlukan 100 μl isipadu plasma yang mengandungi AM. Diclofenac sodium 100 μl dan 100 μl larutan 20 mM ammonium asetat dengan pH 3.9 telah ditambah. Diklorometana digunakan sebagai larutan ekstrak untuk AM dan standard dalaman. Kedua-dua teknik ini telah disahkan dengan kaedah pengesahan bioanalitikal yang diperkenalkan oleh CDER. Bagi parameter pemilihan, kedua-dua teknik ini menunjukkan bahawa tiada kompaun lain terdapat di dalam kompaun AM dan standard dalaman. Graf kalibrasi dengan julat kepekatan antara 250 – 5000 ng ml^{-1} linear dan pekali penentuan (r^2) mencatatkan 0.990 bagi kedua-dua teknik. Had pengesanan adalah 40 ng ml^{-1} dan had kuantifikasi ialah 250 ng ml-1. Sampel kawalan kualiti terdiri daripada empat kepekatan berbeza iaitu LLOQ, 250 ng ml^{-1}, LOQ 400 ng ml^{-1}, MQC 2700 ng ml^{-1} dan HQC 4500 ng ml^{-1}. Ketepatan dan kejituan untuk kedua-dua teknik memenuhi kriteria yang ditetapkan oleh panduan yang disediakan oleh CDER. Kadar perolehan semula AM ialah 80 %. Had pengesanan untuk teknik ekstrak cecair untuk AM ialah 50 ng ml^{-1} dan had kuantifikasi ialah 250 ng ml^{-1}. Kadar perolehan semula AM untuk teknik ini ialah lebih daripada 90 %. Hasil kajian kestabilan AM bagi penyahprotein dan ekstrak cecair menunjukkan AM berkeadaan stabil bagi tempoh masa yang singkat, sewaktu proses beku dan cair, serta untuk tempoh masa yang lama. Kestabilan AM didapati lebih daripada 90 % untuk kesemua sampel kawalan kualiti.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion; it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Pharmaceutical Chemistry.

…………………………………
A.B.M. Helal Uddin
Supervisor

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for a degree of Master of Pharmaceutical Chemistry.

…………………………………
Bappaditya Chartterjee
Examiner

…………………………………
Tan Soo Choon
External examiner

This thesis was submitted to the Department of Pharmaceutical Chemistry and is accepted as a fulfilment of the requirement for the degree Master of Pharmaceutical Chemistry.

…………………………………
Siti Zaiton Mat So'ad
Head, Department of
Pharmaceutical Chemistry

This thesis was submitted to the Kulliyyah of Pharmacy and is accepted as a fulfilment of the requirement for the degree of Master of Pharmaceutical Chemistry.

…………………………………
Siti Hadijah Bt Samsudin
Dean, Kulliyyah of Pharmacy

DECLARATION

I hereby declare that this thesis is the result of my own investigation, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Huda Jamilah Mohamad

Signature…………………..…………. Date ….............................

INTERNATIONAL ISLAMIC UNIVERSITY MALAYSIA

DECLARATION OF COPYRIGHT AND AFFIRMATION OF FAIR USE OF UNPUBLISHED RESEARCH

CHROMATOGRAPHIC METHOD DEVELOPMENT FOR THE DETECTION OF MEFENAMIC ACID IN HUMAN PLASMA USING HPLC WITH UV-VIS DETECTOR

I declare that the copyright holder of this thesis are jointly owned by the student and IIUM

Copyright©2015 by Huda Jamilah Mohamad and International Islamic University Malaysia. All rights reserved.

No part of this unpublished research may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording or otherwise without prior written permission of the copyright holder except as provided below.

1. Any material contained in or derived from this unpublished research may be used by others in their writing with due acknowledgement.

2. IIUM or its library will have the right to make and transmit copies (print or electronic) for institutional and academic purposes.

3. The IIUM library will have the right to make, store in a retrieval system and supply copies of this unpublished research if requested by other universities and research libraries.

By signing this form, I acknowledge that I have read and understand the IIUM Intellectual Property Right and Commercialization policy

Affirmed by Huda Jamilah Mohamad

……..……..…………… ……………………..

Signature Date

To my beloved parents

Mak and ayah

Mohamad bin Hassan

Fatimah @ Maimun bt Yusof @ Jusoh

ACKNOWLEDGEMENTS

Firstly I thank Allah for giving me strength to complete this study. Without Your help and guidance, I am clueless in this research. Secondly, thank you to my dear mother Fatimah Yusof and father Mohamad bin Hassan for your motivation, worry and unconditional love towards me. Your strength in dealing in anything is my motivation to keep living.

A million thanks to my supervisor Assoc. Prof. Dr A.B.M Helal Uddin, for your patience in introducing research world to me. Your advice and all of your guidance will be kept as a guide for my future insyaAllah. May Allah reward more patience and strength in educate ummah for future.

Special thanks to my siblings Hazmi Aminuddin, Asiah Normah, Nor Sakinah, Nor Rashidah, Mohd Husni, Ahmad Rusydi, Ahmad Amin, Ismail Sabri and Nasibah, brothers and sister in laws, and niece and nephew for your indirect motivation for me to survive in this research world. You are all awesome for me. Alhamdulillah.

Also not forget to lecturers and staff of Department Pharmaceutical Chemistry, especially Sr Nurzihan Karim, Sr Fatimah, Sr Azura Yunos, Br Razif, Br Izaha, and Br Najib, may Allah easy your journey in life. Thanks a lot for helping a lot.

Staff of Department of Pharmacetical Technology, Sr Zaililah, Sy Haryanti and Br Dzadil, as well as staff of IKOP Sdn Bhd especially Sr Hanim Rabihah and Br Asri, my prayers always goes to you. Special thanks toast. Prof. Dr Juliana Jaffri, Asst. Prof. Dr Kausar and Assoc. Prof. Dr Farahidah for special support for this work.

To my lab mate, Mohamed Alaama and Noorsyafawati, I am very appreciate all your help and guidance. Your presence really colours the research world. May Allah will protect you both and your family.

Lastly, to my dear friends, Nadia, Hamizah, Izati, Syuhada, Farah, Fadilah, Hawa, Ain, Maryam, Huwaida, Abdurrahman, Reem, Ahmad Zaiter, Maryam Saadah, Asween Roweena, Kak Ida, Mohosina, Farya, Tg Faris, Anugerah and Anas Abdillah, Fahmi, Solahuddin, and Fahim, may have success in your life and thank you for the support throughout this study.

May Allah accept this journey of master degree; give sincerity, and benefits to other.

TABLE OF CONTENTS

Abstract ii
Abstract in Arabic iii
Abstrak in Bahasa iv
Approval Page v
Declaration vi
Copyright vii
Dedication viii
Acknowledgements ix
List of Tables xiv
List of Figures xv
List of Abbreviation xviii

CHAPTER ONE: INTRODUCTION 1
1.1 Background of Study 1
1.2 BIO Analysis and Its Role in Drug Development 1
1.3 Application of Bioanalysis 2
1.3.1 Pharmacokinetic Study and Bioavailability of Drugs 2
1.3.2 Bioequivalence of Drug Molecule 5
1.3.3 Therapeutic Drug Monitoring 6
1.3.4 Anti-Doping in Sport and Role of Bioanalysis 7
1.3.5 Forensic Science 8
1.3.6 New Drug Development and Bioanalysis 9
1.4 Generic Drugs 12
1.4.1 The Regulatory History of Generic Drug 13
1.5 Malaysia Drug Regulatory 14
1.6 Problem Statement 16
1.7 Hypothesis 17
1.8 Research Objectives 17

CHAPTER ONE: LITERATURE REVIEW 18
2.1 Introduction 18
2.2 Mefenamic Acid 19
2.3 Pharmacokinetics Parameters of Mefenamic Acid 20
2.4 High Performance Liquid Chromatography (HPLC) 20
2.4.1 Mobile Phase Resevoir 21
2.4.2 Solvent Delivery Systems (PUMP) 22
2.4.3 Sample Introduction in HPLC 22
2.4.4 Stationary Phase 23
2.4.5 Detectors 28
2.4.6 High Performance Liquid Chromatography (Separations) 30
2.4.6.1 Normal-Phase Chromatography 30
2.4.6.2 Reversed Phase Chromatography 31
2.4.7 Column Efficiency 31
2.4.7.1 Capacity Factor 32
2.4.7.2 Resolution 32

2.4.7.3 Peak Tailing 33
2.4.7.4 Theoretical Plate Number 35
2.5 Bioanalytical Method Development 36
2.6 Bioanalytical Sample Preparation 39
2.6.1 Protein Precipitation Technique 39
2.6.2 Liquid liquid extraction 40
2.6.3 Solid Phase Extraction 40
2.7 Bioanalytical Method Validation 41
2.7.1 Selectivity 42
2.7.2 Accuracy 43
2.7.3 Precision 43
2.7.4 Range 44
2.7.4.1 Calibration Procedure 45
2.7.5 Stability 46
2.7.5.1 Freeze and Thaw Cycle 47
2.7.5.2 Short-term Temperature Stability 47
2.7.5.3 Long-term Stability 47
2.7.5.4 Stock solution Stability 47
2.7.5.5 Post-preparative Stability 48
2.8 Analytical Technique for Detection of Mefenamic Acid 48
2.8.1 Drugs in Pharmaceutical Dosage Form 48
2.8.2 Analysis of MA in Different Biological Matrix 51
2.8.3 Analytical Method for Internal Standard Candidates 58
2.8.4 Study of MA and Other Drugs Using Instruments 62

CHAPTER THREE: METHODOLOGY 69
3.1 Introduction 69
3.2 Chemicals and Reagents 69
3.3 Drug Free Plasma 70
3.4 Instruments 70
3.5 Glassware 71
3.6 Treament of Glassware 72
3.7 HPLC Consumables 72
3.8 Buffer Preparation 72
3.8.1 Preparation of Triethylamine (TEA) Buffer 73
3.8.2 Preparation of 20mM Ammonium Acetate Buffer 73
3.8.3 Preparation of 10mM Phosphoric Acid Buffer 74
3.8.4 Preparation of 1M Phosphoric Buffer 74
3.8.5 Preparation of 0.1M of Glacial Acetic Acid Buffer 74
3.8.6 Preparation of 50mM Potassium Phosphate Buffer 74
3.9 Preparation of Protein Precipating Agent 74
3.9.1 Preparation of 10% v/v of Trichloroacetic Acid 75
3.9.2 Preparation of 6% v/v of Perchloric Acid 75
3.10 Optimisation of HPLC Parameters for Analysis of MA 75
3.11 HPLC Column Maintenance 77
3.12 Optimisation of Chromatographic Condition for MA Standard and Internal Standard 77
3.12.1 Preparation of Standard Stock Solution 78
3.13 Sample Preparation for HPLC Analysis using Spiked MA Plasma 79

3.13.1 Protein Precipitation Techniques 79
3.13.2 Liquid Liquid Extraction Technique 81
3.14 Bioanalytical Method Validation (Protein Precipation Technique And Liquid Liquid Extraction Technique 83
3.14.1 Preparation of Sample for Bioanalytical Method Validation Following CDER Guideline 83

CHAPTER FOUR: RESULTS AND DISCUSSIONS 88
4.1 Introduction 88
4.2 Optimisation of the Chromatographic Method for Detection of MA in Spiked Plasma 89
4.2.1 Chromatographic Stationary Phase (Column) 89
4.3 Optimum UV Wavelength 90
4.4 Optimisation of Mobile Phase 91
4.4.1 Acetonitrile with Phosphoric Acid Buffer (60:40 v/v) 91
4.4.2 Acetonitrile with Ammonium Acetate Buffer pH 3.5 (60:40 v/v) 92
4.4.3 Acetonitrile and 50mM Phosphate Buffer pH 3.5 (60:40 v/v) 93
4.4.4 Acetonitrile and Trimethylamine (TEA) (60:40 v/v) 94
4.5 Screening of Internal Standard 97
4.5.1 Diclofenac Sodium 98
4.5.2 Indomethacin 100
4.5.3 Flufenamic Acid 101
4.6 Sample Preparation Techniques for Mefenamic Acid Drug in Huma Plasma 102
4.6.1 Chromatographic Method Development for Protein Precipitation Technique 102
4.6.1.1 Acid as Protein Precipita Reagent 102
4.6.1.2 Organic Solvent as Protein Precipitant Agent 103
4.6.2 Chromatographic Method Development for Liquid Liquid Extraction 106
4.6.2.1 Types of Solvent 106
4.6.2.2 Buffer 108
4.7 Method Validation for Protein Precipitation Technique 112
4.7.1 Selectivity 113
4.7.2 Linearity 114
4.7.3 Limit of Detection (LOD) and Lower Limit of Quantitation (LOQ) 117
4.7.4 Precision and Accuracy 117
4.7.5 Recovery 120
4.7.6 Stability 121
4.8 Method Validation for Analysis of Spiked MA in Plasma Sample by Liquid Liquid Extraction Technique 123
4.8.1 Selectivity 123
4.8.2 Linearity 125
4.8.3 Limit of Detection (LOD) and Limit of Quantification (LOQ) 127
4.8.4 Accuracy and Precision 127
4.8.5 Recovery 130
4.8.6 Stability 130

CHAPTER FIVE: CONCLUSIONS **133**
5.1 Future Study 137

REFERENCES **138**

APPENDIX I **PEAK PURITY OF MA AND DICLOFENAC SODIUM WITH DIFFERENT BUFFER** **148**
APPENDIX II **EXAMPLE OF LINEARITY** **154**
APPENDIX III **LIMIT OF DETECTION CHROMATOGRAM** **156**
APPENDIX IV **SYSTEM SUITABILITY TESTING** **157**

LIST OF TABLES

Table 3.1	Precipitating agent used for protein precipitation technique and volume added.	80
Table 4.1	System suitability parameters (capacity factor, resolution and tailing factor) for different mobile phase.	96
Table 4.2	Recovery of MA using different volume of acetonitrile	104
Table 4.3	Recovery of MA using different volume of methanol	104
Table 4.4	The recovery of MA and IS using phosphate buffer	109
Table 4.5	The recovery of MA with different concentration of ammonium acetate buffer	110
Table 4.6	The recovery of MA with different pH of buffer.	111
Table 4.7	Intraday and interday accuracy of MA spiked plasma samples that underwent protein precipitation technique.	118
Table 4.8	Intraday and interday precision of MA spiked plasma samples that underwent protein precipitation technique	118
Table 4.9	Intraday and interday retention time for MA spiked plasma in protein precipitation technique	119
Table 4.10	Recovery (%) results for every QC concentration (n=8)	120
Table 4.11	Results of different stability scheme of MA spiked plasma samples stored at -400C	121
Table 4.12	Accuracy for intraday (n=8) and interday (n=6) of MA spiked plasma sample by liquid liquid extraction technique	128
Table 4.13	Intraday (n=8) and interday (n=6) precision of MA spiked plasma sample by liquid liquid extraction technique	129
Table 4.14	Retention time for intraday and interday spiked MA samples for liquid liquid extraction technique.	129
Table 4.15	Recovery (%) of MA for liquid liquid extraction technique	130
Table 4.16	Results of different stability programme conducted for spiked MA plasma sample	131

LIST OF FIGURES

Figure 1.1	Single compartment model of plasma drug concentration time curves	4
Figure 1.2	Timeline of drug discovery	9
Figure 1.3	Timeline of generic drugs	13
Figure 2.1	Chemical structure of mefenamic acid	19
Figure 2.2	Schematic diagram of HPLC (Robards et al, 2002)	21
Figure 2.3	Silanols and siloxane bridges.	24
Figure 2.4	Chlorosilane reaction with the silanols on the silica	25
Figure 2.5	Example of reaction of silica surface with hexamethyldisilazane.	26
Figure 2.6	Example of silane with functional groups.	26
Figure 2.7	Hydrocarbon bonded phase	27
Figure 2.8	Another example for normal phase bonded silica.	28
Figure 2.9	Schematic diagram of system suitability parameters (Synder et al, 2010)	32
Figure 2.10	Separation peaks as indicated by Rs values (CDER, 1994)	33
Figure 2.11	Definitions of peak asymmetry and tailing factors (As and TF) (Snyder et al., 2010)	34
Figure 2.12	Peak shape as a function of asymmetry (As) and tailing (TF) factors (Snyder et al, 2010).	34
Figure 2.13	Measurement of peak width and height of chromatogram (Snyder et al, 2010).	35
Figure 2.14	HPLC Agilent 1100 Series	38
Figure 3.1	Drug free plasma obtained from Hospital Tengku Ampuan Afzan	70
Figure 3.2	Schematic diagram of HPLC method of MA	81
Figure 4.1	UV Spectra of MA Standard	90
Figure 4.2	UV spectra of diclofenac sodium (IS) standard	90

Figure 4.3 Spectral of MA at the liquid liquid extraction analysed by HPLC by using combination of phosphoric acid and acetonitrile mobile phase (40:60 v/v pH 3.2). 92

Figure 4.4 Retention time of MA using TEA buffer with similar pH and different concentration. 94

Figure 4.5 Retention time of MA from same concentration of TEA buffer but with different pH. 95

Figure 4.6 Chromatogram of internal standard candidates ; flufenamic acid (--), indomethacin (--), and diclofenac sodium (--). 97

Figure 4.7 Structure of ibuprofen 98

Figure 4.8 Structure of salicylic acid 98

Figure 4.9 Structure of diclofenac sodium 99

Figure 4.10 Peak purity of internal standard candidates ; indomethacin, flufenamic acid and diclofenac sodium 100

Figure 4.11 Structure of indomethacin 100

Figure 4.12 Structure of flufenamic acid 101

Figure 4.13 MA spiked plasma with methanol as organic solvent 105

Figure 4.14 MA spiked plasma with acetonitrile as organic solvent. 105

Figure 4.15 Chromatograms of three different extracting solvent for MA in spiked plasma. 107

Figure 4.16 The chromatogram of 6 different blank plasma that underwent protein precipitation technique with IS and MA. 113

Figure 4.17 The chromatogram of MA in standard ( ) and spiked plasma ( ). 114

Figure 4.18 Chromatogram of spiked plasma samples of six different calibration concentration prepared by protein precipitation technique 115

Figure 4.19 Chromatogram of spiked plasma samples of six different calibration concentration prepared by protein precipitation technique. 116

Figure 4.20 Linear calibration curve of MA spiked plasma samples using protein precipitation techniques 116

Figure 4.21 Chromatogram of six different drug free plasma prepared by liquid liquid extraction technique 124

Figure 4.22 Selectivity for spiked plasma 5000 ng ml-1 MA and its corresponding standard, 2500 ng ml-1 MA 124

Figure 4.23 Chromatograms from three different spiked plasma, drug free plasmazero blank ( ), and plasma spiked with MA ( ) and internal standard 125

Figure 4.24 Compilation of the chromatograms of 6 different concentrations ranging from 250 – 5000 ng ml-1 of MA with 2000 ng ml-1 IS 126

Figure 4.25 Example of a linear calibration curve of MA spiked plasma sample using liquid liquid extraction technique 127

LIST OF ABBREVIATION

ANDA	Abbreviated new drug applications
BDS	Base deactivated silanol
BE	Bioequivalence
BSTFA	Bis(trimethylsilyl)trifluoroacetamide
C 18	Column 18
CAD	Collision activated dissociation
CDER	Center for Drug Evaluation and Research
CV	Coefficient of variation
CZE	Capillary zone electrophoresis
DCA	Drug Control Authority
EMA	European Medicines Agency
FDA	Food and Drug administration
FEP	Flourinated ethylene-propylene
GC	Gas Chromatography
GC-MS	Gas chromatography mass spectrometry
GMP	Good Manufacturing Practice
HCl	Hydrochloride
HIV	Human Immunodeficiency Virus
HPLC	High Performance Liquid Chromatography
HPLC MS	High performance liquid chromatography mass spectrometry
HQC	High Quality Control
i.d	Internal diameter
ICH	International Conference of Harmonization
ITP	Isotachophoretic
kDa	Kilo dalton
KH_2PO_4	Potassium dihydrogen phosphate
LC MS MS	Liquid chromatography with tandem mass spectrometry
LLOQ	Lower limit of quantification
LQC	Low Quality Control

MA	Mefenamic Acid
MADRAC	Malaysian Adverse Drug Reactions Advisory Committee
MCR-ALS	Multivariate curve resolution coupled to alternative least squares
MeOH	Methanol
MQC	Medium Quality Control
MS/MS	Mass spectrometry / mass spectrometry
MTBE	Methyl tert-butyl ether
NACE	Non-aqueous capillary electrophoresis
NaH_2PO_4	Sodium dihydrogen phosphate
NaOH	Sodium hydroxide
NDA	New Drug Application
NMR	Nuclear Magnetic Resonance
NPCB	National Pharmaceutical Control Bureau
NSAIDs	Non-steroidal Anti-inflammatory drugs
O.D	Outer diameter
ODS	Octa Decyl Silane
PAD	Photo array detector
QC	Quality control
RP-HPLC	Reversed phase high performance liquid chromatography
Rpm	Revolutions per minute
RSD	Relative standard deviation
SAX	Strong anion exchanger
SDS	Sodium dodesyl sulfate
SPE	Solid phase extraction
TDM	Therapeutic Drug Monitoring
TEA	Triethylamine
TLC	Thin Layer Chromatography
TPAB	Tetra-pentyl ammonium bromide
US	United States
USD	United States Dollar
USP	United States Pharmacopoieal
UV	Ultraviolet

UV-vis	Ultraviolet visible
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF STUDY

1.2 BIOANALYSIS AND ITS ROLE IN DRUG DEVELOPMENT

Bioanalysis is the quantitative determination of drugs and their metabolites in biological fluid (Wells, 2003). Examples of biological fluids are blood, plasma, urine and tissues. It is different from the conventional analysis. Inherent complex characteristics of the bio-matrices make it more complicated compared to conventional analysis. The challenge lies in the removal of the matrix interference that limits the detection of analyte. Recently, the term bioanalysis is also used to define analytical techniques applied in the quantification and characterization of biologicals (Hill, 2009). This includes enzymes, proteins, monoclonal antibodies, cytokines and vaccines (Mikkelsen and Corton, 2004). In bio-analytical method validation guidelines, there are two types of bio-analysis discussed in two different categories, namely the chemical method and the ligand binding assay method (Center for Drug Evaluation and Research (CDER) and European Medicines Agency (EMA)). For this study, the scope was mainly focused on the bioanalysis in a biomatrix, namely plasma. Bioanalysis plays an important role in the field of medicine and pharmacy. These are closely related to human wellbeing. Primarily, it is instrumental in new drug discovery and the development to construct the toxicological, bioequivalence, bioavailability, pharmacokinetic, as well as therapeutic drug monitoring studies. Moreover, fields like forensic and doping in sports largely depend on bioanalysis to produce reliable results.

1.3 APPLICATION OF BIOANALYSIS

1.3.1 Pharmacokinetic Study and Bioavailability of Drugs

Pharmacokinetics of drug molecule is defined as the movements of drugs inside the body. It involves the absorption, distribution, metabolism and excretion of drugs from the body (Jambhekar and Breen, 2012). In drug development, pharmacokinetics profile of the drug molecule is very critical. It helps to evaluate the preclinical toxicity testing and animal pharmacology as well as to decide the appropriate dosing for pivotal phase III study of efficacy (Rang, Dale, Ritter and Flower, 2007). In absorption, the drug must pass through the cell membrane to gain access to the cell, though this process depends on the chemical properties of the drugs. At the cellular level, membrane layer consists of lipid materials which allow lipophilic drugs to be easily absorb to the membrane. However, if the drug is in an ionized form, it will modify according to the environment of stomach and gastrointestinal gut. If the drug is in an amphipathic form, whereby it possesses both lipophilic and hydrophilic properties, the absorption of this compound will be much easier. Few drugs can be absorbed by using active transport available in the membrane layer (Galbraith, 2001). After absorption, drugs enter into the blood circulation. The environment of the blood circulation is in aqueous medium. There are several mechanisms that allow transportation of drugs in the blood, which is referred as drug distribution. Protein binding is one mechanisms of drug transportation (Katzung, Masters and Trevor, 2009). If the molecules of the drugs are hydrophobic, they can easily attach themselves to blood proteins, also known as protein bound. Proteins have amphiphatic characteristics as they can render both lipophilic and lipophobic molecule. The protein-bound drug must be in equilibrium with the free drug inside the plasma. Proteins will stay inside the bloodstream and never enter the tissue. For the drugs to

act on the target tissues, they have to be in a free drug form. This equilibrium shift will play an important role in distributing the drugs to the body. Liver plays an important role in drug distribution where after absorbing the drug from the gastrointestinal tract, the hepatic portal system will take it and carry it to the liver. The liver will metabolize the drugs before distributing them to the rest of the body. Such drug is said to have a high hepatic first pass. Knowledge about volume of distribution is important; the approximate volume of plasma in an average 70 kg adult is 3 L. When the drug is administered orally, the plasma is measured to get the concentration of drug in the blood. However, the concentration of drugs in the plasma is not same as the dose given. This is because the drugs can diffuse from blood to the tissues. The distribution of a drug inside a human body can be calculated. To calculate this, a known amount of drugs is injected intravenously, and if the drug is able to stay within the plasma, the amount of drug is equivalent to the drug dissolved in litters of liquid. If the drug is distributed in the body, the concentration will be equivalent to the drug dissolved in 40 L, which is the total volume in the body's fluid compartments. Drug metabolism can detoxify the drugs and also change the drugs to its active form. Besides that, metabolism of drugs will change the drugs to be more hydrophilic so that they will not be re-absorbed into the system during the excretory process. In an excretion process, the majority of drugs are excreted either as the same molecule as they were administered, or as metabolites in the urine or bile. For renal drug excretion, it involves three processes which are glomerular filtration, active tubular secretion and passive diffusion across tubular epithelium. Furthermore, drug can leave the body other natural routes, such as saliva, sweat, tears and breathe (Galbraith, 2009; Rang et al., 2007).

Bioavailability is defined as the unchanged drug that reaches the systemic circulation by any route of administration. It is measured by the measure of area under the blood concentration-time curve (AUC), as shown in Figure 1.1. Different route of administration such as intramuscular, intravenous and rectal administration will give different bioavailability (Katzung et al., 2009). Half-life, denoted as $t^{1/2}$, can be defined as the time required to change the drug in the body by one-half during elimination. The parameters in pharmacokinetics that can be used to measure bioavailability are absorption and liver pass effect (Galbraith, Bullock, Manias, Hunt and Richards, 2007).

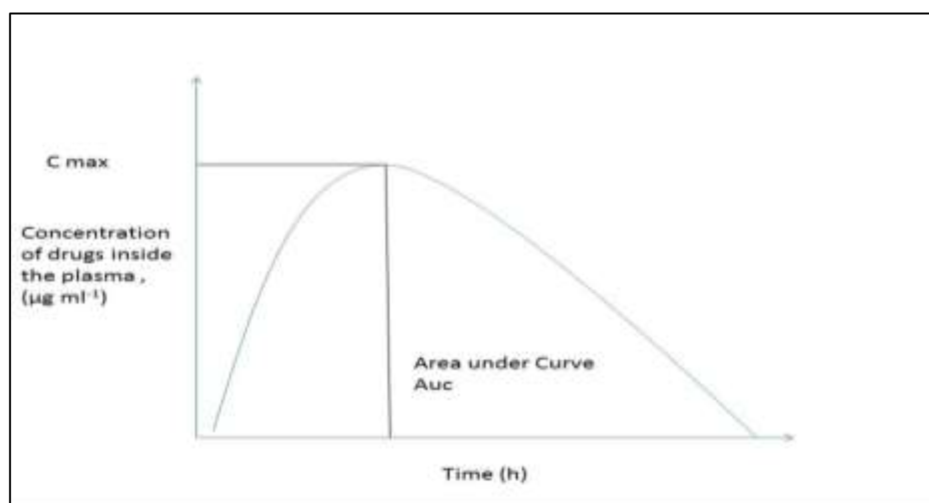

Figure 1.1 Single compartment model of plasma drug concentration time curves

Drug preparation is not the only factor that influences bioavailability. Differences in enzyme activity of gut wall or liver as well as the pH of gastric or intestinal motility can also affect bioavailability (Rang et al., 2007).

Understanding of pharmacokinetic and bioavailability of a drug gives a clue on how to quantitate the drugs in human body. Accordingly, bioanalysis knowledge is