

**EVALUATION OF THE OXIDATIVE  
METABOLITES OF ARACHIDONIC ACID AND  
THEIR IMPLICATION IN THE PROGRESSION OF  
INFLAMMATORY DISEASES: HYPERTENSION  
AND RHEUMATOID ARTHRITIS**

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By

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## ABSTRACT

Inflammation is implicated in diseases such as hypertension and rheumatoid arthritis (RA). A mechanistic understanding of inflammatory processes as it relates to the disease state and injury needs to be developed. Specifically, the role and modulation of inflammation needs to be assessed, as well as the mechanism that produces arachidonic acid (AA) metabolites (eicosanoids). Eicosanoids are specific biomarkers of inflammation. Their biosynthesis from arachidonic acid can be catalyzed by either free radicals or enzymes such as lipoxygenases (LOX), cyclooxygenase-2 (COX-2) and cytochrome P450. Depending on the pathway or parent molecule, different distributions of eicosanoids are found. The oxidation of AA gives hydroxyeicosatetraenoic acids (HETEs), dihydroxyeicosatetraenoic acids (DHETEs), epoxyeicosatetraenoic acids (EETs), prostaglandins (PGs), isoprostanes (Isops) and thromboxanes (TXs). It is our hypothesis that AA metabolites will help in understanding the progression of inflammatory diseases. To confirm this hypothesis, analytical methods including HPLC-UV and LC-MS were developed.

The developed and validated HPLC method was applied to study the effect of acute exercise on prostanoids in hypertensive African American subjects. It was our theory that urinary 6-keto  $\text{PGF}_{1\alpha}$  and 11-dehydro  $\text{TXB}_2$  can be used to assess the role of exercise in hypertension. Moreover, we assume that 8-iso  $\text{PGF}_{2\alpha}$  levels can be used as an indicator to determine the relationship of oxidative stress and endothelial dysfunction in hypertension. The HPLC method involved separating urinary 8-iso  $\text{PGF}_{2\alpha}$ ,  $\text{PGE}_2$ ,  $\text{PGD}_2$ ,  $\text{PGF}_2$ , 6-keto  $\text{PGF}_{1\alpha}$  and 11-dehydro  $\text{TXB}_2$  on a SymmetryShield Rp18 column (250mm

× 4.6mm) by an isocratic elution of 17 mM phosphoric acid and acetonitrile in the ratio of 65:35 and at a flow rate of 1.3 ml/min. The wavelength used for detection was 196 nm. Specificity was confirmed by LC-MS. The method was fully validated and was found to be having sufficient sensitivity (limit of quantification - 7.5 ng - 30 ng) for many biological matrices and applications. The accuracy and precision were within bioanalytical method validation limits (90.3 to 112.8 % and RSD < 10%, respectively) and the method was linear over three orders of magnitude. In addition, a HPLC-UV method for the simultaneous determination of urinary creatinine and prostanoids was also developed and validated as it is necessary to monitor creatinine levels in addition to biomarkers when the measurement is done in urine. The method was found to be linear over three orders of magnitude and is sensitive enough for the analysis of creatinine and prostanoids in urine. The advantage of this method was that one can determine the levels of these prostanoids normalized by urinary creatinine in a single analysis and in less than 17 min.

The LC- ESI (electrospray ionization) MS method, on the other hand was used to determine the role of HETEs in the initiation, progression and resolution phases of inflammation in RA. It is our assumption that 12/15 HETE can be used as novel targets for the treatment of RA. The separation was performed on a C<sub>18</sub> column using a gradient elution of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The flow rate was 1 ml/min and the run time was 75 mins. The method was found to be specific, sensitive and precise. This LC-MS method was also used to develop a retention model for complex regioisomers. Quantitative structure- (chromatographic) retention relationship (QSRR) was used to develop a predictive retention model for fatty acid

metabolites. Retention behaviors of the lipid biomarkers were characterized by application of QSRR analysis utilizing Austin Model 1 mode semi-empirical computations. The retention data of these fatty acids were obtained from an RP-HPLC method utilizing a Symmetry C<sub>18</sub> column under gradient elution. Molecular descriptors that take into account the polarity; chemical reactivity and hydrophobicity of the analytes were calculated using the semi-empirical AM1 mode. It is our hypothesis that QSRR will give insight into molecular mechanism of separation of lipid biomarkers operating in a given chromatographic system and can predict retention of a new analyte and/or to identify unknown analytes.

## ACKNOWLEDGMENTS

More than 300 years ago, Issac Newton is thought to have famously remarked, “*If I have seen a little further it is by standing on the shoulders of Giants.*” How true this statement is even today.

I would like to thank all the people who have contributed to make me realize this dream. This journey would not have been possible without the assistance and support of innumerable people. This is my humble attempt to pay homage to some of them.

Firstly, I would like to express sincere gratitude to my research advisor, mentor and friend, Dr. Susan Jansen Varnum. Her understanding, logical ways of thinking and personal guidance have been instrumental in my research accomplishments. I have learnt from her, both consciously and subconsciously, how to be a good researcher and a student of the subject. She is also a prime example of how to balance a successful academic career and manage a household. Being a woman, I can appreciate how difficult and challenging that can be. I admire and respect what she has achieved and it will be a constant source of inspiration for me.

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some day...Or is it the other way around...Don't worry.... I can match the sport to the team!

Group members Anthony and Michael who were great resources in my early and my last days at Temple University respectively. I had some good healthy discussions with both of them.

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To the various collaborators through the years, who have shared ideas and work with me, to the faculty members at Temple University and my school, college and University in India. I am here today because of what they all have taught me. I will forever be indebted to them. I would also like to thank all the support staff in the Chemistry Department at Temple University.

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Lastly and most importantly, I want to thank my husband, for having confidence in me and seeing me through this and standing strong besides me through difficult times and my beautiful daughter who is the greatest joy of my life and makes it all worthwhile.

## **DEDICATION**

To my family particularly my husband, Harish and my daughter, Ananya

## TABLE OF CONTENTS

ABSTRACT .....	iii
ACKNOWLEDGMENTS .....	vi
DEDICATION .....	ix
LIST OF TABLES .....	xiv
LIST OF FIGURES .....	xvii
CHAPTER	
1. FATTY ACIDS AND THEIR METABOLIES AS BIOMARKERS OF INFLAMMATION .....	1
1.1 Inflammation .....	1
1.2 Polyunsaturated Fatty Acids (PUFA's) .....	2
1.3 Arachidonic Acid Metabolism.....	5
1.3.1 COX-2 Metabolites (Prostaglandin Synthases).....	5
1.3.2 Free Radical per-oxidation .....	10
1.3.3 LOX metabolites.....	11
1.3.4 Cytochrome (CYP) 450 metabolites.....	15
1.4 EPA and DHA Metabolites .....	16
1.5 Structures Of Lipid Biomarkers.....	18
1.6 References.....	22
2. ANALYTICAL TECHNIQUES AND INSTRUMENTATION.....	30
2.1 Introduction .....	30
2.2 Liquid Chromatography and Its Underlying Principles .....	30
2.3 Reverse Phase Hplc .....	32
2.4 Ultra High Pressure Liquid Chromatography (UHPLC).....	34
[Reproduced from Bioanalysis, December 2010, Vol 2, No.12, Pages 2019-2034 with Permission of Future Science Ltd] .....	34
2.4.1 Theory.....	38
2.4.2 System design.....	41
2.5 LC-MS (Liquid Chromatography-Mass Spectrometry) .....	43
2.5.1 Principles of mass spectrometry .....	43
2.5.2 Ion Source: Electrospray Ionization (ESI) .....	44
2.5.3 Mass analyzer: Single quadrupole analyzer.....	45
2.5.4 Ion Detector: Electron Multiplier (EM).....	46
2.5.5 Sample preparation: Solid phase extraction (SPE).....	46
2.6 References.....	48
3. DEVELOPMENT OF AN HPLC-UV METHOD FOR THE DETERMINATION OF PROSTANOIDS IN URINE MATRIX.....	50
3.1 Introduction .....	50

3.2. Experimental.....	51
3.2.1. Chemicals and Materials.....	51
3.2.2. Instruments and operating conditions.....	51
3.2.3. Preparation of standard solutions and quality control (QC) samples .....	52
3.2.4 Urine sample preparation.....	52
3.2.5 Method Validation.....	52
3.3 Results And Discussion.....	55
3.3.1 Chromatography and UV detection.....	55
3.3.2 Method validation .....	55
3.4 Conclusion.....	62
3.5 References.....	67
4. DEVELOPMENT OF AN HPLC-UV METHOD FOR THE SIMULTANEOUS DETERMINATION OF CREATININE AND PROSTANOIDS.....	69
4.1 Introduction.....	69
4.2. Experimental.....	71
4.2.1. Chemicals and Materials.....	71
4.2.2. Instruments and operating conditions.....	71
4.2.3. Preparation of standard solutions and quality control (QC) samples .....	72
4.2.4 Urine sample preparation.....	72
4.2.5 Method validation .....	72
4.2.6 ELISA Method (Jaffe Method) for creatinine determination.....	74
4.3 Results and Discussion .....	75
4.3.1 Method Validation.....	78
4.4 Conclusion.....	87
4.5. References .....	88
5. DEVELOPMENT OF A LC-MS METHOD FOR THE DETERMINATION OF LIPID BIOMARKERS IN BIOLOGICAL TISSUES.....	90
5.1 Introduction.....	90
5.2. Experimental.....	91
5.2.1 Materials .....	91
5.2.2 LC-MS method and conditions.....	92
5.2.3 Sample preparation.....	94
5.2.4 Selectivity, sensitivity and system suitability.....	94
5.2.5 Quantitation of the lipid biomarkers .....	95
5.3. RESULTS AND DISCUSSION.....	95
5.3.1 Chromatography.....	95
5.3.2 Selectivity, sensitivity and system suitability.....	96
5.4. Conclusion.....	99
5.5 References.....	100
6. QSRR PREDICTION OF CHROMATOGRAPHIC RETENTION BEHAVIOR OF POLYUNSATURATED FATTY ACIDS FROM SEMI-EMPIRICALLY COMPUTED MOLECULAR DESCRIPTORS .....	103

6.1 Introduction .....	103
6.2 Retention Modeling In Rp Hplc.....	104
6.2.1. Thermodynamic-based models.....	104
6.2.2 Molecular-interaction models-Quantitative structure retention relationship (QSRR).....	107
6.3 Experimental.....	110
6.3.1 Chemicals and Materials.....	110
6.3.2 LC-MS method and conditions.....	110
6.3.3 Sample preparation procedure.....	111
6.3.4 QSRR analysis and molecular descriptors .....	111
6.3.5 Statistical Analysis.....	112
6.3.6 Identification of unknown analyte .....	112
6.4 Results And Discussion.....	112
6.5 Conclusion .....	127
6.6 References.....	140
<b>7. COMPARISON OF AN ULTRA HIGH PRESSURE LIQUID CHROMATOGRAPHY-UV METHOD WITH A CONVENTIONAL HPLC-UV METHOD FOR THE SIMULTANEOUS DETERMINATION OF PROSTANOIDS</b>	
“Are the methods equivalent in terms of their accuracy and precision?” .....	142
7.1 Introduction .....	142
7.2 Experimental .....	144
7.2.1 Materials .....	144
7.2.2 Instrumentation.....	145
7.2.3 Chromatographic conditions.....	145
7.2.4 Standard Solutions .....	146
7.2.5 Validation .....	146
7.2.6 Matrix Effect.....	147
7.2.7 Statistical methods .....	147
7.3 Results.....	148
7.3.1 Validation .....	148
7.3.2 Matrix effect .....	155
7.3.3 Comparison Studies.....	155
7.4 Conclusion.....	167
7.5 References.....	169
<b>8. DETERMINING THE ROLE OF URINARY PROSTANOIDS IN HYPERTENSIVE MIDDLE-AGED AFRICAN AMERICAN POPULATION FOLLOWING ONE BOUT OF EXERCISE.....</b>	
8.1 Introduction .....	171
8.2 Protocol.....	174
8.2.1 Participants.....	174
8.2.2 Urine collection .....	175
8.2.3 Acute Exercise Session .....	175
8.2.4 Statistics .....	176

8.3 RESULTS .....	176
8.4 Discussion .....	180
8.5 Conclusion .....	183
8.6 Acknowledgment.....	184
8.7 References.....	185
<b>9. EVALUATION OF THE ROLE OF LOX METABOLITES OF ARACHIDONIC ACID IN THE PROGRESSION OF RHEUMATOID ARTHRITIS .....</b>	<b>189</b>
9.1 Introduction .....	189
9.2 Protocol.....	192
9.2.1 Animal studies and Induction of Arthritis.....	192
9.2.2 Sample preparation.....	192
9.2.3 Statistics .....	193
9.3 RESULTS .....	193
9.4 Discussion.....	194
9.5 Conclusion .....	202
9.6 Acknowledgment.....	203
9.7 References.....	204
<b>BIBLIOGRAPHY.....</b>	<b>208</b>
<b>GLOSSARY OF TERMS .....</b>	<b>235</b>
<b>ABBREVIATIONS .....</b>	<b>238</b>
<b>APPENDIX A .....</b>	<b>241</b>
<b>DEVELOPMENT OF AN HPLC-UV METHOD FOR THE DETERMINATION OF SEX HORMONES IN BIOLOGICAL MATRICES .....</b>	<b>241</b>
A.1 Introduction .....	241
A.2. Experimental.....	243
A.2.1. Chemicals and Materials .....	243
A.2.2. Instruments and operating conditions.....	243
A.2.3. Preparation of standard solutions and quality control (QC) samples .....	244
A.2.4 Sample preparation- Solid phase extraction .....	244
A.2.5 Method Validation.....	245
A.3.1 Chromatography.....	246
A.3.2 Method validation .....	246
A.4 Conclusion.....	255
A.5 References.....	256
<b>APPENDIX B: COPYRIGHT CLEARANCE CERTIFICATES .....</b>	<b>258</b>

**LIST OF TABLES**

Table	Page
2.1 List of commercially available UHPLC instruments with their operating pressures .....	37
3.1 Method Validation Results: Selectivity.....	59
3.2 Method Validation Results: Sensitivity & Linearity.....	60
3.3 Method Validation Results: Accuracy.....	63
3.4 Method Validation Results: Intra assay and inter assay precision.....	64
3.5 Method Validation Results: Stability study of 8-iso PGF <sub>2α</sub> in mobile phase.....	65
3.6 Method Validation: Stability studies in mobile phase.....	66
4.1 Method Validation Results: Selectivity.....	79
4.2 Method Validation Results: Sensitivity and Linearity.....	79
4.3 Method Validation Results: Accuracy levels.....	80
4.4 Method Validation Results: Spiked Recovery. ....	80
4.5 Method Validation: Instrument Precision Intra-Assay and Inter-Assay precision.....	82
4.6 Method Validation: Stability studies.....	82
4.7 Comparison data for creatinine levels in urine obtained from HPLC-UV and ELISA.....	84
4.8 Comparison data for % spiked recovery of creatinine from standard solutions and urine matrix. “Urine Blank” is the urine from a normotensive patient.....	85

5.1	SIM time gradient for LC-MS analysis.....	93
5.2	Internal standards used for quantifying these lipid biomarkers.....	96
5.3	Retention times and the m/z charge ratio for all lipid biomarkers.....	98
6.1	Partition theory: Clog P for all lipid biomarkers.....	115
6.2	Adsorption theory: Dipole moment and polarizability of all lipid biomarkers.....	117
6.3	Hydrophobic theory: Solvation energies of all lipid biomarkers.....	121
6.4	Chemical Reactivity: HOMO and LUMO energies of all lipid biomarkers.....	128
6.5	Coefficients of the molecular descriptors.....	136
6.6	% Accuracy using individual molecular descriptors for all lipid biomarkers.....	137
6.7	% Accuracy using individual molecular descriptors for all lipid biomarkers.....	138
6.8	Values of molecular descriptors for possible candidates of an unknown HETE at RRT of 31.58 min.....	139
7.1	Method Validation Results: Sensitivity and Linearity- Eq. of the line with the weighting factors.....	153
7.2	Method Validation Results: Recovery Accuracy.....	154
7.3	Matrix effect.....	156
7.4	Comparison of Precision: p values (F-test) (2-tailed).....	157
7.5	Comparison of Accuracy: p values (T-test) (2-tailed).....	160

7.6	Values for slope, intercept, 95% CI and r obtained from Ordinary Linear Regression analysis.....	161
7.7	Values for $S_{y/x}$ and $S_{a\ tot}$ from Ordinary linear regression analysis.....	162
7.8	Values for slope and intercept obtained from Deeming regression analysis.....	165
7.9	Summary of the statistical analysis.....	168
8.1	Average urinary levels of 6-keto PGF1 $\alpha$ and 11-dehydro TXB2 in terms of ng/ $\mu$ g of creatinine.....	178
8.2	Average urinary levels of 8-iso PGF2 $\alpha$ in terms of ng/ $\mu$ g of creatinine.....	179
8.3	Ratio of urinary levels of 6-keto PGF1 $\alpha$ /creatinine and 11-dehydro TXB2 /creatinine.....	182
A.1	Method Validation Results: Selectivity.....	248
A.2	Method Validation Results: Sensitivity & Linearity.....	252
A.3	Method Validation Results: Accuracy.....	253
A.4	Method Validation Results: Intra assay and inter assay precision.....	254

## LIST OF FIGURES

Figure	Page
1.1 Chemical structure of Polyunsaturated fatty acids.....	2
1.2 Biosynthesis of $\omega$ -6 and $\omega$ -3 PUFA's.....	4
1.3 Oxidative pathway of Arachidonic Acid by COX-2 enzyme.....	8
1.4 Oxidative pathway of Arachidonic Acid by LOX enzymes.....	12
1.5 Oxidative pathway of Arachidonic Acid by CUP450 enzymes.....	17
3.1 Structure of prostanoids.....	57
3.2 Representative chromatogram of blank solvent and standard solution of prostanoids.....	58
4.1 Structure of creatinine.....	76
4.2 Representative chromatogram of creatinine and prostanoids in solvent and urine matrix.....	77
4.3 Bland Altman plot comparing the HPLC-UV method and the Jaffe method for creatinine measurement.....	86
5.1 Representative LC-MS chromatogram of all lipid biomarkers using SIM as defined in Table 5.1.....	97
6.1 Number of papers published from 1990 to 2011 in the field QSRR.....	109

6.2	Partition theory: Clog P vs. RRT (min).....	116
6.3	Adsorption theory: Dipole moment vs. RRT (min).....	119
6.4	Adsorption theory: Polarizability vs. RRT (min).....	119
6.5	Hydrophobic theory: Solvation energy vs. RRT (min).....	122
6.6	Chromatographically determined RRT (min) vs. theoretically determined RRT (min) for 41 lipid biomarkers.....	123
6.7	Chemical reactivity: LUMO energies vs. RRT (min).....	129
6.8	Chromatographically determined RRT (min) vs. theoretically determined RRT (min) for 41 lipid biomarkers.....	130
6.9	Change in % acetonitrile (ACN) and dielectric constant with time(min).....	131
6.10	Chromatographically determined RRT (min) vs. theoretically determined RRT (min) for compounds with RRT <10 min.....	132
6.11	Chromatographically determined RRT (min) vs. theoretically determined RRT (min) for compounds with RRT (10-25 min).....	133
6.12	Chromatographically determined RRT (min) vs. theoretically determined RRT (min) for compounds with RRT (25-50 min).....	134
6.13	Chromatographically determined RRT (min) vs. theoretically determined RRT (min) for compounds with RRT (50-70 min).....	135

6.14 Relationship between retention time and position of OH substitution for selective HETEs.....	139
7.1 Flow chart/ decision tree representation of the statistical analysis performed.....	149
7.2 Representative HPLC chromatogram of all the prostanoids.....	151
7.3 Representative UHPLC chromatogram of all the prostanoids.....	153
7.4 Bland Altman plot for 8-Isoprostane.....	166
8.1 Representative HPLC-UV chromatogram of prostanoids in control urine.....	177
9.1 Time point study: Amount of 15-HETE and LXA4 in terms of pg/mg of tissue in the initiation, progression and resolution phase of inflammation.....	196
9.2 Time point study: Amount of 12-HETE, 15-HETE and LXA4 in terms of pg/mg of tissue in the initiation, progression and resolution phase of inflammation.....	197
9.3 Inhibition study: Amount of 12-HETE, 15-HETE and LXA4 in terms of pg/mg of tissue in the initiation, progression and resolution phase of inflammation.....	198
9.4 Inhibition study: Amount of 15-HETE and LXA4 in terms of pg/mg of tissue in the initiation, progression and resolution phase of inflammation.....	199

9.5	Comparison of PGJ2 (Time point study) with 15-HETE (Inhibition study).....	201
A.1	Structure of sex hormones.....	247
A.2	Representative chromatogram of standard solution of sex hormones .....	249
A.3	Representative chromatogram of sex hormones in extracted liver tissue.....	250

## CHAPTER 1

### 1. FATTY ACIDS AND THEIR METABOLITES AS BIOMARKERS OF INFLAMMATION

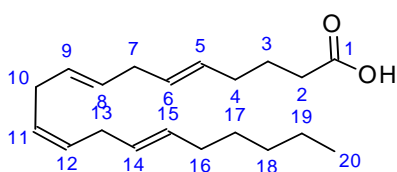
#### 1.1 Inflammation

Inflammation is part of human body's complex biological response to harmful stimuli such as pathogens and irritants. It is necessary, in order for the body to deliver effector molecules and cells to site of damage, to form a physical barrier in order to stop the spread of damage and to heal and repair damage [1]. A poorly regulated inflammatory response can destroy healthy tissues and can cause more injury. Inflammatory cascade is a dynamic process consisting of the following components: inducers (such as bacteria, viruses), sensors (receptors on mast cells, dendritic cells, macrophages) and mediators (C-reactive protein, cytokines, bradykinin, histamine, polyunsaturated fatty acids) [2]. Analysis of C-reactive protein (CRP) is the general test performed to check for inflammation in the body. CRP is considered to be a good inflammatory biomarker as its level increases by 1000 folds in the presence of infection/inflammation [3]. Moreover, it has a long plasma life ( $\approx$  19hours) [3]. The major disadvantage of CRP is that it is a non-specific biomarker. This means that it cannot pinpoint the exact site/source of inflammation [3]. This may be due to the fact that CRP is synthesized in the liver. Arachidonic Acid, Eicosapentaenoic acid and Docosahexaenoic acid and their metabolites are polyunsaturated fatty acids that are released at the site of inflammation and hence thought to act as specific biomarkers of inflammation. The main objective of

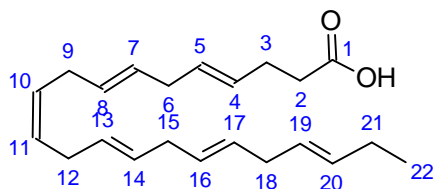
this research was to identify metabolites of polyunsaturated fatty acids that can act as specific biomarkers of inflammation.

## 1.2 Polyunsaturated Fatty Acids (PUFA's)

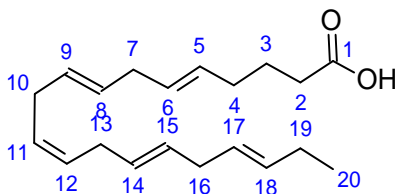
Polyunsaturated fatty acids are carboxylic acids with long, unbranched, unsaturated aliphatic chains. Arachidonic Acid (AA) ( $\omega$ -6 PUFA), eicosapentaenoic acid (EPA) ( $\omega$ -3 PUFA) and docosahexaenoic acid (DHA) ( $\omega$ -3 PUFA) (Figure 1.1) are among the fatty acids that are stored in the phospholipid bilayer, an important component of the cell membrane. Phospholipid bilayers are the basic structure of the membrane that provides a barrier between the outside and the inside of the cell [3,4]. The lipid bilayers contain proteins, fatty acids, enzymes, receptors and ion channels that plays important parts in the communication between the cells [4,5].



Arachidonic Acid ( $C_{20}H_{32}O_2$ )



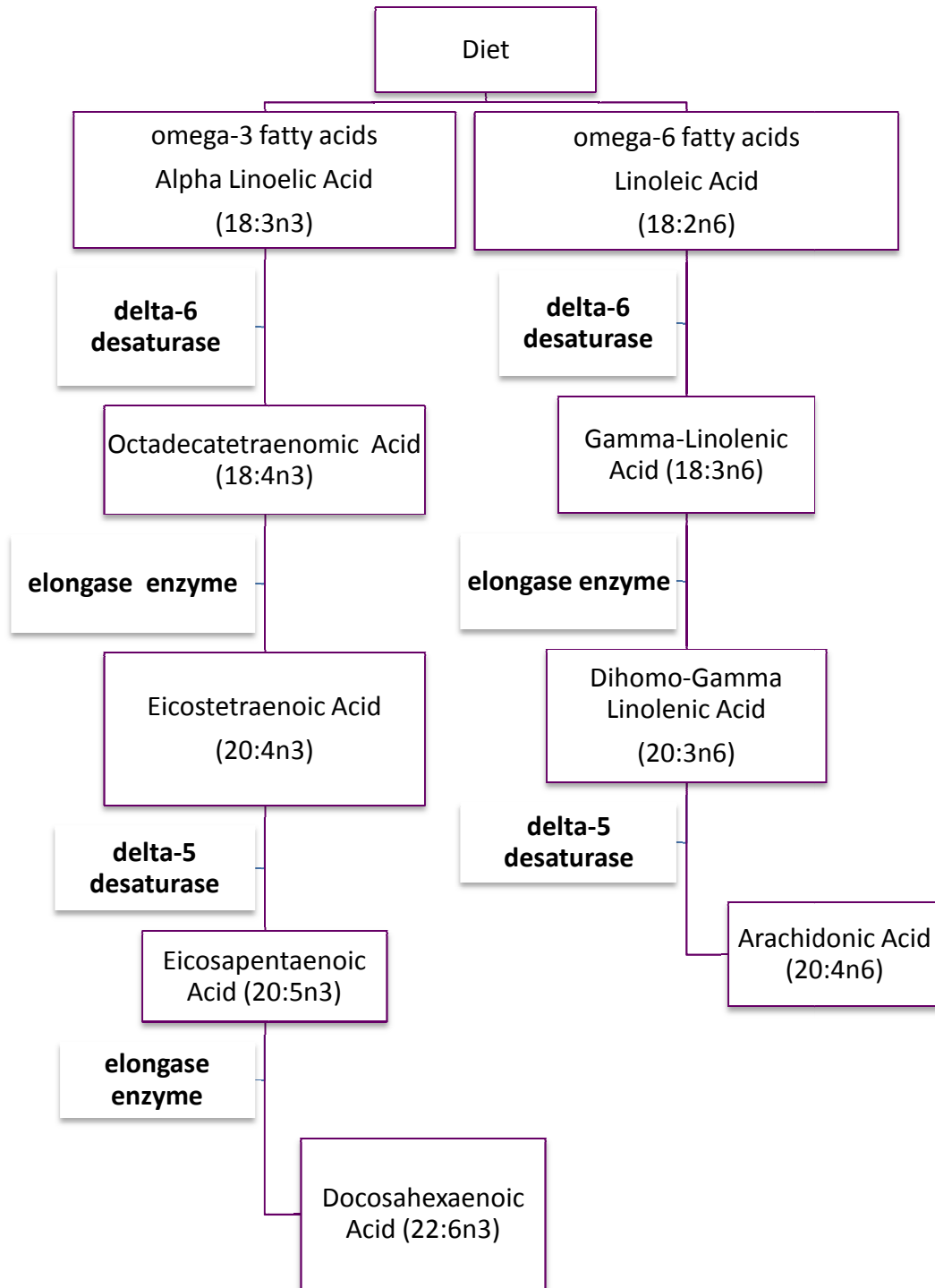
Docosahexaenoic Acid ( $C_{22}H_{32}O_2$ )



Eicosapentaenoic Acid ( $C_{20}H_{30}O_2$ )

**Figure 1.1. Chemical structure of polyunsaturated fatty acids**

The main source of these fatty acids is from diet [6]. Most of the  $\omega$ -6 PUFA's are consumed in the diet from vegetable oils such as soybean oil and corn oil. Linoleic acid, which is converted into AA through a series of steps, as outlined in Figure 1.2, is a major constituent of the vegetable oils [6]. The major sources of  $\alpha$ -linolenic acid (ALA), the precursor of EPA and DHA ( $\omega$ -3 PUFA's) are flax seed and fish [6]. As seen from Figure 1.2, ALA must be first converted to EPA and then to DHA. When these PUFA's are consumed they are first incorporated in the cell membranes of tissues in the body. Hence, any change in the diet will change the composition of the PUFA's in the cell membranes. This can alter the cell functions, as membrane lipids are precursors for the synthesis of important signaling molecules involved in the cell growth, development and inflammation. When the cells are stimulated by an external factor, arachidonic acid is released from the cell membrane through the action of phospholipase A2 (PLA<sub>2</sub>) [7,8]. The dietary EPA and DHA compete with AA and can displace it from the cell membranes. They also compete for the enzymes (cyclooxygenase, lipoxygenase and Cytochrome P450) that convert AA to the eicosanoids [9]. DHA and EPA are metabolized to resolvins (RVs) of the D series and E series respectively. They are known to have anti-inflammatory properties and also they play an important role in the resolution phase of the inflammatory cycle [9].



**Figure 1.2. Biosynthesis of  $\omega$ -6 and  $\omega$ -3 PUFA's [6] [Nomenclature of fatty acids: e.g. -18:3n3 denotes an 18 carbon chain with three double bonds and the first double bond is on carbon 3 from the methyl group end]**

for brain-mediated functions [10]. The three PUFA's and their metabolites are discussed in this chapter.

### **1.3 Arachidonic Acid Metabolism**

AA (cis 5,8,11,14-eicosatetraenoic acid), a  $\omega$ -6 polyunsaturated fatty acid, is present in the phospholipid bilayers (especially phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositides) of cell membranes. It is abundant in the brain, muscles, and liver. It is one of the essential fatty acids required by most mammals. The production of arachidonic acid from linoleic acid occurs primarily in the liver, as the activity of the desaturases is highest in the liver. AA in the esterified form is then transported in blood, in lipids of various lipoproteins (VLDL-very low-density lipoprotein, LDL- low-density lipoprotein, HDL- high-density lipoprotein) bound to albumin [11]. Albumin bound AA in plasma is avidly taken up by cells. The mechanism of uptake is not entirely clear [11]. Incorporation of arachidonic acid into phospholipid first requires activation to the acyl-coenzyme A (CoA). Arachidonoyl-CoA is then incorporated into phospholipids [11]. In the presence of external stimuli, esterified AA is released from the phospholipid bilayer usually by a  $\text{Ca}^{+2}$  regulated cytosolic phospholipase  $A_2$  (cPLA<sub>2</sub>) [12]. It then undergoes metabolism by three enzymatic pathways: cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 and a non-enzymatic free radical peroxidation.

#### **1.3.1 COX-2 Metabolites (Prostaglandin Synthases)**

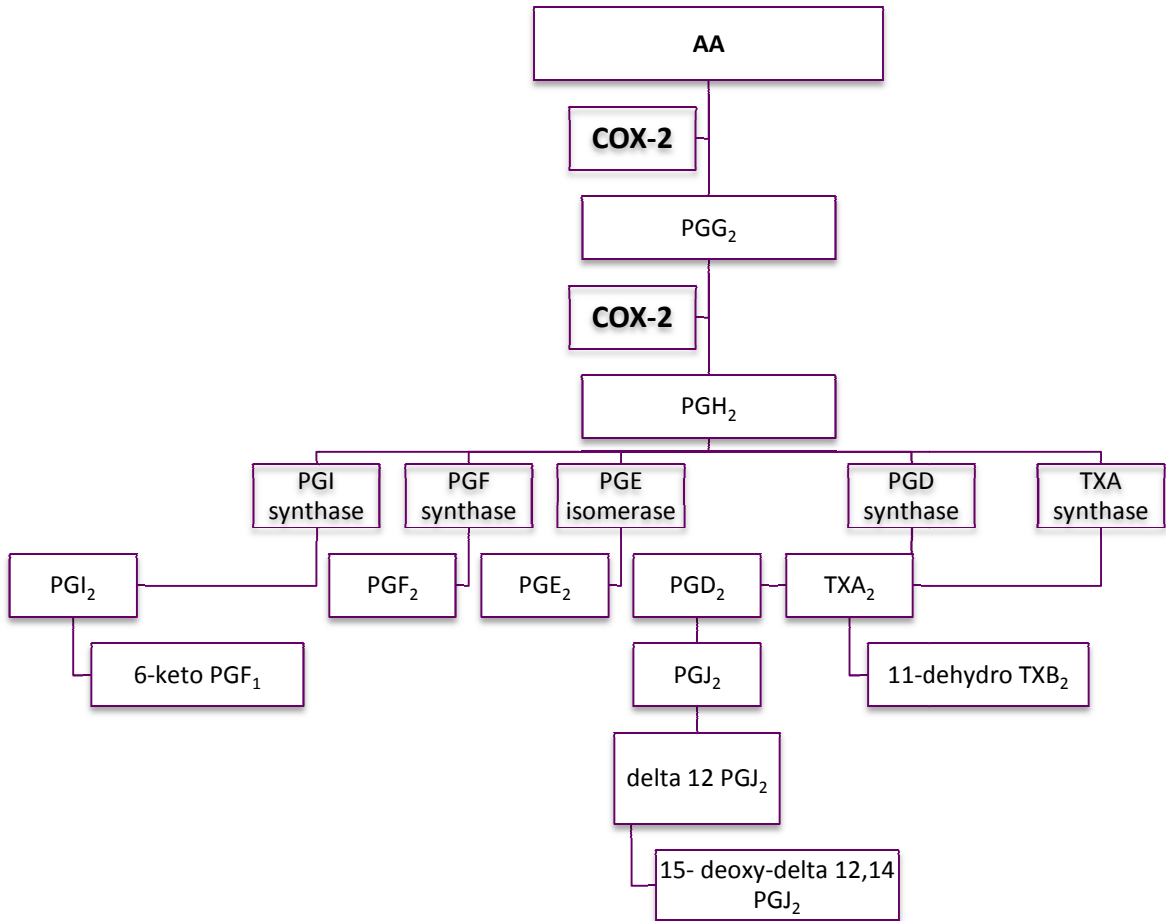
Three isozymes of COX exist in the human body: constitutive COX-1, inducible COX-2 and COX-3. COX-1 is responsible for maintaining homeostasis in various physiological functions in the body whereas COX-2 is involved in numerous

inflammatory responses [5]. COX-2 is induced by various stimuli such as cytokines and mitogens [13]. COX-1 and COX-2 enzymes are 63% alike and 77% comparable at the amino acid level [14]. The major difference is that COX-2 has a larger pocket at the active site due to the isoleucine to valine substitution [14]. COX-3, which was discovered in 2002 is thought to be an intron-splice variant of COX-1 [15]. COX-1 and COX-3 have similar but not identical amino acid sequences [15]. The role of COX-3 is the subject of considerable interest and not fully understood. The current research concentrates on the role inflammatory COX-2 metabolites of AA.

Prostaglandin synthases 2 (COX-2) catalyzes two reactions: the first is the addition of molecular oxygen to AA to form the unstable hydroperoxy endoperoxide (PGG<sub>2</sub>) and the second is the conversion of PGG<sub>2</sub> to a more stable hydroxy endoperoxide (PGH<sub>2</sub>) (Figure 1.3) [14,16]. PGH<sub>2</sub> is then transformed to primary prostanoids by a range of enzymes (Figure 1.3) [14,17]. These prostanoids can be classified into three main groups: prostaglandins (PGE<sub>2</sub>, PGF<sub>2α</sub>, PGD<sub>2</sub>), prostacyclins (PGI<sub>2</sub>) and thromboxanes, (TXA<sub>2</sub>) (Figure 1.3). Prostanoids are produced by most cells in the human body and act as autocrine and paracrine lipid mediators i.e., they signal at/adjacent to the site of synthesis [18,19]. They are then released from these cells and transported through a known prostaglandin transporter (PGT) and by other unknown transporters [20]. There are 9 known prostaglandin receptors in human and mouse, four of which bind PGE<sub>2</sub> (EP1–EP4) (21–24), two bind PGD<sub>2</sub> (DP1 and DP2) (25–27), and the remaining bind PGF<sub>2</sub>, PGI<sub>2</sub>, and TxA<sub>2</sub> (FP, IP, and TP, respectively) [18]. The majority of the known physiological/pathological functions of the prostanoids are summarized below.

*PGI<sub>2</sub> (Prostacyclin)* - Prostacyclin is a potent vasodilator. It inhibits platelet aggregation and leukocyte activation [28]. It is produced in the endothelial cells by the action of prostacyclin synthase [28]. It is unstable at physiological pH and has a half-life of less than 42 seconds [29]. It is converted rapidly into an inactive hydration product, 6-keto PGF<sub>2</sub>. Within the vascular system, it functions as a vasodilator and inhibits platelet aggregation as well as vascular smooth cell proliferation [30,31]. In the renal system, prostacyclin is known to regulate blood flow and glomerular filtration rate [32]. Furthermore, according to Idzko *et al.* PGI<sub>2</sub> reduces pulmonary blood pressure [33]. Prostacyclin has both pro- and anti-inflammatory properties depending on the type of tissue and the disease model being studied [30]. It acts as a pro-inflammatory marker in musculoskeletal disorders such as rheumatoid arthritis and osteoarthritis but it has anti-inflammatory properties in atherosclerosis [30].

*TXA<sub>2</sub> (Thromboxane)*- Thromboxane is a vasoconstrictor. It is produced by activated platelets by the action of thromboxane A synthase [34]. It is unstable at the physiological pH and is converted rapidly into 11-dehydro TXB<sub>2</sub>. In addition to causing irreversible platelet aggregation, TXA<sub>2</sub> causes bronchial and vascular smooth muscles to contract [34]. There is numerous evidence in literature that suggests that platelet activation determined by measuring the urinary excretion of 11-dehydro TXB<sub>2</sub> are associated with increased risk in cardiovascular disorders, coronary and cerebral vascular syndromes and atherothrombosis[35].



**Figure 1.3. Oxidative pathway of Arachidonic Acid by COX-2 enzyme [5]**

*PGE<sub>2</sub>* - PGE<sub>2</sub> is a pro-inflammatory molecule that is responsible for the redness, pain and swelling in inflammatory diseases such as rheumatoid arthritis. It is almost ubiquitous in humans. During inflammation, chemical mediators invoke PGE<sub>2</sub> synthesis (by PGE synthase) in fibroblasts, endothelial cells, monocytes, and neutrophils at the site of inflammation [36]. It is also a known vasodilator. It regulates renal water re-absorption and plays a role in development of fever and hyperalgesia [36]. It is thought to be involved in tumor-induced inflammation, Alzheimer's and other neurological diseases [37-40].

*PGF<sub>2α</sub>* - PGF<sub>2</sub> is a responsible for wide range of bodily functions such as contraction and relaxation of smooth muscles and controlling of the blood pressure [5]. Furthermore, it is important in the uterine and ovarian function [41]. It is also thought to play a role in cardiac muscle growth [42]. PGF<sub>2</sub> levels are found to be elevated in tumors suggesting it can be a good prognostic index [43]. It has been implicated in cardiovascular diseases, hypertension and cancers.

*PGD<sub>2</sub>* - PGD<sub>2</sub> is a prostaglandin produced by mast cells. It is thought to have wide variety of role in vivo. It is considered to be an unstable pro-inflammatory molecule [44]. It is readily converted into wide variety of metabolites in vivo. PGD<sub>2</sub> metabolites of the J series, PGJ<sub>2</sub>, Δ<sup>12</sup>-PGJ<sub>2</sub>, and 15d-PGJ<sub>2</sub> are bioactive and are thought to have anti-inflammatory properties [44]. In summary, PGD<sub>2</sub> and its metabolites have pro and anti-inflammatory properties respectively. There is evidence in the literature, which suggests that low levels of PGD<sub>2</sub> (nano-molar concentrations) recruit and activate leukocytes whereas greater concentrations (micro-molar concentrations) inhibit activation and

induce apoptosis [44].  $\text{PGD}_2$  and its metabolites have been implicated to be involved in the resolution of carrageenin-induced pleural inflammation [45,46].

### 1.3.2 Free Radical per-oxidation

Free radicals have been implicated in the patho-physiology of diseases such as cancer, atherosclerosis, Alzheimer's and the normal aging process [47]. One of the well known targets of the free radicals are lipids. Morrow *et.al.* in 1990, reported a series of prostaglandin like compounds that are produced by the free radical peroxidation of arachidonic acid independent of the COX enzyme [48]. These prostaglandins like compounds are known as isoprostanes. The mechanism of formation of F2-isoprostanes from AA involves abstraction of allylic hydrogen atom to yield arachidonyl radical followed by insertion of oxygen to form peroxy radical [47]. Four different peroxy radical can be formed depending on the site of abstraction of hydrogen and addition of oxygen. The peroxy radicals then undergo endocyclization followed by addition of oxygen to yield bicycloendoperoxide regioisomers. These are then reduced to form F2-isoprostanes. 8-iso  $\text{PGF}_{2\alpha}$  (15 F2-Isop) is the most abundantly produced isoprostane in vivo [49]. Apart from being a marker of lipid peroxidation, it can also be a marker of oxidative stress. Oxidative stress is a condition in which there is an imbalance between the production of reactive oxygen species and the biological system's ability to detoxify them. 8-iso  $\text{PGF}_{2\alpha}$  is a potent vasoconstrictor and is found in of many organs such as kidney, lung, heart and brain [50,51]. Moreover, it induces the release and proliferation of vascular smooth muscles [52]. Increased levels of 8-iso  $\text{PGF}_2$  have been found in

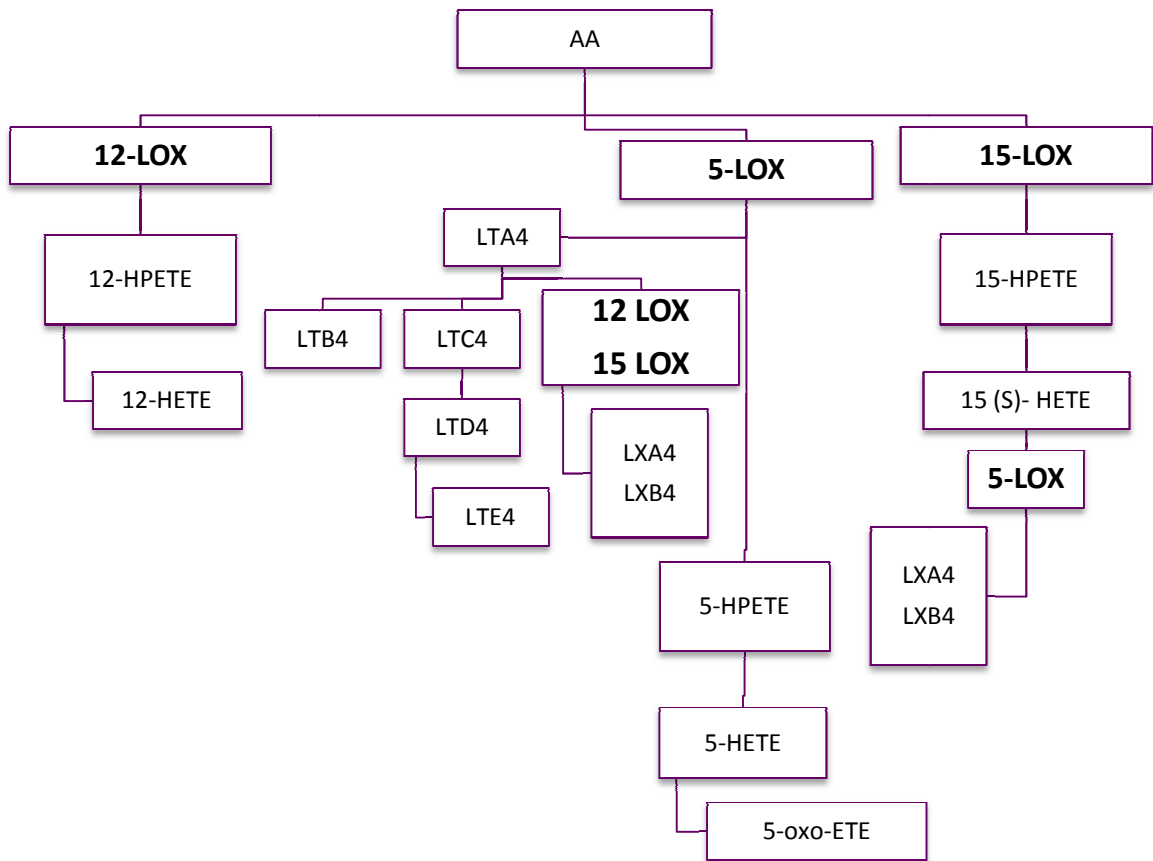
numerous diseases such as diabetes, atherosclerosis, obesity and chronic obstructive pulmonary disease (COPD) [52].

### 1.3.3 LOX metabolites

Lipoxygenase are a group of enzymes that catalyzes the addition of oxygen to AA producing hydroperoxyeicosatetraenoic acid (HPETEs) [53,54]. HPETE undergoes reduction to give leukotrienes (LTs), hydroxyeicosatetraenoic acid (HETEs) and lipoxins (LXs) (Figure 1.4) [55]. LOX enzymes are classified into 5-, 8-, 12- and 15-LOX depending on the site of insertion of oxygen onto AA. The LOX metabolites of AA have been implicated in the pathophysiology of diseases such as cancer, rheumatoid arthritis, atherosclerosis and Alzheimer's [56].

*5-LOX metabolites:* Borgeat *et. al.* [57] described 5-LOX enzyme as a 78 kDa protein that catalyzes the biosynthesis of LTs and 5-HETE from AA. AA is first converted to an unstable epoxide, leukotriene A<sub>4</sub> (LTA<sub>4</sub>), which is then converted to LTB<sub>4</sub> via LTA<sub>4</sub> hydrolase or LTC<sub>4</sub> via LTC<sub>4</sub> synthase [58]. LTB<sub>4</sub> was the first leukotriene to be isolated [56]. It is involved in number of inflammatory responses such as leukocyte activation, chemotaxis, and degranulation [56]. BLT1 and BLT2 are the two LTB<sub>4</sub> receptors [58-60]. BLT1, which is high affinity receptor, is expressed in inflammatory cells such as leukocytes [59]. On the other hand, the low affinity receptor BLT2 is expressed in variety of tissues [60,61]. There are evidences that suggest that LTB<sub>4</sub> stimulates generation of ROS thus playing a role in oxidative stress [56]. It is also implicated in diseases such as rheumatoid arthritis, inflammatory bowel diseases and asthma [62]. The

cysteinyl leukotrienes (LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub>) are important mediators of allergic inflammation such as asthma and allergic rhinitis [56, 58, 63].



**Figure 1.4. Oxidative pathway of Arachidonic Acid by LOX enzymes [5]**

They are produced by number of cells including eosinophils, basophils, macrophages, mast cells, T cells and endothelial cells [63]. They are involved in allergic inflammatory responses such as bronchoconstriction, wheezing, and increased mucus secretion [64-66]. In the vascular system, they act as vasodilators and increases the vascular permeability thus resulting in tissue edema [65,66]. 5-HETE, which is also a 5-LOX metabolite of AA, is not a potent mediator of inflammation [67]. It is enzymatically oxidized to 5-oxo-ETE in the presence of NADP [67]. 5-oxo-ETE is more potent than 5-HETE. It is a chemoattractant for eosinophils, neutrophils, basophils and monocytes [68]. Though the patho-physiology of 5-oxo-ETE is not well understood, it is thought to play a vital role in allergic diseases, cancer and cardiovascular diseases [68].

*12-LOX metabolites:* 12-LOX enzyme catalyzes the oxygenation of AA to 12 (S)-hydroperoxyeicosatetraenoic acid which subsequently is converted into a more stable 12(S)-hydroxyeicosatetraenoic acid (12-HETE). Three types of 12-LOX have been characterized: platelet, leukocyte and epidermal type 12-LOX [56]. The platelet type 12-LOX is known to synthesize 12(S)-HETE [56]. On the other hand, leukocyte type 12-LOX also known as 12/15 LOX enzyme, synthesizes both 12(S)-HETE and 15(S)-HETE [56]. An increase in 12-LOX activity has been observed in hypertension, thrombosis and skin tumors [69]. 12/15 LOX have been implicated in the progression of atherosclerosis and diabetes I [70]. 12-HETE, the 12-LOX metabolite of AA has pro-inflammatory properties and is thought to be involved in chemotaxis and regulation of leukocyte adherence [70].

*15-LOX metabolites:* 15 LOX catalyzes the addition of oxygen to C15 of AA to give 15-HETE. Two types of 15-LOX enzymes have been characterized, reticulocyte type (15-

LOX-1) and the epidermal type (15-LOX-2) [71-73]. 15-LOX-1 preferentially metabolizes linoleic acid to 13 (S)-HODE [55]. It also converts AA into 15(S)-HETE [56]. 15-LOX-2, which is expressed in the skin, lungs, cornea and prostate glands, is primarily responsible for the metabolism of AA to 15(S) HETE [56]. There are contradictory reports on the role of 15(S)-HETE in inflammation. It is a known anti-inflammatory molecule. It decreases the production of LTB<sub>4</sub> in arthritis [74-76]. There are also results that indicate that 15-HETE has pro-inflammatory action by enhancing the expression of TNF $\alpha$  [77].

*5-LOX, 12-LOX and 15-LOX metabolites- Lipoxins:* Serhan and colleagues were the first to identify lipoxins [78]. In the last two decades there has been tremendous efforts in identifying the physiological functions of lipoxins in inflammatory responses. They are thought to act as braking signals in inflammation [79]. Lipoxins found in human body are 5S, 6R, 15S-trihydroxy-7, 9,13-trans-11-cis-eicosatetraenoic acid (LXA<sub>4</sub>) [80] and its positional isomer 5S, 14R, 15S-trihydroxy-6, 10,12-trans-8-cis-eicosatetraenoic acid (LXB<sub>4</sub>) [81]. 15-epi-LXs are a class of lipoxins that are triggered by aspirin and are 15 R enantiomers of LXA<sub>4</sub> and LXB<sub>4</sub> [82]. There are two major routes for LX formation. The first involves the conversion of AA to LTA<sub>4</sub> via the 5-LOX pathway. The LTA<sub>4</sub> is then taken up by the platelets and converted to LXA<sub>4</sub> by the action of 12-LOX enzymes [83,84]. The second route involves the formation of 15 HPETE via the 15-LOX enzyme in the epithelial cells. This is then converted into an unstable epoxide intermediate 5,6-epoxytetraene via the 5-LOX enzyme in the neutrophil [82,84]. This intermediate is then converted to LXA<sub>4</sub> and LXB<sub>4</sub> through the actions of epoxide hydrolases in leukocytes [78]. Aspirin induces acetylation of COX-2, thus shifting the AA metabolism from

COX-2 to LOX [82]. This results in the formation of 15 (R) HETE, which is then transformed by the leukocyte 5-LOX to 15-epi-LXA4 and 15-epi-LXB4 [82]. There is still some ambiguity in the role of lipoxins in the patho-physiology of inflammatory diseases. There is evidence that suggests that lipoxin production decreases in diseases such as asthma, liver disease and leukemia whereas it increases in juvenile periodontitis, arteriosclerotic plaque rupture, and nasal polyps [79].

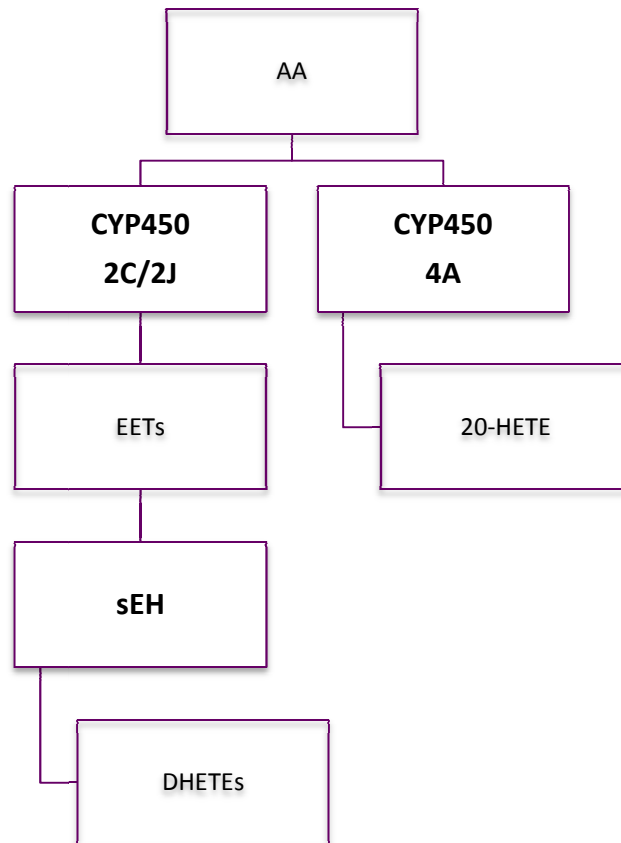
### **1.3.4 Cytochrome (CYP) 450 metabolites**

AA can undergo metabolism by CYP450 primarily CYP 450 2C and 2J to give epoxyeicosatrienoic acid (EETs) (Figure 1.5). CYP enzyme produces four regioisomers of EET from AA: 5,6-, 8,9-, 11,12-, and 14,15-EET [85]. EETs have anti-inflammatory properties. It is a cardio-protective molecule and is known to regulate blood pressure [86]. They are produced in the blood vessels by CYP enzyme present in the endothelial cells [87]. They are also known to inhibit inflammatory responses induced by cytokines [85]. EETs are rapidly metabolized to DHETEs in presence of soluble epoxide hydrolase enzyme (sEH) [88,89]. sEH is regioselective. 14,15-EET is the preferred substrate followed by 11,12- EET, 8,9-EET and 5,6-EET [85]. DHETEs are more polar and less active than EETs [88,89]. They have been implicated in adult respiratory disorders. The other major product of CYP 450 is 20-HETE. 20 HETE is an  $\omega$ -hydroxylation product of AA synthesized in the kidney. CYP 450 4A is primarily responsible for the synthesis of 20-HETE [90]. It is a potent vasoconstrictor in the arterioles of renal, skeletal and cerebral muscles [91]. Information regarding the patho-physiological role of 20-HETE in humans is still limited. One reason for this is because 20-HETE is excreted in urine as

glucuronide conjugates which can be measured by analytical techniques after hydrolysis with glucuronidase [92]. It is thought to play a vital role in hypertension [92].

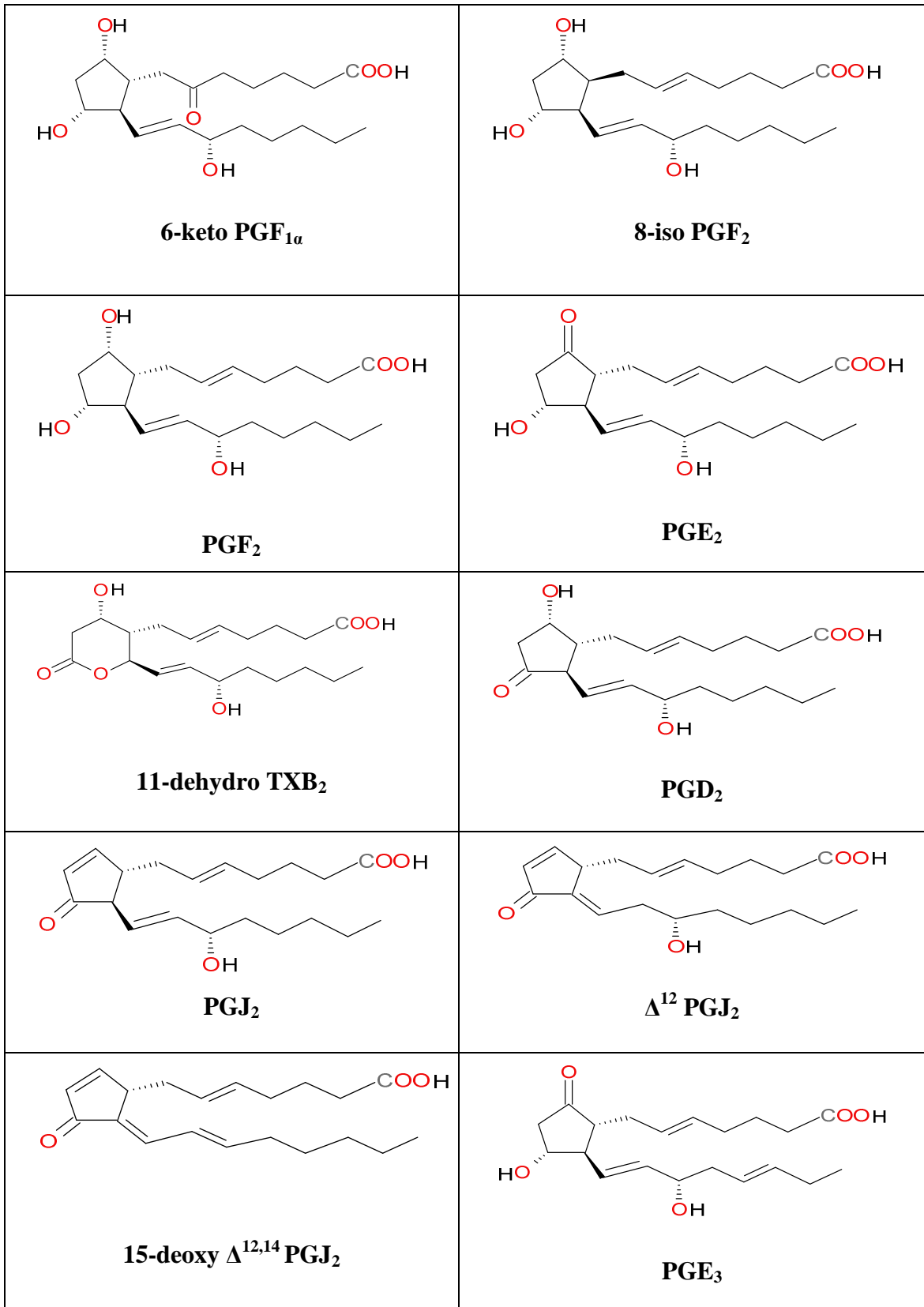
#### **1.4 EPA and DHA Metabolites**

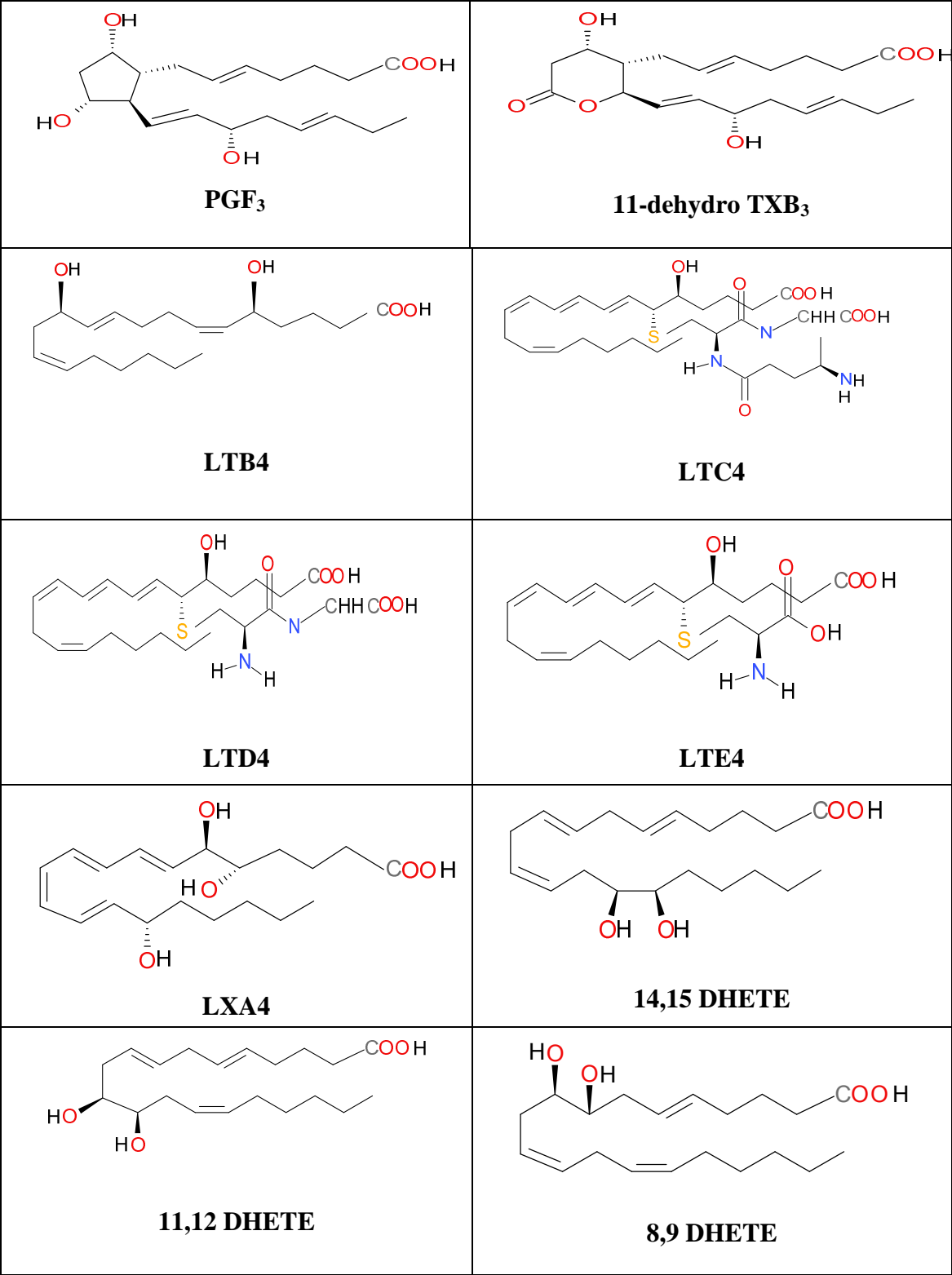
It was found that  $\omega$ -3 PUFA's when administered at daily high doses (milligram and gram) have favorable actions in many inflammatory diseases [93]. The three major LOX enzymes found in humans (5-, 12-, 15-LOX) can convert  $\omega$ -3 PUFA's to various oxygenated products [94]. The oxygenated metabolites of EPA and DHA are named as *resolvins* (resolution phase interaction products) as they participate in the resolution of inflammation [93,95-96]. They are termed as resolvins of E series (RvE) and resolvins of D series (RvD) derived from EPA and DHA respectively [93]. Resolvins are predominately generated in whole human blood and their generation increases in the presence of aspirin [95,96]. They exert potent anti-inflammatory properties in diseases such as atherosclerosis, asthma, cardiovascular diseases, cancer, mental depression and preventing sudden death after myocardial infarction [97-99]. EPA can also undergo metabolism via COX-2 enzyme to give prostaglandins of the 3 series (PGE<sub>3</sub>, PGF<sub>3</sub>, 11-dehydro TXB<sub>3</sub>).

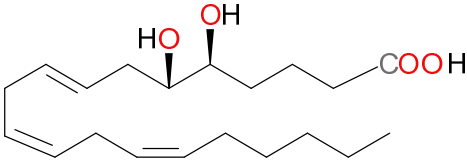
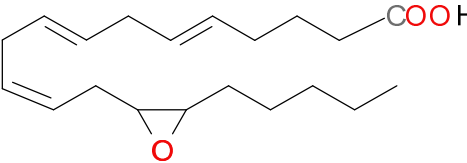
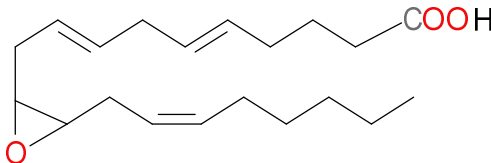
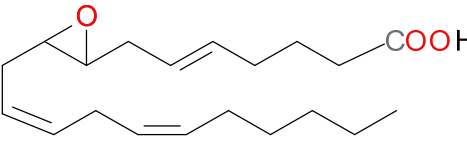
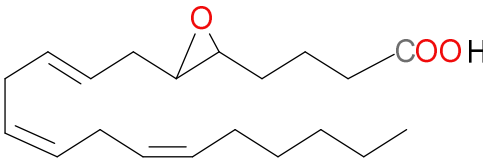
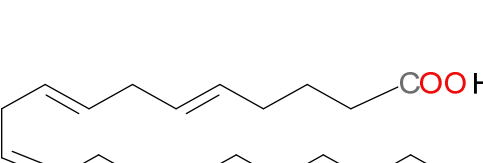
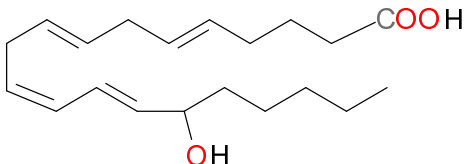
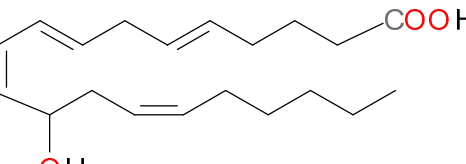
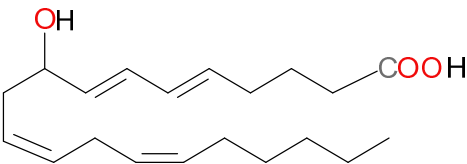
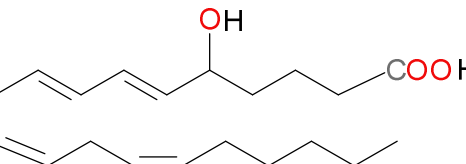


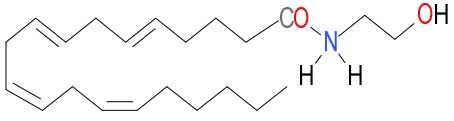
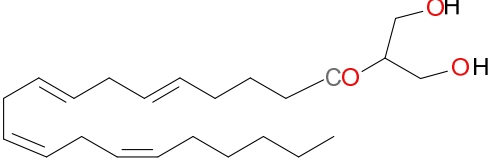
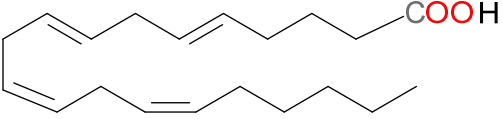
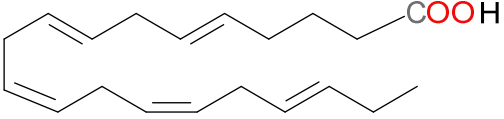
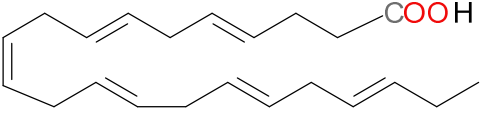
**Figure 1.5. Oxidative pathway of Arachidonic Acid by CYP 450 enzymes [5]**

## 1.5 Structures Of Lipid Biomarkers





 <p style="text-align: center;"><b>5,6 DHETE</b></p>	 <p style="text-align: center;"><b>14,15 EET</b></p>
 <p style="text-align: center;"><b>11,12 EET</b></p>	 <p style="text-align: center;"><b>8,9 EET</b></p>
 <p style="text-align: center;"><b>5,6 EET</b></p>	 <p style="text-align: center;"><b>20-HETE</b></p>
 <p style="text-align: center;"><b>15-HETE</b></p>	 <p style="text-align: center;"><b>12-HETE</b></p>
 <p style="text-align: center;"><b>9-HETE</b></p>	 <p style="text-align: center;"><b>5-HETE</b></p>

 <p style="text-align: center;"><b>Anandamide</b></p>	 <p style="text-align: center;"><b>Arachidonyl Glycerol</b></p>
 <p style="text-align: center;"><b>Arachidonic Acid</b></p>	 <p style="text-align: center;"><b>Eicosapentaenoic Acid</b></p>
 <p style="text-align: center;"><b>Docosahexaenoic Acid</b></p>	

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## CHAPTER 2

### 2. ANALYTICAL TECHNIQUES AND INSTRUMENTATION

#### 2.1 Introduction

One of the objectives of this research was to develop analytical methods to separate and quantitate fatty acids and their metabolites. The focus of this chapter is on the techniques and instrumentation involved in the development of these methods. Emphasis has been given to the principles of liquid chromatography and the instrumental design of HPLC (high pressure liquid chromatography), UHPLC (Ultra high pressure liquid chromatography) and LC-MS (Liquid chromatography- mass spectrometry).

#### 2.2 Liquid Chromatography and Its Underlying Principles

Reverse phase HPLC, UHPLC and LC-MS are liquid chromatographic techniques that work on the same chromatographic principles. They all fall under the broad category of liquid-solid chromatography, which is a separation technique that utilizes, chemical and/or physical interactions between the analytes transported in the liquid mobile phase and a solid stationary phase. The separation of the analytes occurs due to one of the four mechanisms: Van der Waals, dipole-dipole, hydrogen bonding or ionic interactions and this depends on the nature of the analyte and the stationary and mobile phases used [1]. These interactions coupled with the distance travelled by the individual analytes through the column dictates the time it takes to reach the detector. Therefore we can say that retention behavior is a direct reflection of the distribution of the analytes between the stationary and the mobile phases. Equilibrium constant is a parameter that directly

reflects the distribution of the analytes between the stationary and the mobile phase. The equilibrium constant ( $K$ ) for a given analyte (solute) is defined as the ratio of the moles of the analyte in the stationary phase to the moles in the mobile phase [2]:



$$K = C_S/C_M \dots (2.1)$$

$C_S$  = Concentration of analyte in stationary phase;  $C_M$  = Concentration of analyte in mobile phase;  $K$  = equilibrium constant

The retention factor (capacity factor), which is the ratio of the retention times of the retained and un-retained species, is directly proportional to the equilibrium constant as can be seen in eq. 2.2.

$$t_R - \frac{t_0}{t_0} = k' = K\phi \dots (2.2)$$

$\phi$  = phase ratio of the column (ratio of the volume of the stationary phase to that of the mobile phase);  $k'$  = retention factor;  $t_R$  = retention time of the analyte;  $t_0$  = retention time of the mobile phase (un-retained species) [3].

The acceptable  $k'$  values are between 1 and 10.  $k'$  values higher than 10 wastes valuable analytical time whereas less than unity do not provide adequate resolution [3]. Capacity factor is very useful for comparing results obtained from two different systems since it is not directly dependent on the column length and flow rate. When more than one analyte is present, determining the relative retention, more widely known as selectivity factor ( $\alpha$ )

is important. It is defined as the ratio of the capacity factor of the analytes to be separated.

$$\alpha = k'_A/k'_B \dots (2.3)$$

Therefore, by definition  $\alpha$  is always greater than 1. Greater values than 1 for  $\alpha$  will represent a better separation of the analytes [3]. The degree of separation or resolution of two adjacent analytes is defined as the distance between the peaks divided by the average peak width. As per the FDA guidance to the industry, for any bioanalytical validation [4], resolution between two adjacent analytes should be greater than 1.5 [4]. The other chromatographic parameter that is of importance is the efficiency of the column, which is expressed as a dimensionless quantity called as the plate number ( $N$ ). It reflects the number of times the analyte (solute) partitions between the stationary and mobile phase as it passes through the column and is given as [5]:

$$H = L/N \dots (2.4)$$

$H$ = plate height;  $L$  = length of the column;  $N$  = number of the theoretical plates.

Greater the number of plates, smaller is the plate height ( $H$ ). Smaller the plate height better is the efficiency.

### **2.3 Reverse Phase Hplc**

Reverse Phase HPLC (RP-HPLC) is one of the most common HPLC technique employed by analytical chemists. It employs a polar mobile phase and a non-polar stationary phase, typically a long chain hydrocarbon. The more polar analytes are less retained by the stationary phase and elute out of the column much faster. In contrast, the

non-polar analytes that are retained much longer on the stationary phase due to the hydrophobic interactions between the largely hydrophobic surfaces of the bonded stationary phase.

The HPLC system consists of four main components that are the pump, injector, column, and detector. Fundamentally, the liquid mobile phase is drawn from the reservoir to the stationary phase by a reciprocating pump [3]. A ball check-valve maintains a unidirectional flow. The typical operating pressure for the HPLC systems is around 6000 psi with the flow rates ranging from 0.1-10 ml/min [3]. The sample is injected into the system through an automatic or a manual injector. These injectors are capable of delivering sample volumes ranging from less than 1 to 100  $\mu\text{L}$  [3,5]. The analytical columns act as stationary phase in HPLC. The type of column used for the HPLC analysis is dependent on the type of analytes and the application of the analysis. A majority of HPLC columns are packed with silica particles. The particle size ranges from 3 to 50  $\mu\text{m}$ , with 5  $\mu\text{m}$  being the most common [3,5]. The silica surface provides a place where hydrophobic chains ( $\text{C}_8$ ,  $\text{C}_{18}$ ) can be bonded by reaction with an organochlorosilane. Only a fraction of the silanol groups can be bonded with the hydrophobic chain due to steric hindrance. The residual silanols can cause problems especially to basic analytes as it can cause secondary reactions and affect chromatographic behavior (asymmetry). The occurrence of the residual silanols can be reduced by a process called end capping. The final part of the HPLC system is the detector. Different types of detectors utilizing techniques such as ultraviolet (UV), fluorescence, refractive index and mass to charge ratio (mass spectrometry) can be used. UV detector is the most common and popular HPLC detector.

## 2.4 Ultra High Pressure Liquid Chromatography (UHPLC)

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There is significant emphasis on developing high throughput assays, which not only reduce the analysis time but also decrease the total cost of the analysis. High throughput chromatographic separations can be achieved by increasing the flow rate [6, 7], decreasing the particle size [8] decreasing the column length [9] or increasing the column temperature (increases the viscosity of mobile phase). Ultra high pressure chromatography (UHPLC) is a technique that reduces the analysis time by using columns with a packing particle size less than 2  $\mu\text{m}$  at elevated pressures. McNair *et.al.* [6,7] were the first to manufacture and demonstrate the use of UHPLC. The basic principles of chromatographic technique are based on the theories and assumptions by Van Deemter [8], Giddings [9], and Knox [10,11]. According to these theories, the column efficiency, resolution and sensitivity improve with decreasing particle size. The downside of decreasing particle size is the significant increase in the column backpressure. The column backpressure increases inversely with the square of the particle size at constant linear velocity and the cube of the particle size at optimum linear velocity [10]. As a result, the use of smaller particles (sub 2  $\mu\text{m}$ ) increases the operating pressure in many cases to 18,000 psi, which is beyond the pressures of conventional HPLC systems. Hence, it was necessary to develop systems (pumps, injection systems, detectors, and columns) that can handle higher pressures and generate reproducible and accurate chromatographic data. One of the biggest challenges of this technology was the development of columns with sub 2  $\mu\text{m}$  particles that could not only withstand high

pressure but could also maintain similar retention and capacity as the HPLC under sustained usage [12]. Another crucial parameter in UHPLC is sample introduction. The injection valves (automatic and manual) that are conventionally used in HPLC systems cannot withstand the pressures generated in UHPLC. There is also a need for a fast pulse free injection cycle to protect the column from large pressure fluctuations and to maintain the speed of the UHPLC technique [12]. To maintain sensitivity, it is necessary to have low volume injections with minimal carryover. The detectors coupled with UHPLC must have a fast sampling rate in order to obtain a sufficient number of data points across the analyte peak [12]. The analyte peak can be integrated accurately and reproducibly only if there are a large number of data points. The detectors that have commonly been used with the UHPLC are Ultra-Violet (UV), Mass Spectrometry (MS) and Tandem Mass spectrometry (MS/MS).

Apart from these engineering challenges, high pressures and flow rates in liquid chromatography can generate heat. The heat generated can be calculated in terms of power (W) by using the following equation:

$$Power = \Delta PF \dots\dots\dots (2.5)$$

Where,  $\Delta P$  = pressure drop across the column ( $N/m^2$ ) and  $F$  = flow rate ( $m^3/s$ ).

Heat is generated by low column permeability due to small particle size. If the dissipation of this heat is poor as is the case when using large bore columns, the retained heat can increase the temperature of the mobile phase in the column. Poor heat dissipation and frictional heating of the mobile phase causes temperature gradients inside the column that affect chromatographic separation and column efficiency. Reducing the

column diameter improves the dissipation of frictional heat as the column surface area to volume ratio increases. Capillary columns (30-100  $\mu\text{m}$  ID) are primarily used in UHPLC, as they are better in dissipating heat. This is due to their large surface area to volume ratio and the reduced flow rates dictated by the dimensions of the column. However, reduction of the column internal diameter decreases sample capacity. Theoretically, sample capacity is proportional to the square of the column diameter. Reducing the column internal diameter decreases the sample capacity and concomitantly the sensitivity.

It is therefore not surprising that, although known as early as 1975, that smaller particle size resulted in faster separation, it was only in 2004 that the first UHPLC system was commercially available [12]. This UHPLC system was developed by Waters Corporation and is known as the ACQUITY UPLC<sup>TM</sup> system. Since then UHPLC systems have been manufactured by other companies. A list of commercially available UHPLC systems is summarized in Table 2.1.

**Table 2.1. List of commercially available UHPLC instruments with their operating pressure**

<b>Company</b>	<b>Model of Commercially available UHPLC</b>	<b>Maximum Operating Pressure</b>
<b>Agilent</b>	1200 Rapid resolution LC 1290 Infinity	17,400 psi
<b>Dionex</b>	UltiMate <sup>®</sup> 3000 Rapid Separation LC (RSLC) UltiMate <sup>®</sup> 3000 x2 Dual Rapid Separation LC	11,600 psi
<b>Hitachi</b>	LaChromUltra High Speed	8702.2 psi
<b>Jasco</b>	X-LC	15,000 psi
<b>PerkinElmer</b>	Flexar	10,000 psi (FX-10) 15,000 psi (FX-15)
<b>Shimadzu</b>	Prominence HT HPLC Nexera UHPLC	9572.4 psi 18,854 psi
<b>Thermo Scientific</b>	Rheos ALLEGRO UHPLC Accela High Speed LC	14,500 psi 18,129 psi
<b>Waters</b>	ACQUITY UPLC	15,000 psi

### 2.4.1 Theory

The relationship between the column efficiency and the linear velocity of the mobile phase was introduced for the first time by Van Demeter's equation. This equation (6) relates the height equivalent theoretical plates (HETP) to the linear velocity of the mobile phase [6]:

$$H = A + \frac{B}{u} + Cu \quad \dots (2.6)$$

Where,  $H$  is the HETP (mm),  $u$  is the linear velocity (cm/s) and  $A$ ,  $B$  and  $C$  represents different sources of peak dispersion in the column.

$A$  describes Eddy diffusion, which arises from multiple pathways due to uneven packing.  $B$  describes longitudinal diffusion, which results due to the diffusion of the analyte molecules in the forward and backward direction as the band moves along the column.  $C$  is the mass transfer resistance in the stationary phase ( $C_s$ ) and the mobile phase ( $C_m$ ), which occurs due to the slow equilibration between the two phases. For packed columns,  $A$  and  $C$  terms contribute more to the peak dispersion than the  $B$  term. Also, the mass transfer resistance in the stationary phase ( $C_s$ ) is minimal when bonded or thin stationary phase films are used in liquid chromatographic separations. Hence the dispersion in packed columns primarily results from Eddy diffusion and mass transfer resistance in the mobile phase ( $C_m$ ).

$$H = \frac{L}{N} = 2\lambda d_p + \frac{2\gamma D_m}{u} + \frac{(f)k d_f^2}{D_s} u + \frac{(f)k d_p^2}{D_m} u \quad \dots (2.7)$$

Where,  $L$  = length of the column (cm),  $N$  = number of theoretical plates,  $dp$  = diameter of the packing material ( $\mu\text{m}$ ),  $\lambda$  = constant which depends on column packing,  $\gamma$  = obstruction factor ( $>0.6$  for packed columns),  $D_m$  = diffusion coefficient of the analyte in the mobile phase ( $\text{cm}^2/\text{s}$ ),  $k$  = retention factor of the analyte,  $d_f^2$  = effective film thickness of the stationary phase ( $\mu\text{m}$ ),  $D_s$  = diffusion coefficient of the analyte in the stationary phase ( $\text{cm}^2/\text{s}$ ).

From equation (2.7) it is evident that the  $C_m$  and  $A$  terms depend on the diameter of the packing material. Thus, small uniform packing particles reduce the peak dispersion and enhance the efficiency of the column. Fast and efficient separations can therefore be obtained using smaller packing particles (equation 2.8).

$$t = \frac{(1 + k)Ld_p}{D_m v} \dots (2.8)$$

where,  $t$  = analysis time (s) and  $v$  = reduced velocity( $\text{cm}/\text{s}$ )

Very high pressure is required to push the mobile phase through a column packed with smaller diameter particles while maintaining the linear velocity. However, there can be a significant pressure drop ( $\Delta P$ ) across the column, which is given by [13]:

$$\Delta P = \frac{\Phi \eta u L}{d_p^2} \dots (2.9)$$

$\Delta P$  = pressure drop ( $\text{dyne}/\text{cm}^2$ ),  $L$  = length of the column (cm),  $\phi$  is the dimensionless flow resistance parameter, and  $\eta$  and  $\mu$  are the viscosity ( $\text{g}/\text{s cm}$ ) and the velocity ( $\text{cm}/\text{s}$ ) of the mobile phase respectively.

The optimum linear velocity ( $u_{opt}$ ) according to the reduced parameter analysis is inversely proportional to the particle size [6]. It is given by the equation:

$$u_{opt} = \frac{3D_m}{d_p} \dots (2.10)$$

We can conclude from equations (2.6) to (2.10) that smaller particle size decreases peak dispersion, enhances column efficiency and reduces analysis time with an increase in the pressure [14, 15].

#### *Effect of injection volume on efficiency*

Extra column band broadening needs to be reduced to improve efficiency. The maximum extra column broadening that is allowed for LC systems is given as [15]:

$$\sigma_{\epsilon}^2 (acc) \leq 0.10 \sigma_c^2 \leq 0.10 \pi^2 L^2 r^4 \epsilon^2 (1+k)^2 / N \dots (2.11)$$

Where,  $\sigma_{\epsilon}^2 (acc)$  = maximally acceptable variance due to extra coulumn band broadening,  $\sigma_c^2$  = peak variance caused by chromatographic process,  $L$  =length of the coulumn (cm),  $r$  = column radius ( $\mu\text{m}$ ),  $e$  = coulumn porosity,  $k$  = retention factor and  $N$  = efficiency of the column.

The variance due to injection volume is given as:

$$V_s^2 \approx 12 \sigma_{\epsilon}^2 (acc) \dots (2.12)$$

Where,  $V_s^2$  = variance due to injection volume

From equations 2.11 and 2.12 we can say that the injection volume decreases rapidly with a decrease in diameter and the length of the column. Efficiency also increases with decreasing injection volume.

## 2.4.2 System design

### a. *Pumps*

The main criterion for the pumps used in UHPLC is that they should deliver solvents reproducibly and smoothly at high operating pressures. They should also compensate for solvent compressibility (relative volume change with respect to pressure) in both the isocratic and gradient modes. Most of the commercially available systems automatically compensate for solvent compressibility. The other criterion is the delay volume. When using a gradient in very high-pressure systems, a small gradient delay volume is necessary. The delay volume in the commercially available UHPLC systems ranges from <10  $\mu\text{L}$  to 120  $\mu\text{L}$ .

### b. *Column and packing materials*

Packing materials can be divided into two major types: nonporous and porous silica particles. The properties of porous and non-porous particles such as pressure drop, sample loading, capacity and separation power were compared by Wu *et al.* [14,15]. According to Wu and coworkers, the nonporous particles can withstand higher pressures and have lower mass transfer resistance and higher efficiency than the porous particles. Despite these disadvantages, the porous particles have larger surface area and a high sample loading capacity. They also suggested that porous particles provide better separation of compounds that elute early whereas the compounds that elute later are

separated more efficiently by nonporous particles. The third stationary phase is the newly developed superficially porous or fused core silica particles. They are formed by fusing a 0.5 $\mu\text{m}$  porous silica layer onto a solid 1.7 $\mu\text{m}$  core ( $d_p = 2.7 \mu\text{m}$ ). These particles enable the use of higher flow rates with a decrease in the axial diffusion when compared to the totally porous particles [15]. De Steffano et al [16] have compared the performances of the fused core silica particles with sub 2  $\mu\text{m}$  particles. The authors found that higher efficiency is obtained for the fused core silica particles when compared to the totally porous particles. These authors also compared the reduced plate height (plate height divided by the particle diameter) of columns with totally porous particles and fused core particles. The reduced plate height was around 1.5 for the column with fused core particles (2.7  $\mu\text{m}$ ) as compared to 2 for the totally porous particles (3.5  $\mu\text{m}$  and 3  $\mu\text{m}$ ). This increase in the efficiency when using the fused core particles was attributed to the narrow particle size distribution of the material. The standard deviation for the particle size distribution was around 5-6% for the fused core particles and around 19% for the good quality 3 $\mu\text{m}$  totally porous particle. The other advantage of using the fused core particle is that it operates at a much lower backpressure (one half to one third) as compared to the other particles (due to the large particle size). This also allows the use of higher flow rates. The authors also studied the column loading capacity and the stability of the packed bed.

### *c. Sample injectors/Injection Valve*

A static split injection system was used by MacNair *et al.* [6, 7]. Although this injection valve enhanced column efficiencies and could withstand pressure as high as 100,000 psi, it had some disadvantages. Large sample volume and irreproducible and

long injections are a few of the limitations of the static split injection technique. Wu *et al.* [15] used a pressure balance injection system. This was found to be more efficient, reproducible and required a lower sample volume when compared to the static split technique. Also the injection process lasted for only a few seconds (1-3 s). The pressure balance injection system can withstand pressure only up to 15,000 psi. The third type of injection system is the passive feedback-switching valve, which is similar to the pressure balance system. This valve can withstand pressure up to 10,000 psi and has a lifetime greater than 4 months. Xiang *et al.* [17] used a new injection valve assembly that could operate at pressures as high as 30,000 psi. The authors found that the new injection valve was easier to operate, had better reproducibility, smaller injection volume and a shorter injection time when compared to the static split system.

## **2.5 LC-MS (Liquid Chromatography-Mass Spectrometry)**

### **2.5.1 Principles of mass spectrometry**

Mass spectrometric detection involves detection of analytes on basis of their mass to charge ratio. Mass spectrometric analysis consists of the following steps: producing gas-phase ions of the analytes (ion source) followed by separating the ions according to their masses (analyzer), fragmenting ions by collision (optional), detecting ions and measuring their mass/charge ratio (detector) and finally processing the data (computer) [18]. The ion source, analyzer and the detector used in this research are explained below. Mass spectrometry functions under high vacuum. This is because collisions against the walls would result in ions losing their charge, as well as, causing a deviation in the ion trajectory. Also, a poor vacuum can result in ion-molecule collisions producing

unwanted reactions and products. An approximate mean free path of an ion in a mass spectrometry can be obtained by using the following equation [18]:

$$\dots (2.13)$$

$L$  = length in cm and  $p$  = pressure in pascals (Pa). The mean free path of an ion in mass spectrometry should be at least 1 m.

### 2.5.2 Ion Source: Electrospray Ionization (ESI)

Electrospray ionization involves applying a strong electric field to a liquid (HPLC eluent in case of LC-MS) flowing through a capillary tube under atmospheric conditions. This electric field is obtained by applying a potential difference (3-6 kV) between the capillary tube and the counter electrode. The distance between the capillary tube and the counter electrode is between 0.3- 2 cm. The electric field causes charge accumulation at the liquid surface at the end of the capillary, which will break to form highly charged droplets. The drying gas evaporates the solvent in the droplets causing them to shrink. When the repelling forces come close to the cohesion forces, there is a columbic explosion. The droplets undergo series of ruptures resulting in smaller and smaller droplets. This breakdown of the droplets continues till a point where the repulsive columbic forces are great enough to overcome the forces of the surface tension of the droplet (Rayleigh limit) (equation 2.14).

$$q^2 = 8 \pi^2 \epsilon_0 \gamma D^3 \dots (2.14)$$

$q$  = charge;  $\epsilon_0$  = permittivity of the environment;  $\gamma$  = surface tension;  $D$  = diameter of spherical droplet

The ions then travel into the mass analyzer.

### **2.5.3 Mass analyzer: Single quadrupole analyzer**

Once the ions are produced, they are separated according to their masses in the mass analyzers. Quadrupole analyzers use the stability of the ion trajectories to separate them according to the mass/ charge ratio. The quadrupole analyzers are made up of four cylindrical rods and each pair of rods is connected electrically. A time dependent direct current (DC) and oscillating radio frequency RF voltages are applied to the rods. The RF voltages applied to the each pair of the rods are  $180^\circ$  out of phase. Typically, the DC voltage will vary from 5000 to 200 V whereas the RF voltage varies from zero to 3000 V. Paul and Steinweger at Bonn University were the first to describe the quadrupole principles in 1953 [19]. The ions travelling in the z-axis through the quadrupole are subjected to forces induced by electric fields in the x and y directions. This will cause the ions to travel in a three-dimensional sine wave through the center of the quadrupole. The equations to describe this propagation were established by Mathieu in 1866 [20]. These equations are not described in detail as it is beyond the scope of this dissertation. They mentioned as it establishes a relationship between time and the coordinates of an ion. Only ions with certain m/z have stable trajectory through the quadrupole. The other ions will strike the rods and discharge itself and will not be detected. Once the ions leave the mass analyzer they are sent to the ion detector.

#### **2.5.4 Ion Detector: Electron Multiplier (EM)**

Once the ion beam exits the mass analyzer, it enters the detector where it is detected and transformed into a usable signal. There are several methods for ion detection. But the most common detector is the electron multiplier (EM). The EM is shaped as a horn with either beryllium oxide or aluminium oxide coating on the surface [18]. When the ions strike the detector plate (conversion dynode), the energy is enough to dislodge electrons from the surface. These electrons move further into the EM, striking the walls, causing emission of more electrons as they pass through the gradient potential. A cascade of electrons is thus created which results in a measurable current at the end of the EM [18].

#### **2.5.5 Sample preparation: Solid phase extraction (SPE)**

Biological fluids, serum, plasma and urine cannot be directly injected into the LC system because of the presence of large amounts of proteins in these matrices. The total concentration of protein in the blood plasma and serum is approximately 6-8 g/dL [21]. The proteins can undergo denaturation and bind irreversibly to the column, causing reduced flow. The required sample preparation is assumed to be the rate-limiting step of any bioanalysis assay. Sample preparation involves multiple extraction procedures (centrifugation, filtration, solid liquid extractions), which are generally tedious and time consuming. The commonly used extraction techniques are Solid Phase Extraction (SPE), Liquid -Liquid extraction (LLE) and Protein Precipitation (PPT). SPE and LLE are predominantly used as they are robust and give a much cleaner extract. In this research, solid phase extraction was used. SPE is a technique in which the analytes are removed

by selective partitioning between solid and liquid phases. The separation principles are similar to those in HPLC. One major difference between the two techniques is that in HPLC there is a constant flow of the mobile phase whereas in SPE there is sequential loading and elution. The second difference being that the analytes in HPLC, migrate according to the  $k$  value (ideal Range: 2 -10) leading to their separation. In case of SPE, there is no separation of the analytes. The  $k$  values are several magnitudes greater which causes the analytes to be removed as a tight band. The SPE process generally consists of the following parts: conditioning, equilibration, loading, adsorption, washing and elution. The elution of the analytes is generally done in a volatile solvent like ethyl acetate. The solvent is then evaporated to dryness under a drying gas like nitrogen. The analytes are then dissolved in the appropriate solvent prior to its analysis in the LC-MS.

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## CHAPTER 3

### 3. DEVELOPMENT OF AN HPLC-UV METHOD FOR THE DETERMINATION OF PROSTANOIDS IN URINE MATRIX

#### 3.1 Introduction

Common laboratory methods for the analysis of prostanoids require multiple ELISA assays and/or application of variety of chromatographic methods. The common analytical methods used for the analysis of the prostanoids are immunoassays [1], GC-MS [2], LC-MS [3] and LC-MS/MS [4, 5]. The immunoassays though popular, suffer in reproducibility. They cannot be used for the simultaneous profiling of all the metabolites under study. GC-MS methods, on the other hand, have good sensitivity but require derivatization, which is laborious and time consuming. LC-MS/MS is considered to be the gold standard method for the analysis of the prostanoids. However, LC-MS/MS instrumentation can be very expensive and require significant expertise. In the last few decades there have been several simple HPLC-UV methods that have been published for the determination of a few of these prostanoids [6-10]. However, no methods exist for the panel of interest. Assays of AA acid metabolites are limited by availability, reliability and expense of instrumentation.

In this chapter, we report a single, sensitive and a straightforward HPLC assay for the rapid profiling of 8-iso PGF<sub>2α</sub>, PGE<sub>2</sub>, PGD<sub>2</sub>, PGF<sub>2</sub>, 6-keto PGF<sub>1α</sub> and 11-dehydro TXB<sub>2</sub>. We have applied our efforts to develop a chromatographic method that can be applied to this series of prostanoids important in the assessment of inflammatory disease.

## **3.2. Experimental**

### **3.2.1. Chemicals and Materials**

The prostaglandins (6-keto PGF<sub>1α</sub>, PGF<sub>2α</sub>, PGE<sub>2</sub>, 11-dehydro TXB<sub>2</sub> and PGD<sub>2</sub>) were purchased from Biomol (Plymouth meeting, PA, USA). 8-iso PGF<sub>2α</sub> was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Ortho Phosphoric acid, HPLC water, and HPLC acetonitrile were purchased from Fisher Scientific (Waltham, MA, USA).

### **3.2.2. Instruments and operating conditions**

The Jasco HPLC system consisting of the following parts was used; Jasco pumps (PU-980), a Jasco UV-VIS detector (UV-975) (Jasco Incorporated Easton, MD, USA) and a Rheodyne manual injector (Rheodyne LLC, Rohnert Park, CA, USA). Jasco-Borwin software (version 3.3.5) was used for data collection. SymmetryShield Rp18 4.6 x 250 mm column with 5 μm particle size (Waters Corporation, Milford, MA, USA) was used for the analysis. The HPLC method employed an isocratic elution of 17mM phosphoric acid (pH = 2.11) (solvent A) and acetonitrile (solvent B) in the ratio of 65:35 with a flow rate of 1.3 ml/min. The analytes were separated at ambient temperature with an injection volume of 100 μL. 196 nm was used for the detection. The run time for the method was 16.5 min. The Agilent 1100 series liquid chromatography-electrospray ionization-mass selective detector system was used for peak confirmation. The mass spectrometric parameters were as follows: drying gas flow rate 12 L/min, gas temperature 350 °C, nebulizer pressure 40 psig, fragmentor voltage 120 V and capillary voltage 3000 V. The chromatographic parameters were the same as in the HPLC-UV method. The prostanoids were detected in the negative ionization mode as (M-H)<sup>-</sup>.

### 3.2.3. Preparation of standard solutions and quality control (QC) samples

Working standards for all the prostanoids were prepared from the respective stock solutions by proper dilutions with the solvent (17mM phosphoric acid and acetonitrile in the ratio of 1:1). Calibration standards for the prostanoids were prepared at mass concentrations of 0.1, 0.2, 0.3, 0.5, 1, 2, 3, 5 and 10 ng/μL from the respective stock solutions. Quality control (QC) samples were prepared in triplicate for all the analytes representing low, medium and high concentrations in linearity curve.

### 3.2.4 Urine sample preparation

Prior to the analysis the urine samples were allowed to thaw at room temperature. 75μL of the thawed, filtered urine sample was diluted to 500 μL with the solvent (17mM phosphoric acid and acetonitrile in the ratio of 1:1) before injecting into the chromatographic system.

### 3.2.5 Method Validation

Given below are the method validation criteria as per the FDA guidance for the Industry: Bioanalytical method validation [11]:

1. The method should be *selective* so as to differentiate and quantify the analytes in presence of other endogenous compounds in the sample.
2. The *standard curve (linearity)* should include the entire range of expected concentrations.
3. *Accuracy and precision* of the mean should not exceed 15%.

4. *Recovery* of the analytes need not be 100% but should be precise and reproducible. A spiked recovery value between 80 and 120 % is considered acceptable.

5. *Matrix effect* and *stability* of the analytes at intended storage temperature should be evaluated.

#### 3.2.5.1 Selectivity

Five different urine samples were analyzed by both HPLC and LC-MS to determine peak selectivity. Solvent blanks were also analyzed to investigate possible interferences.

#### 3.2.5.2 Linearity and Sensitivity

The linearity for all the analytes was established by injecting in triplicate; standard solutions with mass concentration ranging from LOQ to 10 ng/ $\mu$ L. The linearity curve was obtained by plotting the peak areas of the prostanoids with the respective theoretical column loading (ng). The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as the lowest concentrations that give the signal to noise ratio as 3:1 and 10:1 respectively.

#### 3.2.5.3 Accuracy and Precision

Five solutions of each mass concentration of the QC samples were prepared and injected in triplicate to determine the accuracy (n = 15). The calculated concentrations for each of the samples were then compared to the theoretical concentration to determine the % accuracy for each of the compounds. Inter-assay and intra-assay precision were determined by injecting in triplicate; five replicate preparations of three mass

concentration of each analyte spiked in urine for a single day and for four consecutive days respectively.

#### 3.2.5.4 Spiked recovery and matrix effect

The recovery for the prostanoids was determined at three concentrations (low, medium and high) by spiking the urine samples from a control subject. Obtained concentrations were corrected for the presence of endogenous prostanoids. The recoveries were then calculated by comparing the corrected concentrations with the spiked theoretical concentrations.

To study the matrix effect three different lots of urine samples from control subjects were used. The matrix effect was examined by comparing the detector responses of the analytes in the spiked urine samples (B) to the corresponding detector response in the standard solutions (A) at three concentrations (low, medium and high). The absolute matrix effect was calculated using the formula  $(B/A \times 100)$  [12]. F test and the student's T test were also performed between the detector responses of the analytes spiked in urine and solvent to determine if the differences were statistically significant.

#### 3.2.5.5 Stability

Urine samples spiked with the prostanoids at two different concentrations were used for the stability study in mobile phase. The stability of 8-iso PGF<sub>2α</sub> in urine and mobile phase for three freeze thaw cycles, 24 hr at room temperature and on ice for 5 hr was studied. Stability on ice for 5 hr was studied for all the prostanoids. Stability of the stock solutions at room temperature and at -80°C was also evaluated.

### **3.3 Results And Discussion**

#### **3.3.1 Chromatography and UV detection**

6-keto PGF<sub>1α</sub> and 11-dehydro TXB<sub>2</sub> were used for the method development and validation instead of PGI<sub>2</sub> and TXA<sub>2</sub>. PGI<sub>2</sub> and TXA<sub>2</sub> are unstable at physiological pH and undergo hydrolysis to form 6-keto PGF<sub>1α</sub> and 11-dehydro TXB<sub>2</sub> respectively. Since PGI<sub>2</sub> and TXA<sub>2</sub> are present at very low concentrations due to this conversion, 6-keto PGF<sub>1α</sub> and 11-dehydro TXB<sub>2</sub> have been used as reliable biomarkers for their estimation [13, 14]. The prostanoids have weak UV chromophores (Figure 3.1) but it is possible to detect them at 200 nm. A wavelength of 254 nm was used up to 3 min to prevent the strong absorbance of highly polar urinary compounds such as creatinine and creatine phosphates and buffer solvent compositions, from obscuring the prostanoids because of signal scaling. At 3 min the wavelength is set to 196 nm for detection of prostanoids.

#### **3.3.2 Method validation**

##### **3.3.2.1 Selectivity**

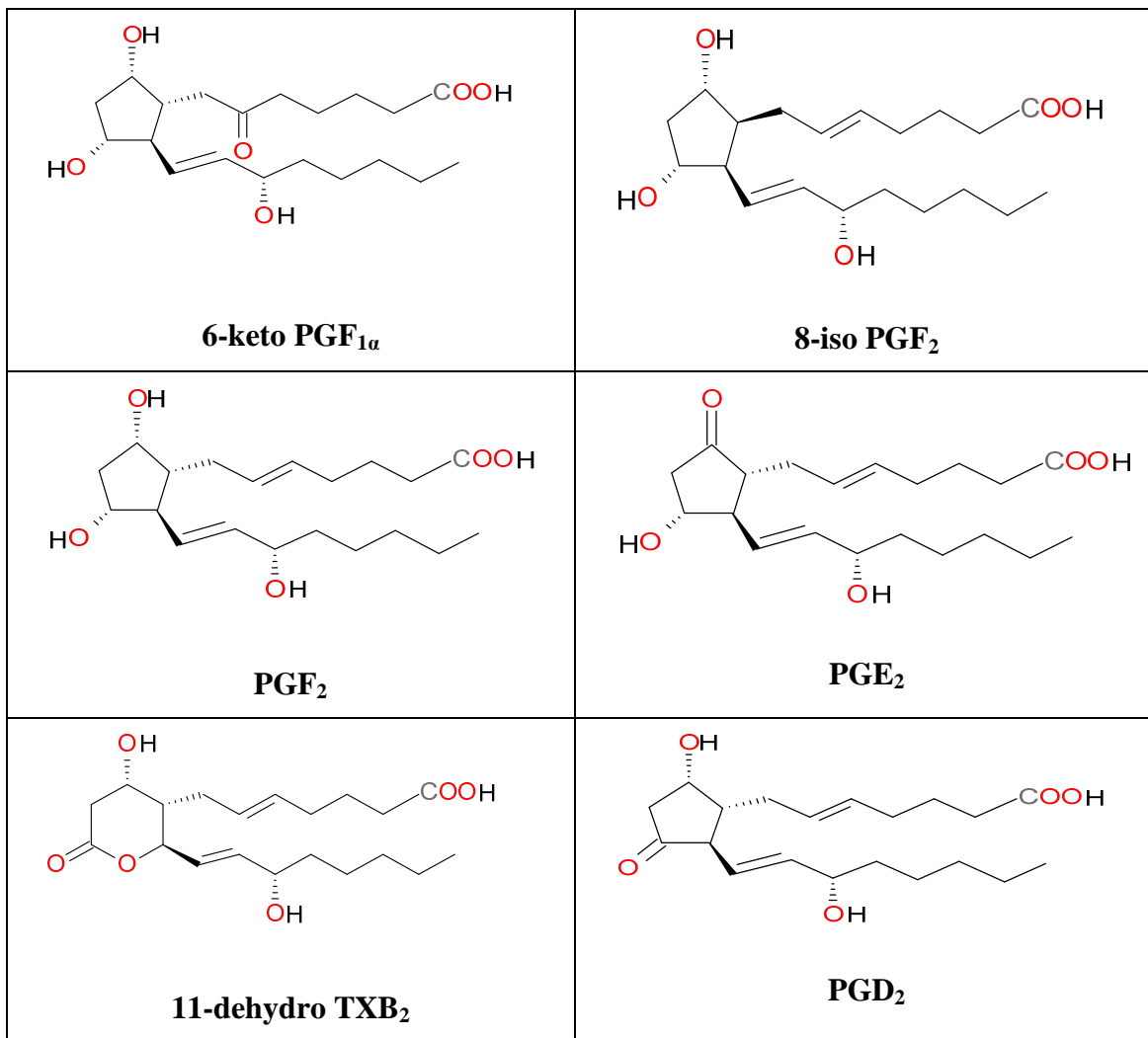
There are no interfering endogenous compounds in the urine at the retention times of the prostanoids. This was also confirmed by LC-MS. Peak purity analyses were performed for each prostanoid. All met the 99.99% purity criteria. A representative HPLC chromatogram of the blank solvent and standard solution are shown in Figure 3.2. The resolution, asymmetry and the retention times of each prostanoids in the solvent and the urine are summarized in Table 3.1.

### 3.3.2.2 Linearity and Sensitivity

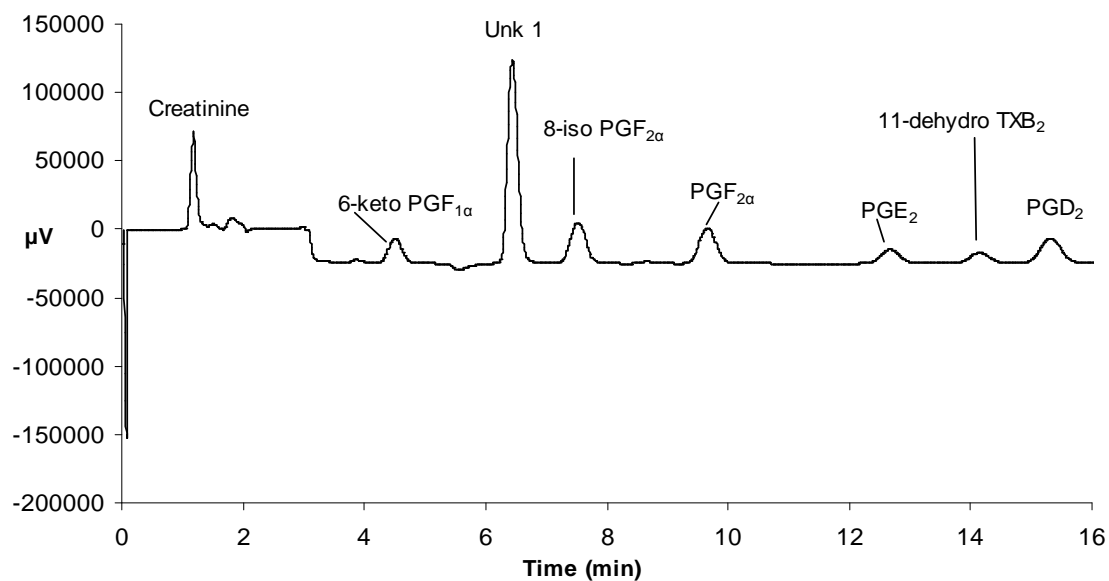
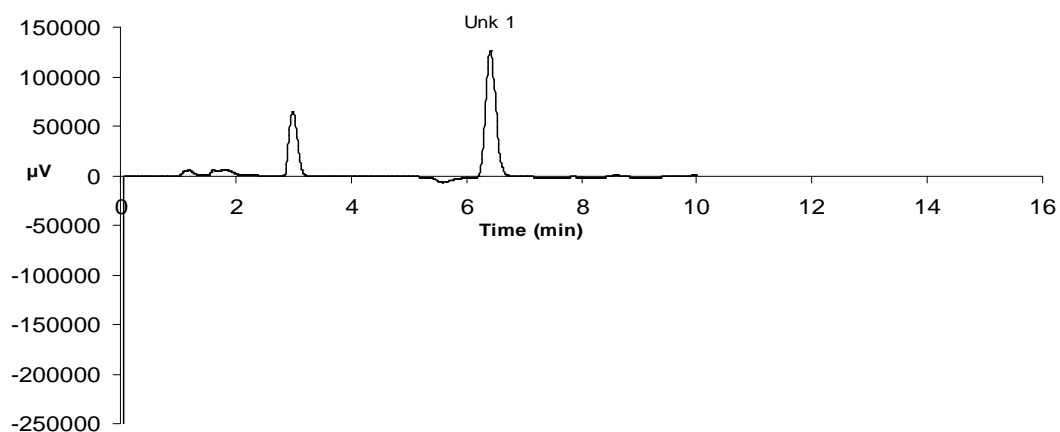
All the compounds show a good linear response with  $R^2 > 0.999$  in the linear range as mentioned in Table 3.2. The LOD and the LOQ are given in terms of concentrations (ng/  $\mu$ L). The sensitivity for all the analytes are in the nanogram range demonstrating that this HPLC method can be used to analyze prostanoids as their concentrations are sufficiently high in urine. However, if necessary, urine samples can be concentrated easily, reducing concerns about method sensitivity.

### 3.3.2.3 Accuracy and Precision

The % accuracy for all the prostanoids were between 90.3 - 112.8 % (Table 6.3), meeting the requirement as per the FDA guidance for the Industry: Bioanalytical Method Validation [10]. The method meets precision criteria as the % RSD for the system suitability, intra-assay (Table 3.4) and inter-assay precision (Table 3.4) for all analytes lie within 10%.



**Figure 3.1 Structure of prostanoids**



**Figure 3.2. Representative chromatogram of blank solvent and standard solution of prostanoids. (Note-Wavelength change from 254nm to 196 nm causes shift in baseline at approximately three minutes)**

**Table 3.1. Method Validation Results: Selectivity**

Compound	RT (min)		Asymmetry ( $A_s$ )		Resolution	
	Solvent	Urine	Solvent	Urine	Std's	Urine
6-keto PGF <sub>1α</sub>	4.51	4.34	1.03	1.45	10.53	3.54
8-iso PGF <sub>2α</sub>	7.53	7.37	1.08	1.15	7.99	3.79
PGF <sub>2</sub>	9.66	9.09	1.06	1.73	4.43	6.84
PGE <sub>2</sub>	12.67	12.60	1.04	1.15	5.34	4.74
11-dehydro TXB <sub>2</sub>	14.1	13.89	1.02	1.25	2.25	1.64
PGD <sub>2</sub>	15.32	15.52	1.05	1.37	1.60	1.65

**Table 3.2. Method Validation Results: Sensitivity & Linearity**

<b>Compound</b>	<b>LOD (ng/ μL)</b>	<b>LOQ (ng/ μL)</b>	<b>No.of data points (n)</b>	<b>Linear range</b>	<b>Equation of the line</b>	<b>R<sup>2</sup></b>
6-keto PGF <sub>1α</sub>	0.12	0.2	10	0.12- 10.52	y = 907.5x - 7699.7	0.999
8-iso PGF <sub>2α</sub>	0.10	0.27	9	0.30- 10.00	y = 1584.0x + 1899.5	0.999
PGF <sub>2</sub>	0.22	0.08	10	0.11- 10.52	y = 2189.6x - 4975.1	1.000
PGE <sub>2</sub>	0.10	0.25	8	0.26- 10.52	y = 848.8 + 9.7	1.000
11-dehydro TXB <sub>2</sub>	0.08	0.30	8	0.30- 10.00	y = 699.3x + 10154.0	0.999
PGD <sub>2</sub>	0.04	0.13	9	0.10- 10.00	y = 1450.4x + 8921.1	0.999

#### 3.3.2.4 Spiked Recovery and matrix effect

The % recoveries for all prostanoids at three concentration levels were within 95-108%. These values fall within  $\pm 15\%$ , which is the valid limit established by FDA [11]. The absolute matrix effect of the analytes was also found to be within  $\pm 15\%$ . The values obtained for the matrix effect indicate the changes in the response of the analytes in presence of other compounds in the matrix. They are found to be within the acceptable limits of recovery. There is no specific acceptable limit for the matrix effect in biological samples [11]. Also, the  $p$  values obtained from the student's  $t$  test for all the analytes in the matrix compared with the solvent were found to be greater than 0.05 indicating that the differences are not statistically significant.

#### 3.3.2.5 Stability

We studied the stability of 8-iso  $\text{PGF}_{2\alpha}$  in urine and mobile phase for three freeze thaw cycles, 24 hr at room temperature and on ice for 5 hr. The results obtained demonstrated that 8-iso  $\text{PGF}_{2\alpha}$  is stable in all the three conditions in urine matrix but only stable on ice for 5 hr in mobile phase (Table 3.5). 8-iso  $\text{PGF}_{2\alpha}$  in the urine matrix is found to be stable up to 10 days at  $-80^\circ\text{C}$  [15]. Since all the prostanoids have similar structures and properties and are known to be stable up to 48 hours in urine, our study focused on laboratory conditions that is stability in mobile phase. Therefore, stability at  $0^\circ\text{C}$  of the prostanoids in mobile phase was determined as a necessary condition of the method. The results shown in Table 3.6 demonstrate that the prostanoids are stable on ice for 5 hrs. The differences fall within the limits of precision of the method ( $\pm 15\%$ ) [10]. Also, since

the method relies on manual injection, post preparative stability i.e. auto-sampler stability is not studied. Stock solution of all the prostanoids are found to be stable up to 12 hrs at room temperature and upto 48 hrs at -80°C.

### **3.4 Conclusion**

The HPLC UV method for the simultaneous determination of urinary prostanoids (8-iso PGF<sub>2α</sub>, PGE<sub>2</sub>, PGD<sub>2</sub>, PGF<sub>2</sub>, 6-keto PGF<sub>1α</sub> and 11-dehydro TXB<sub>2</sub>) was developed and validated. All the prostanoids can be determined in a single assay in less than 17 minutes using UV detection. These conditions make this a feasible and widely applicable method. This method can be performed in any common laboratory or clinical setting where basic HPLC instrumentation is available. Common stationary and mobile phases were used as well. 8-iso PGF<sub>2α</sub>, PGE<sub>2</sub>, PGD<sub>2</sub>, PGF<sub>2</sub>, 6-keto PGF<sub>1α</sub> and 11-dehydro TXB<sub>2</sub> were separated on a SymmetryShield Rp18 column (250mm × 4.6mm) by an isocratic elution of 17 mM phosphoric acid and acetonitrile in the ratio of 65:35 and at a flow rate of 1.3 ml/min. The wavelength used for detection was 196 nm. Specificity was confirmed by LC-MS. The method was fully validated and was found to be having sufficient sensitivity (limit of quantification - 7.5 ng - 30 ng) for many biological matrices and applications. The accuracy and precision were within bioanalytical method validation limits (90.3 to 112.8 % and RSD < 10%, respectively) and the method was linear over three orders of magnitude. According to our knowledge, compared to the techniques available or published, this method is cheaper and simpler and can be used by all.

**Table 3.3. Method Validation Results: Accuracy levels I, II &III represents low, medium & high concentration respectively of the linearity range**

Compound	Accuracy Level I		Accuracy Level II		Accuracy Level III	
	Conc. (ng/ μL)	%Accuracy (n=15)	Conc. (ng/ μL)	%Accuracy (n=15)	Conc. (ng/ μL)	%Accuracy (n=15)
6-keto PGF <sub>1α</sub>	0.53	98.2	0.79	98.9	1.05	101.5
8-iso PGF <sub>2α</sub>	0.50	102.9	5	103.5	8	99.8
PGF <sub>2</sub>	0.53	97.0	1.05	97.3	5.25	99.8
PGE <sub>2</sub>	0.53	97.3	1.05	107.7	5.25	100.5
11-dehydro TXB <sub>2</sub>	0.50	100.1	1.00	90.3	5.00	92.8
PGD <sub>2</sub>	0.53	102.1	1.05	106.2	5.25	112.8

**Table 3.4. Method Validation Results: Intra assay and inter assay precision**

<b>Compound</b>	<b>Conc. (ng/<math>\mu</math>L)</b>	<b>Intra-Assay precision %RSD</b>	<b>Inter-Assay precision %RSD</b>
6-keto PGF <sub>1<math>\alpha</math></sub>	0.53	8.22	5.61
	0.79	5.34	4.89
	1.05	4.16	2.22
8-iso PGF <sub>2<math>\alpha</math></sub>	0.50	8.15	4.92
	5.00	2.40	2.96
	8.00	3.02	2.19
PGF <sub>2</sub>	0.53	7.05	1.83
	1.05	2.62	4.20
	5.25	8.91	9.42
PGE <sub>2</sub>	0.53	3.74	5.31
	1.05	6.21	2.82
	5.25	3.89	4.02
11-dehydro	0.50	6.60	9.01
TXB <sub>2</sub>	1.00	3.06	4.21
	5.00	2.97	5.44
PGD <sub>2</sub>	0.53	2.17	2.30
	1.05	2.67	2.97
	5.25	2.30	6.17

**Table 3.5. Method Validation Results: Stability study of 8-iso PGF<sub>2α</sub> in mobile phase**

	<b>Spiked value</b>	<b>Room Temperature (24 hrs)</b>	<b>On Ice (5 hrs)</b>	<b>Freeze Thaw (3 cycles)</b>
Compound	Conc. (ng/μL)	%loss/gain (n=9)	%loss/gain (n=9)	%loss/gain (n=9)
8-iso PGF <sub>2α</sub>	0.30	-19.2	+2.3	-41.9
	2.50	-37.7	+8.7	-36.1

**Table 3.6. Method Validation: Stability studies in mobile phase**

<b>Compounds</b>	<b>Spiked value</b>	<b>On Ice</b>
	<b>Conc.</b>	<b>(5 hrs)</b>
	<b>(ng/<math>\mu</math>L)</b>	<b>%loss/gain</b>
		<b>(n=9)</b>
6-keto PGF <sub>1<math>\alpha</math></sub>	10.00	-4%
	1.00	-2%
8-iso PGF <sub>2<math>\alpha</math></sub>	0.30	+2.3
	2.5	+8.7
PGF <sub>2</sub>	10.00	-5%
	0.10	+2%
PGE <sub>2</sub>	10.00	-9%
	1.00	-13%
11-dehydro TXB <sub>2</sub>	10.00	-0.3%
	1.00	+9%
PGD <sub>2</sub>	10.00	-14%
	1.00	+5%

### 3.5 References

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## CHAPTER 4

### 4. DEVELOPMENT OF AN HPLC-UV METHOD FOR THE SIMULTANEOUS DETERMINATION OF CREATININE AND PROSTANOIDS

#### 4.1 Introduction

Chapter 4 concentrates on the determination of creatinine by the chromatographic method developed and validated for the estimation of prostanoids (Chapter 3).

It is necessary to monitor creatinine levels in addition to biomarkers, when the measurement is done in urine. Creatinine is formed by non-enzymatic dehydration and loss of phosphate from creatine and creatine phosphate, which are generally found in the muscle and blood [1]. It is excreted through the kidney at a nearly constant rate. As a result, measurement of the creatinine levels in urine is considered to be a reliable marker in assessing the kidney function [2]. Since the volume of urine does not remain constant, the concentration of the compounds in urine will vary. Because of normal concentration variations due to varying patient levels of hydration, the ratio of 8-isoprostane to creatinine provides a valid assessment of oxidative stress [3]. The normal range of creatinine levels as per the World Health Organization (WHO) is 30mg/dL to 300 mg/dL [4]. High creatinine levels generally indicate that the kidneys are not functioning properly. On the other hand low creatinine levels (< 20mg/dL) indicate possible dilution of the urine sample or kidneys can be damaged in the course of diseases that include diabetes and hypertension. Thus, the creatinine levels can indicate the state of kidney health and risk.

There are several methods that have been reported for the estimation of creatinine and prostanoids individually. The determination and the quantification of prostanoids are explained in great detail in Chapter 3. Creatinine can be analyzed by enzyme less electrosensor [5], ion chromatography [6], capillary electrophoresis [7], HPLC [8], LC-MS [9], and LC-MS/MS [10]. Jaffe reaction is the commonly used method for the estimation of urinary creatinine. This method involves the colorimetric determination of a complex formed as result of the reaction between creatinine and picric acid under alkaline conditions [11]. The method, though very simple, is less selective [10]. GC-MS with isotope dilution procedure is also frequently used for the determination of urinary creatinine. The drawback of this method is that it is time consuming as it involves derivatization of creatinine and then separation by cation exchange column. The LC-MS and LC-MS/MS methods on the other hand do not require derivitization or any pretreatment before the introduction into the mass spectrometer but these methods involve the use of internal standards that can be expensive. The general practice for the determination of urinary prostanoids involves separate determination of prostanoids and creatinine by different analytical and then reporting the amount in terms of creatinine (ng/mg of creatinine).

In this chapter, we report a single, sensitive and a straightforward HPLC assay for the rapid profiling of prostanoids and creatinine in urine. The advantages of the HPLC method are that it can be used for the simultaneous determination of creatinine and prostanoids from a single urine sample, thus saving time and also the UV detection makes the method transportable and cheap. As a single assay, in which the sample is directly analyzed, risk of contamination is minimal. To our best knowledge, this is one of

the first fully validated HPLC UV methods that have been reported for the simultaneous determination of creatinine and prostanoids in urine.

## **4.2. Experimental**

### **4.2.1. Chemicals and Materials**

Creatinine was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ortho Phosphoric acid, HPLC water, and acetonitrile were purchased from Fisher Scientific (Waltham, MA, USA). ELISA assay kits for creatinine were purchased from R & D systems (Minneapolis, MN 55413).

### **4.2.2. Instruments and operating conditions**

The Jasco HPLC system consisting of the following parts was used; Jasco pumps (PU-980), a Jasco UV-VIS detector (UV-975) (Jasco Incorporated Easton, MD, USA) and a Rheodyne manual injector (Rheodyne LLC, Rohnert Park, CA, USA). Jasco-Borwin software (version 3.3.5) was used for data collection. SymmetryShield Rp18 4.6 x 250 mm column with 5  $\mu$ m particle size (Waters Corporation, Milford, MA, USA) was used for the analysis. The HPLC method employed an isocratic elution of 17mM phosphoric acid (pH = 2.11) (solvent A) and acetonitrile (solvent B) in the ratio of 65:35 with a flow rate of 1.3 ml/min. The analytes were separated at ambient temperature with an injection volume of 100  $\mu$ L. The wavelength gradient employed was 254 nm, 0.0- 3.0 min and 196 nm, 3.1 -16.5 min. The run time for the method was 16.5 min. The Agilent 1100 series liquid chromatography-electrospray ionization-mass selective detector system was used for peak confirmation. The mass spectrometric parameters were as follows:

drying gas flow rate 12 L/min, gas temperature 350 °C, nebulizer pressure 40 psig, fragmentor voltage 120 V and capillary voltage 3000 V. The chromatographic parameters were the same as in the HPLC-UV method. Creatinine was detected in the positive ionization mode (M-H)<sup>+</sup>.

#### **4.2.3. Preparation of standard solutions and quality control (QC) samples**

Calibration standards for creatinine were prepared at concentrations of 5, 10, 50, 100, 200, 300, 500, 750, 1000, 2000, 2500, 3000 and 4000 ng/μl from a stock solution of 10,000 ng/μl by proper dilution with the 1:1 mixture. Quality control (QC) samples were prepared in triplicate for all the analytes representing low, medium and high concentrations in linearity curve.

#### **4.2.4 Urine sample preparation**

Prior to the analysis the urine samples were allowed to thaw at room temperature. 75μL of the thawed, filtered urine sample was diluted to 500 μL with the solvent (17mM phosphoric acid and acetonitrile in the ratio of 1:1) before injecting into the chromatographic system.

#### **4.2.5 Method validation**

The method was validated as per the FDA guidance for Industry: Bioanalytical method validation [12].

#### 4.2.5.1 Selectivity and Sensitivity

Selectivity was established by both HPLC and the LC-MS. Peak selectivity was confirmed by injecting creatinine dissolved in the 1:1 mixture. Solvent blanks were also analyzed. Peak selectivity of creatinine in urine was confirmed by using the same method on the Agilent 1100 series Liquid chromatography - electro spray ionization-mass selective detector system. Creatinine was detected as  $(M+H)^+$  ( $m/z = 114$ ) in the positive ionization mode. The sensitivity of the method is demonstrated by estimating the limit of detection (LOD) and the limit of quantification (LOQ) for creatinine. LOD and LOQ were defined as the concentration where the peak response was three times and ten times that of the noise levels respectively.

#### 4.2.5.2 Linearity, Accuracy and Spiked Recovery

Creatinine was validated for linearity from LOQ to the detector maximum. The correlation coefficient and the equation of the line were obtained by plotting the peak areas of the standard solutions against the respective theoretical concentrations. Accuracy was determined by the analysis of the Quality control samples. Three solutions of each mass concentration were prepared and injected in triplicate. Recovery for each concentration was determined by using the equation of the line obtained from the linearity data. Analytical recoveries for creatinine were obtained at three different concentrations levels, 0.3 ng/ $\mu$ l, 1 ng/ $\mu$ l and 2.5 ng/ $\mu$ l (low, medium and high) by spiking the urine sample already containing these analytes. Recovery rates were calculated by using the equation  $[C_{\text{calculated}} / C_{\text{nominal}}] \times 100$ .

#### 4.2.5.3 Precision

Instrument precision was determined by injecting six times a single mixture containing all the compounds, and then calculating the %RSD. Intra-assay precision was also performed by injecting in triplicate; three different standard preparations of two mass concentrations for each of the analyte. In addition, inter assay precision was determined by injecting in triplicate a single mass concentration of each analyte for four consecutive days.

#### 4.2.5.4 Stability Studies

Urine samples spiked with creatinine at two different concentrations were used for the stability studies. The stability of creatinine in urine was tested for 3 freeze thaw cycles. Each freeze thaw cycle consisted of keeping the samples frozen at -80 °C for 24 hrs and then thawing them at room temperature. Stability studies were also carried out at room temperature for 24 hrs and on ice for 5 hrs.

#### **4.2.6 ELISA Method (Jaffe Method) for creatinine determination**

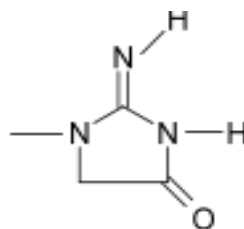
The ELISA assay kit from R&D systems, which uses the principle of the Jaffe colometric method, was used for the comparison study. The basic principle of the Jaffe reaction is the formation of an orange-red complex due to the reaction between creatinine and alkaline picrate solution. We followed the standard ELISA protocol. The standards and urine samples were prepared as per the procedure provided with the kit. The diluted standards and urine samples along with the alkaline picrate reagent were added to the microplate and incubated for around 30 minutes. The optical density, which was measured at 490 nm, is directly proportional to the concentration of creatinine present in

the sample. The unknown concentration in the urine samples was calculated by using the standard curve. Accuracy was determined by analyzing creatinine standard solutions at three different concentrations 12.4 mg/dL, 50 mg/dL and 400 mg/dL). Recovery of each standard solution was determined by using the equation of the line obtained from the standard curve. Analytical recoveries were also obtained by spiking the urine sample at two different concentrations levels (100 mg/dL and 500 mg/dL) and calculating the % recovery by using the equation  $[C_{\text{calculated}} / C_{\text{nominal}}] \times 100$ .

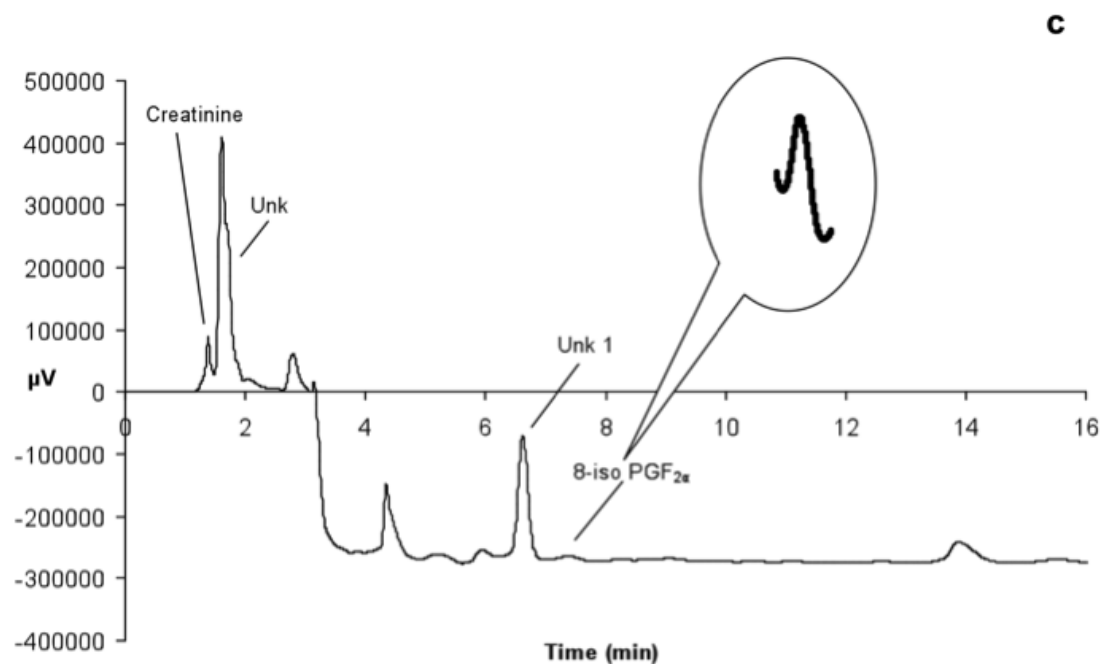
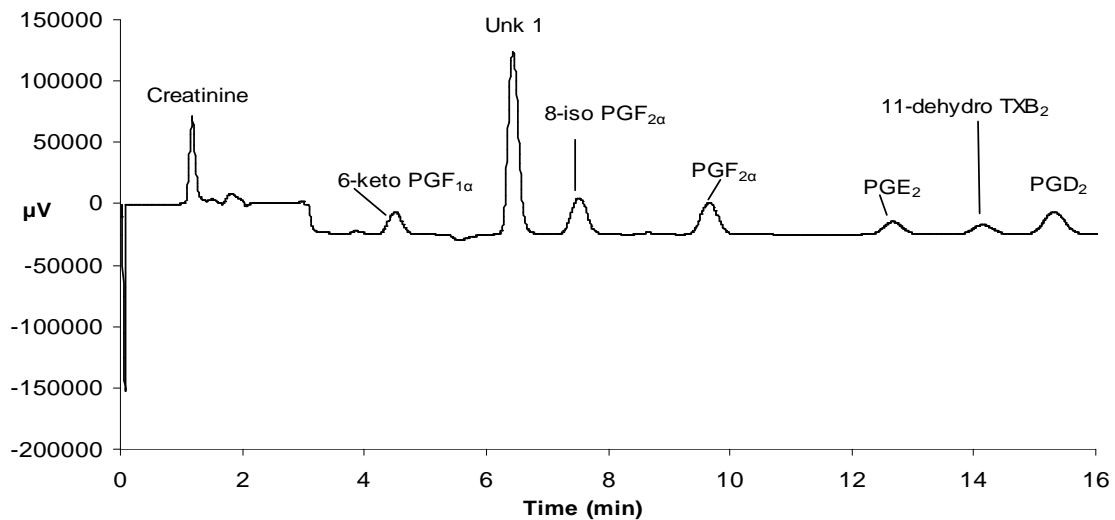
### 4.3 Results and Discussion

The development of an HPLC UV method for simultaneous determination of creatinine and prostanids was challenging due to the differences in their polarity and absorption characteristics. Prostanoids are compounds that do not have very active UV chromophores. This is because of the presence of a five-membered ring structure with no conjugated  $\pi$  bonds. In addition to this, the functional groups such as the hydroxy and keto have very little UV absorbance. Hence, these compounds can be detected only at very low wavelengths ( $< 200$  nm). Creatinine (2-amino-1-methyl-5*H*-imidazol-4-one), (Figure 4.1) on the other hand has very high absorbance at wavelengths below 240 nm such that the absorbance reaches the detector maximum even at extremely low concentrations. Consequently, a wavelength gradient had to be used for the simultaneous determination of these compounds. Creatinine was detected at 254 nm while the prostanoids were detected at 196 nm. 254 nm was chosen as the ideal wavelength of detection so that the method could be validated for the entire concentration range of creatinine, which would normally be present in the urine (30 mg/dL - 400 mg/dL). The other problem faced during the development of the UV method was that most of the

organic solvents used in HPLC have their UV cut -off above 200 nm except for acetonitrile, which has a UV cut -off of 190 nm. Hence, acetonitrile was used as the organic phase. Phosphoric acid solution (pH =2) was used as the aqueous phase as it gave much better chromatographic results than acetic acid solution. An acidic solution (pH =2) was necessary to maintain the prostanoids in the neutral state. Creatinine, at pH =2 which is below its pKa value (4.83) will be in the protonated form [13]. Creatinine in this form will be retained for a very short period of time on a non- polar stationary phase compared to prostanoids, which are in the neutral form. The flow rate and ratio of the aqueous and organic phase were chosen to obtain a method with a practical run time and which resulted in good separation of all the compounds. All the chromatograms showed a negative drop at around 3.0 min (Figure 4.2). This is due to the change in the wavelength from 254 nm to 196 nm.



**Figure 4.1. Structure of creatinine**



**Figure 4.2. Representative chromatogram of creatinine and prostanoids in solvent and urine matrix (Note-Wavelength change from 254nm to 196 nm causes shift in baseline at approximately three minutes)**

### 4.3.1 Method Validation

#### 4.3.1.1 Selectivity and Sensitivity

The retention time, resolution and asymmetry for all the peaks in a solution of pure standards and also in the urine sample spiked with the pure standards are summarized in Table 4.1. Figure 4.2 is a representative chromatogram of a standard solution and urine matrix. Solvent blanks were analyzed and they showed no significant interferences in the vicinity of the analyte peaks. There is a slight variation in the retention time of the compounds in the urine matrix as compared to that in the solvent as can be seen from Table 4.1. We compared the retention times statistically by the student's t test [14]. Differences with  $P < 0.05$  were considered to be significant. P values for all except creatinine were found to be greater than 0.05. These variations in retention time of creatinine can be attributed to the changes in the pH of the urine samples [15]. The sensitivity of the method is demonstrated by estimating the limit of detection (LOD) and the limit of quantification (LOQ) for creatinine. LOD and the LOQ values for creatinine in terms of column loading are given in Table 4.2.

#### 4.3.1.2 Linearity and Accuracy and Spiked Recovery

Creatinine showed a good linear response with  $R^2 > 0.999$  in the linear range as mentioned in Table 4.2. The % accuracy for creatinine at three concentration levels are given in Table 4.3. The % recovery for creatinine falls within  $\pm 15\%$  which is an acceptable limit for bioanalytical method validation (Table 4.4).

**Table 4.1. Method Validation Results: Selectivity**

Compound	RT(min)	Asymmetry( $A_s$ )			
		$A_s = W_{2/2} / W_{1/2}$			
		$W_{1/2}$ = peak first half width			
		$W_{2/2}$ = peak second half width at 10%			
	Solvent	Urine	Solvent	Urine	
Creatinine	1.18	1.38	0.80	0.70	

**Table 4.2. Method Validation Results: Sensitivity and Linearity**

Compound	LOD	LOQ	Linear range (ng/ $\mu$ L)	Equation of the line	$R^2$
	Conc. (ng/ $\mu$ L)	Conc. (ng/ $\mu$ L)			
Creatinine	1	3	5- 4000	$y = 17656.9x + 9986.3$	0.998

**Table 4.3. Method Validation: Accuracy Level I, II and III represents the low, medium and high concentrations respectively, of the linearity range**

Compound	Accuracy Level I		Accuracy Level II		Accuracy Level III	
	Conc.	%Recovery	Conc.	%Recovery	Conc.	%Recovery
	(ng/ μL)	(n=9)	(ng/ μL)	(n=9)	(ng/ μL)	(n=9)
Creatinine	50	99.2	1000	93.9	3500	105.3

**Table 4.4. Method Validation: Spiked Recovery Levels I, II and III represents the low, medium and high concentrations respectively, of the linearity range**

Compound	Accuracy Level I		Accuracy Level II		Accuracy Level III	
	Spiked		Spiked		Spiked	
	Conc.	%Recovery	Conc.	%Recovery	Conc.	%Recovery
	(ng/ μL)	(n =9)	(ng/ μL)	(n=9)	(ng/ μL)	(n=9)
Creatinine	0.3	102.4	1	99.7	2.5	88.4

#### 4.3.1.3 Precision

The results obtained for the instrument precision or system suitability, Intra-assay precision and Inter-assay precisions are given in Table 4.5. RSD values for the instrument precision were found to be less than 5% for creatinine and less than 10% for both the intra-assay and the inter-assay precision.

#### 4.3.1.4 Stability Studies

As can be seen from Table 4.6, creatinine in the urine samples is stable at all the three conditions. On an average the change observed over time is around 6%.

#### 4.3.1.5 Method Comparison HPLC vs ELISA

We measured creatinine levels in urine samples from six hypertensive subjects by the HPLC method described in the manuscript and the Jaffe method (Elisa Assay). Urine sample was analyzed in triplicate by both the methods. The results obtained by the two methods were compared statistically by the paired Student's t-test to test the differences between the samples [16]. Differences with  $P < 0.05$  are considered to be significant. As can be seen from Table 4.7, the P values for all the samples were found to be less than 0.05. We also compared the accuracy of the standards and the spiked recovery of creatinine in urine obtained by the two methods. The accuracy of the standards for both the methods are found to be comparable and are given in Table 4.8. The % recovery for creatinine spiked in urine falls within  $\pm 15\%$  for the HPLC method, which is an acceptable limit for bioanalytical method validation. On the other hand, % recovery of creatinine decreases with increasing concentration in the ELISA method.

**Table 4.5. Method Validation: Instrument Precision Intra-Assay and Inter-Assay Precision**

Compound	Conc. (ng/ $\mu$ L)	Instrument	Intra-Assay	Inter-Assay
		precision %RSD (n=6)	precision %RSD (n=15)	precision %RSD (n=15)
Creatinine	25	4.94	5.43	3.55
	500	-	4.94	3.24
	1000	-	1.95	2.67

**Table 4.6. Method Validation: Stability studies**

Compounds	Spiked Conc. (ng/ $\mu$ L)	Room	On Ice	Freeze Thaw
		Temperature (24 hrs)	(5 hrs)	(3 cycles)
		%loss/gain (n=9)	%loss/gain (n=9)	%loss/gain (n=9)
Creatinine	0.1	+ 5.2	+4.7	+1.5
	10	+2.5	+10.7	+12.8

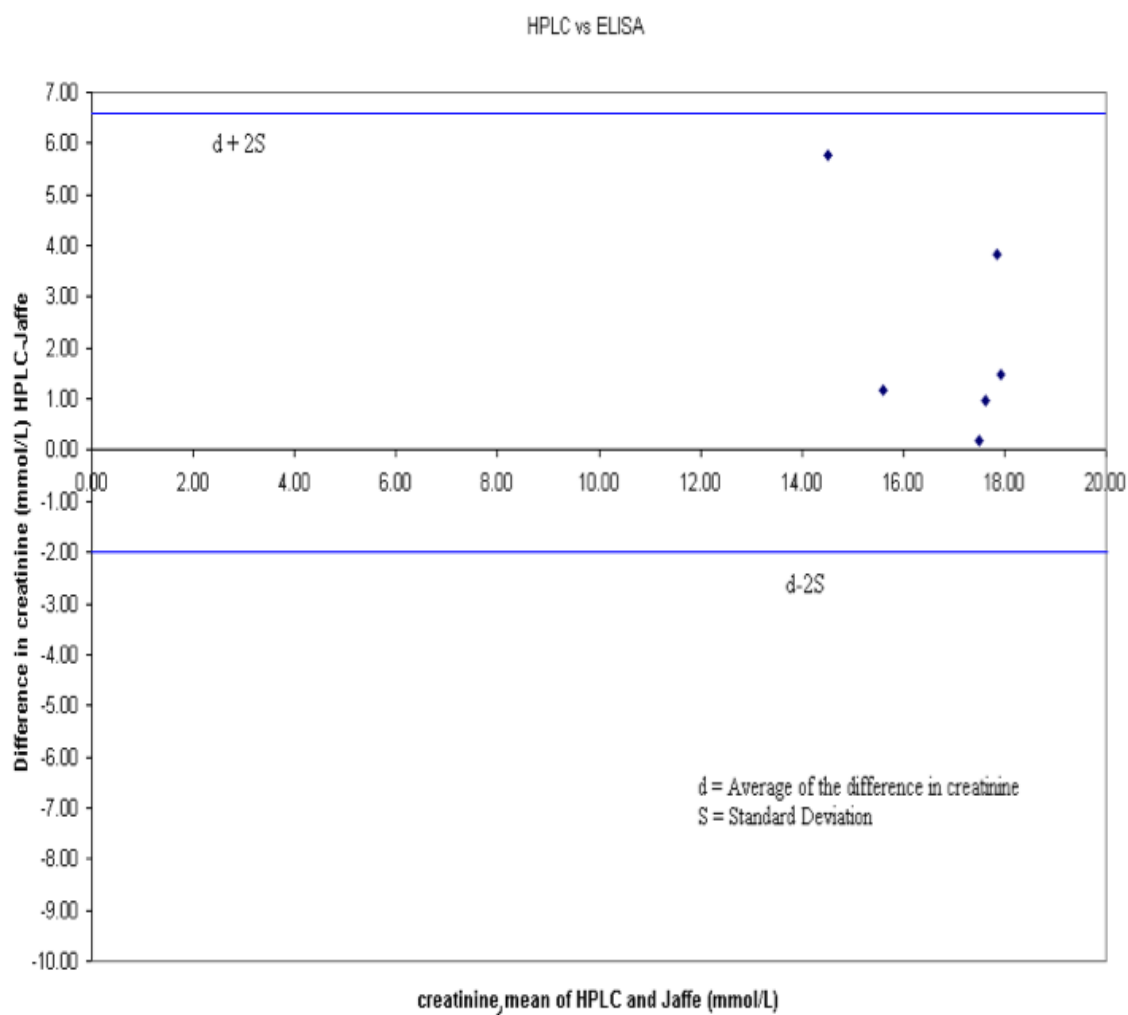
Though the results from both the methods are comparable, urinary creatinine concentrations obtained by Jaffe method was lower than that obtained by the HPLC method. The results (Table 4.7) obtained by the two methods were compared by plotting the difference for creatinine measurement (HPLC-Jaffe) vs. mean concentrations (mmol/L) as per Bland Altman plot (Figure 4.3) [17]. This was done to assess the agreement between the two methods. All measurements lie within the 95% limits of agreement ( $d \pm 2S$ ), which suggests the two methods are in good agreement with each other [17]. The results obtained by the two methods were also compared statistically by the paired t test. As can be seen from the results compiled in Table 7, the t values for two sets of observation exceed the tabulated value,  $t = 2.920$  at 95 % confidence level for two degrees of freedom. We can thus conclude that, the two methods are significantly different and showed an increase in deviation with increasing concentration. The discrepancies observed at higher creatinine concentration can be attributed to the increase in the interfering compounds with the increasing creatinine concentration. The effect of the impurities would result in low values when using the Jaffe method [18]. This may also be the reason for the low recovery value for creatinine in urine at high concentrations. Thus we can conclude that the two methods are significantly different for creatinine determination and that the HPLC method gives much accurate results at higher concentrations in the urine matrix.

**Table 4.7. Comparison data for creatinine levels in urine obtained from HPLC-UV and ELISA (Note:  $t_{\text{tab}} = 2.920$  @ 95% CI)**

Subjects	HPLC-UV			Elisa (Jaffe method)			t value	P value (2 tailed)
	(mg/dL)	(mg/dL)	(mg/dL)	(mg/dL)	(mg/dL)	(mg/dL)		
1	80.77	74.07	76.04	112.33	111.70	108.41	4.44	0.007
2	97.92	97.55	99.38	93.93	93.30	95.51	4.38	0.007
3	190.09	198.08	188.42	171.68	167.42	168.96	4.47	0.007
4	301.51	306.88	305.42	199.95	195.10	200.38	4.23	0.008
5	325.61	304.23	320.09	248.32	250.36	248.32	4.47	0.007

**Table 4.8. Comparison data for % spiked recovery of creatinine from standard solutions and urine matrix. “Urine Blank” is the urine from a normotensive patient.**

Recovery from Standard Solutions				Recovery from “Urine Blank”		
Method	Std Soln Conc. (mg/dL)	Spiked Conc. (mg/dL)	% Recovery (n =9)	Conc in urine (mg/dL)	Spiked Conc. in urine (mg/dL)	% Recovery (n =9)
ELISA	200	50	99.0	195	100	112.4
		400	97.3		500	65.1
HPLC	200	100	93.9	199	70	99.7
		350	105.3		200	88.4



**Figure 4.3. Bland Altman plot comparing the HPLC-UV method and the Jaffe method for creatinine measurement**

#### **4.4 Conclusion**

In this chapter, we report a simple, fast and accurate method for the simultaneous determination of urinary creatinine and prostanoids. The method is linear over three orders of magnitude and is sensitive enough for the analysis of creatinine and prostanoids in urine. The advantage of this method is that one can determine the levels of these prostanoids normalized by urinary creatinine in a single analysis and in less than 17 min.

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## CHAPTER 5

### 5. DEVELOPMENT OF A LC-MS METHOD FOR THE DETERMINATION OF LIPID BIOMARKERS IN BIOLOGICAL TISSUES

#### 5.1 Introduction

The past decade has seen extensive research in determining the role of inflammation in the patho-physiology of diseases like atherosclerosis, Alzheimer's, and rheumatoid arthritis [1,2]. The current chapter focuses on the development of an LC – MS method for the determination and quantitation of biomarkers that play an important role in the progression and the resolution of inflammation.

Common analytical methods that are used for the determination of these lipid biomarkers include HPLC-UV [3-6], HPLC with fluorescence [7-10], capillary electrophoresis-UV [11], GC-MS [12-14], LC-MS and LC-MS/MS [15-26]. Immunoassays were considered to be the standard method for the determination of the lipid markers for many years [2]. Immunoassays (enzyme immunoassays and radio-labeled immunoassays) are sensitive but suffer from poor specificity and reproducibility due to cross-reactivity. HPLC methods mentioned above have a disadvantage that limits their application in bioanalysis. Most of these lipid molecules do not have good chromophores, thus making UV detection difficult. However, it is possible to detect them at 200 nm, which is near the UV cut off of many organic solvents used as HPLC mobile phases. Using fluorescence for detection of the lipid biomarkers involves numerous complicated time-consuming steps as these compounds contains no aromatic or

naturally fluorescing systems. Hence, they need to be derivatized to a complex that fluoresces, to be detected. Likewise, in case of GC analysis, the lipid biomarkers need to be derivatized so that they are volatile enough to enter into the gas phase and thermally stable to withstand degradation. HPLC coupled with mass spectrometry is the more popular choice when it comes to detection of these biomarkers. This is because it is sensitive, specific and does not require derivatization. There are several LC MS methods that have been developed to separate and quantitate endogenous lipid biomarkers. A comprehensive review of the literature revealed no currently published methods that simultaneously separate the  $\omega$ -6 and  $\omega$ -3 polyunsaturated fatty acids and their metabolites. Blewett *et. al.* [15] had previously reported a validated LC-MS method for the analysis of the metabolites of AA in rat tissue. However, this method neglected to include the  $\omega$ -3 fatty acids (EPA and DHA) and their metabolites. The goal of this project was to develop a LC-ESI-MS method to separate and quantitate 35 different inflammatory biomarkers. The separation was performed on a C<sub>18</sub> column using a gradient elution of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The flow rate was 1 ml/min and the run time was 75 mins.

## **5.2. Experimental**

### **5.2.1 Materials**

The prostanoids (PGE<sub>2</sub>, PGF<sub>2</sub>, PGD<sub>2</sub>, 6-keto-PGF<sub>1</sub>, and 11-dehydro-TXB<sub>2</sub>, PGJ<sub>2</sub>,  $\Delta$ 12 PGJ<sub>2</sub>, 15-deoxy  $\Delta$ <sup>12,14</sup> PGJ<sub>2</sub>, 8-Isoprostane), leukotrienes (LTB<sub>4</sub>, LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub>), lipoxins (LXA<sub>4</sub>), HETEs (5-HETE, 9-HETE, 12-HETE, 15-HETE and 20-HETE), EETs (5,6-EET, 8,9- EET, 11,12-EET, 14 and 15-EET), DHETEs (5,6-DHETE,

8,9-DHETE, 11,12-DHETE and 14,15-DHETE), Arachidonic Acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), anadamide, 2- Arachidonyl glycerol, and deuterated eicosanoids used as internal standards (3,3,4,4-<sup>2</sup>H<sub>4</sub>-PGE<sub>2</sub>, 5,6,8,9,11,12,14,15-<sup>2</sup>H<sub>8</sub>-15-HETE, 6,7,14,15-<sup>2</sup>H<sub>4</sub>-LTB<sub>4</sub> and 5,6,8,9,11,12,14,15-<sup>2</sup>H<sub>8</sub>-AA) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Acetonitrile, methanol, and formic acid were purchased from Fisher Scientific (Waltham, MA, USA). The structures of the lipid biomarkers studied in the method are shown in section 1.5.

### **5.2.2 LC-MS method and conditions**

The HPLC used was an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA) with a binary pump, in-line degasser, and a Rheodyne manual injector. The HPLC was coupled to an Agilent G1946 B mass selective detector (MSD). The separation was performed on a Symmetry C18 4.6 mm × 250 mm, 5µm particle size column (Waters Corp., Milford, MA, USA). The mobile phase used was a mixture of acetonitrile with 0.1% formic acid (v/v) (B) and water with 0.1% formic acid (v/v) (A) and the flow rate was set at 1 mL/min. Gradient elution was employed and was used as follows: 40% B for 10 min, 40–60% B from 10 to 25 min, hold 60% B from 25 to 50min, 65–90% B from 50 to 60min, hold 90% B from 60 to 70 min, 5 min re-equilibration at 40% B from 70 to 75 min. The injection volume was 20 µL. Electrospray ionization (ESI) in the negative ion and positive ion mode was used as the ionization source. Nitrogen was used as the nebulizer gas and was maintained at a flow of 12.0 L/min with a nebulizer pressure of 35 psi. The gas temperature was set at 350°C and the capillary voltage was 3000 V. The fragmentor voltage was set at 120 V and the gain was 2.0.

Chromatograms were obtained using SIM (selective ion monitoring) with a time gradient as given in Table 5.1.

**Table 5.1. SIM time gradient for LC-MS analysis**

Time			Time		
(min)	m/z	Ions monitored	(min)	m/z	Ions monitored
0.00	351	PGE <sub>2</sub> , PGD <sub>2</sub>	20.00	319	HETEs, EETs
	353	PGF <sub>2</sub> , 8-isoprostane		327	d <sub>8</sub> -15-HETE
	355	d <sub>4</sub> -PGE <sub>2</sub>		315	15-deoxy- $\Delta^{12,14}$ PGJ <sub>2</sub>
	367	11-dehydro TXB <sub>2</sub>		335	LTB <sub>4</sub>
	369	6 keto PGF <sub>1</sub>		337	DHETEs
	495	LTD <sub>4</sub>		339	d <sub>4</sub> -LTB <sub>4</sub>
	349	PGE <sub>3</sub>		377	Arachidonyl glycerol
	365	11-dehydro TXB <sub>3</sub>		346	Anadamide
	438	LTE <sub>4</sub>		311	d <sub>8</sub> -AA
	624	LTC <sub>4</sub>		303	AA
12.00	336	LXA <sub>4</sub>	61.00	301	EPA
	333	PGJ <sub>2</sub> , $\Delta$ 12 PGJ <sub>2</sub>		327	DHA

### 5.2.3 Sample preparation

Sample preparation was performed as per the extraction protocol published by Blewett *et al.* [2]. An Oasis solid phase extraction cartridge (Waters Corp., Milford, MA) was sequentially preconditioned with 2mL 0.1% formic acid (v/v), 2-mL methanol, and 2-mL ethyl acetate. 1ml of urine sample was loaded on the column and washed with 2mL 0.1% formic acid (v/v) and 2mL 10% methanol with 0.1% formic acid (v/v). The eicosanoids were eluted with 1.5-mL ethyl acetate with 0.01M BHT and 0.5-mL methanol with 0.2% formic acid and 0.01M BHT. 10  $\mu$ L of an internal standard solution (d<sub>4</sub>- PGE<sub>2</sub>: 2.5 ng/  $\mu$ L, d<sub>8</sub>-15-HETE: 500 pg/ $\mu$ L, d<sub>4</sub>-LTB<sub>4</sub>: 1ng/  $\mu$ L, d<sub>8</sub>-AA: 50 $\mu$  g/ $\mu$ L) was added and the samples were evaporated to dryness. Evaporated samples were reconstituted to 100  $\mu$ L with methanol. 20  $\mu$ L of the reconstituted sample was injected into the LC-MS.

### 5.2.4 Selectivity, sensitivity and system suitability

A full method validation (linearity, accuracy recovery) is beyond the scope of this research. Selectivity and sensitivity of the method was performed. Solvent blanks were also analyzed to investigate possible interferences. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as the lowest concentrations that give the signal to noise ratio as 3:1 and 10:1 respectively.

System suitability was performed, by injecting the standard mixture six times consecutively. The %RSD of the six standard ratios (response area of the analytes to that of the internal standards) was determined.

### **5.2.5 Quantitation of the lipid biomarkers**

A single point calibration was used to quantify the lipid biomarkers. To perform this, a mixture consisting of all 35-lipid biomarkers and 4 internal standards was injected thrice. The average response (area) of the analytes to that of the internal standards in the standard solutions was determined. This ratio and the concentrations of the analytes in the standard mixture were used to determine the unknown concentrations in biological tissues. The internal standards that were used for quantifying these lipid biomarkers is given in Table 5.2.

## **5.3. RESULTS AND DISCUSSION**

### **5.3.1 Chromatography**

Individual compounds were analyzed to assess retention and chromatographic behavior. This data was then used to change the chromatographic parameters to optimize the separation. This was important to identify and separate compounds that have similar  $m/z$  ratio such as HETEs and EETS ( $m/z = 319$ ). A mass spectral scan was also performed to ensure the use of the correct  $m/z$  in SIM analysis. Once the initial analysis was performed, a mixture of all 35-lipid biomarkers and 4 internal standards was prepared in methanol. A representative total ion chromatogram (TIC) is shown in Figure 5.1. As can be seen from Figure 5.1, the peaks are sharp and well defined. There is baseline separation between all compounds except for the co-eluting compounds (11-dehydro TXB<sub>2</sub> ( $m/z = 367$ ) and PGD<sub>2</sub> ( $m/z = 351$ ), LXA4 ( $m/z = 336$ ) and LTE4 ( $m/z = 438$ ), 5,6 DHETE ( $m/z = 337$ ), 20-HETE ( $m/z = 319$ ) and 15 deoxy delta 12,14 PGJ<sub>2</sub> ( $m/z = 315$ ), Anandamide ( $m/z = 346$ ) and 5,6 EET ( $m/z = 319$ ) and AA ( $m/z = 303$ ) and

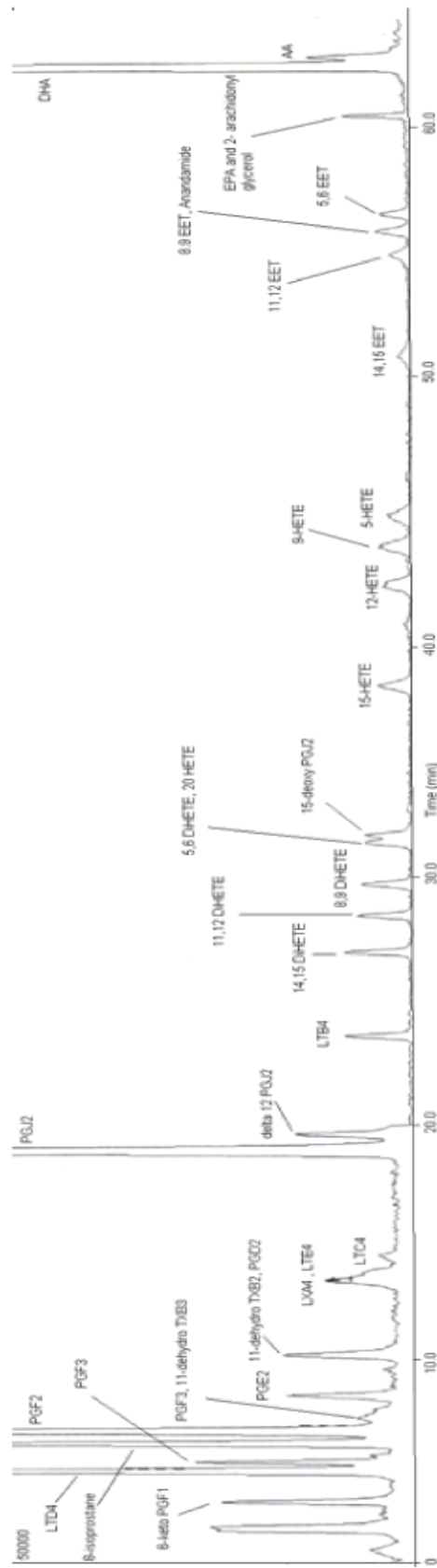
DHA ( $m/z = 327$ ). The co-eluting compounds have different mass/ charge ratios, which can be corrected when using SIM monitoring. Anandamide and Arachidonyl glycerol are detected in the positive ion mode. All the other lipid biomarkers are detected in the negative ion mode. The retention times and the  $m/z$  charge ratio for all the analytes are given in Table 5.3.

### 5.3.2 Selectivity, sensitivity and system suitability

Methanol, which was used as the blank, showed no interferences near the analytes. LOQ for all the analytes are given in Table 3. They range from 0.27  $\text{pg}/\mu\text{L}$  to 11.19  $\text{pg}/\mu\text{L}$ . The method meets precision criteria as the RSD for the system suitability is within 10%.

**Table 5.2. Internal standards used for quantifying these lipid biomarkers**

Internal std	Ions
$d_4$ -PGE <sub>2</sub>	PGE <sub>2</sub> , PGD <sub>2</sub> , PGF <sub>2</sub> , 8-isoprostane, 11-dehydro TXB <sub>2</sub> , 6 keto PGF <sub>1</sub> , PGE <sub>3</sub> , 11-dehydro TXB <sub>3</sub> , PGJ <sub>2</sub> , $\Delta^{12,14}$ PGJ <sub>2</sub> , 15-deoxy $\Delta^{12,14}$ PGJ <sub>2</sub>
$d_4$ -LTB <sub>4</sub>	LTD <sub>4</sub> , LTE <sub>4</sub> , LTC <sub>4</sub> , LTB <sub>4</sub> , LXA <sub>4</sub>
$d_8$ -15-HETE	HETEs, EETs, DHETEs
$d_8$ -AA	Arachidonyl glycerol, Anadamide, AA, EPA, DHA



**Figure 5.1. Representative LC-MS chromatogram of all lipid biomarkers using SIM as defined in Table 5.1**

**Table 5.3. Retention times and the m/z charge ratio for all lipid biomarkers****(continued on next page)**

<b>Lipid Biomarkers</b>	<b>RT (min)</b>	<b>LOQ (pg)</b>
6-keto PGF <sub>1</sub>	4.09	5.5
LTD <sub>4</sub>	5.09	60.2
PGF <sub>3</sub>	5.34	20.0
8-isoprostane	5.70	170.0
PGF <sub>2</sub>	6.56	14.4
PGE <sub>3</sub>	6.92	25.0
11-dehydro TXB <sub>3</sub>	7.17	55.0
PGE <sub>2</sub>	8.59	25.3
11-dehydro TXB <sub>2</sub>	9.84	52.5
PGD <sub>2</sub>	10.34	20.6
LXA <sub>4</sub>	13.49	75.0
LTE <sub>4</sub>	13.90	30.9
LTC <sub>4</sub>	14.57	32.6
PGJ <sub>2</sub>	19.06	10.0
$\Delta$ -12 PGJ <sub>2</sub>	19.55	15.0
LTB <sub>4</sub>	23.70	15.7
14,15 DHETE	27.08	20.0
11,12 DHETE	28.62	20.0
8,9 DHETE	29.89	20.0

5,6 DHETE	31.62	20.0
20-HETE	31.98	23.2
15-deoxy $\Delta$ -12,14 PGJ <sub>2</sub>	32.19	10.0
15-HETE	38.91	24.7
12-HETE	42.4	29.3
9-HETE	43.96	31.3
5-HETE	45.19	43.0
14,15 EET	51.98	84.7
11,12 EET	56.24	223.8
8,9 EET	57.23	35.4
Anadamide	57.28	30.0
5,6 EET	57.96	16.0
Arachidonyl glycerol	60.72	25.0
EPA (eicosapentaenoic acid)	61.75	140.0
DHA (docosahexaenoic acid)	63.92	150.0
AA	64.89	132.0

#### 5.4. Conclusion

The LC-MS method for the simultaneous determination of 35 lipid biomarkers was developed. The method was found to be specific, sensitive and precise. One-point calibration was used to quantify the lipid biomarkers in biological tissues. According to our knowledge, this is the only method that can simultaneously separate  $\omega$ -6 and  $\omega$ -3 polyunsaturated fatty acids and their metabolites.

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## CHAPTER 6

### 6. QSRR PREDICTION OF CHROMATOGRAPHIC RETENTION BEHAVIOR OF POLYUNSATURATED FATTY ACIDS FROM SEMI-EMPIRICALLY COMPUTED MOLECULAR DESCRIPTORS

#### 6.1 Introduction

Chromatography is a technique that utilizes analytes chemical and/or physical interactions with the mobile and stationary phases for their separation. Depending on these interactions, different analytes travel at different rates across the stationary phase. These interactions depend on the chemical structure of the analyte, the physiochemical properties of the mobile phase and the physiochemical properties of the stationary phase. These interactions coupled with the distance travelled by the individual analytes through the column dictates the time it takes to reach the detector. The retention behavior is therefore a direct reflection of the interaction of analytes between the stationary and the mobile phases. Behavior of the analytes in chromatography can be studied using retention modeling techniques.

This chapter describes a retention modeling technique called as Quantitative structure- (chromatographic) retention relationship (QSRR), which was used to develop a predictive retention model for fatty acid metabolites. Retention behaviors of 41 lipid biomarkers were characterized by application of QSRR analysis utilizing Austin Model 1 mode semi-empirical computations. The retention data of these fatty acids were obtained from an RP-HPLC method utilizing a Symmetry C18 column under gradient elution. Molecular descriptors that take into account the polarity; chemical reactivity and

hydrophobicity of the analytes were calculated using the semi-empirical AM1 mode. It is our hypothesis that QSRR will give insight into molecular mechanism of separation of lipid biomarkers operating in a given chromatographic system and can predict retention of a new analyte and/or to identify unknown analytes.

## **6.2 Retention Modeling In Rp Hplc**

In the late 1970's researches realized that majority of chromatographic separations were carried out using RP-HPLC. RP-HPLC utilizes a non-polar stationary phase and a moderately polar mobile phase. Refining this technique drove the researches from the late 1970's to the early 1990's, to develop models to: (1) explain the retention mechanism and (2) predict elution order and thus help in analyte identification. Several models have been developed to explain the retention behavior of analytes in RP-HPLC. They can be broadly classified into two categories: thermodynamic-based models [1] and molecular-interaction models [2]. The thermodynamic based models addresses the retention mechanism in terms of partition and/or adsorption. The molecular-interaction models on the other hand, addresses the retention mechanism in terms of the properties of the analyte, the mobile phase and the stationary phase.

### **6.2.1. Thermodynamic-based models**

Retention in chromatography is controlled by the thermodynamic equilibrium of an analyte (A) between the stationary and the mobile phases [3] (equation 2.1).

The retention factor (capacity factor), which is the ratio of the retention times of the retained and un-retained species, is directly proportional to the equilibrium constant as can be seen in eq. 2.2

Also, according to classical thermodynamics, the change in free energy when the analyte is transferring from one phase to another is given by the following expression:

$$-\Delta G^{\circ} = RT \ln K \dots (6.1)$$

$-\Delta G^{\circ}$  = Gibbs free energy;  $R$  = gas constant;  $T$  = temperature in Kelvin;  $K$  = equilibrium constant

$$\Delta G^{\circ} = \Delta H^{\circ} - T \Delta S^{\circ} \dots (6.2)$$

$\Delta H^{\circ}$  = change in enthalpy;  $\Delta S^{\circ}$  = change in entropy

Substituting equation 6.1 in 6.2 and rearranging we get:

$$\ln K = -\frac{\Delta H^{\circ}}{R} + \frac{\Delta S^{\circ}}{R} + \ln \phi \dots (6.3)$$

$\phi$  = ratio of the volume of mobile phase to that of the stationary phase in the column

[volume of mobile phase = dead time \* flow rate and volume of stationary phase = mass of the stationary phase \* density]

Therefore, theoretically from the standard enthalpy change and entropy change, we can calculate the equilibrium constant and subsequently the retention time of the analyte. However, these properties are difficult to isolate and estimate. The other point to be noted is that though both the classical and the statistical thermodynamic theories take into account most of the intermolecular interactions, assumed to be occurring during separation, neither can accurately describe the retention behavior of the analytes [3]. This is because the thermodynamic properties (entropy, enthalpy) are bulk properties of the physiochemical systems and are a combination of several different molecular effects.

#### 6.2.1.1. Solvophobic theory

The most well known classical thermodynamic theories proposed for modeling the retention mechanism is the solvophobic theory [4]. Professor Horvath of Yale University was the first to put forward a theory to explain the retention behavior/pattern of analytes in RP-HPLC [4] (1976). This was adapted from the original work by Sinanoglu and co-workers [5]. This was called as the “Solvophobic theory”. The solvophobic theory takes into account, only the interactions between the analytes and the mobile phase. According to this theory, the retention time depends on two parameters: (1) contact area of the analyte with the mobile phase; the larger the contact area greater is the retention time and (2) surface tension between the analyte and mobile phase. The theory assumes that when the analytes dissolves in the mobile phase, there is a negative free energy change. The two interactions that are associated with this free energy change are the *Van der Waals interactions*, which is approximately proportional to the molecular surface area of the analyte and the *electrostatic interactions*, which depends on the dielectric constant of the mobile phase and the dipole moment of the analyte molecules [6]. The solvophobic theory has helped in better understanding the physiochemical properties that determine the retention behavior in chromatography. But it suffers as it assumes that the stationary phase is non- interactive whereas we known from literature that there exists interactions between the analyte and the stationary phase (free silanol groups on the stationary phase, pi-pi interactions).

## **6.2.2 Molecular-interaction models-Quantitative structure retention relationship (QSRR)**

Molecular-interaction models assume that retention of the analyte molecules depend on the forces between them and the molecules of the two phases. Pioneering work on quantitative structure activity relationships (QSAR) by Hansch and others provided means to describe activity in terms of solutes molecular structure, using statistical techniques. Hansch *et. al.* [7] used QSAR along with multiple regression analysis to gain insight into the activity of chloramphenical (an inhibitor of protein synthesis). This model has been successfully employed for quantitative structure retention relationship (QSRR) studies. QSRR uses this model to understand the relationship between the retention and the chemical nature of the analyte. QSRR assumes that since all the conditions in chromatography are kept constant; the retention of the analyte is dependent only on it's structure (only independent variable in the system). This technique is capable of correlating chromatographic retention data to the analyte properties using multiple regression analysis [8]. Though the only three papers were published in 1977, QSRR is steadily gaining appreciation with more than 350 papers published since 2001 (Figure 6.1).

### **6.2.2.1 Types of QSRR**

There are three main types of QSRR:

#### **1. QSRR based on partition coefficient**

The oldest is the one that relates logarithm of retention factors ( $\log k$ ) to the logarithm of partition coefficients ( $\log P$ ) [9].

## 2. Linear Solvation- Energy Relationships (LSER)

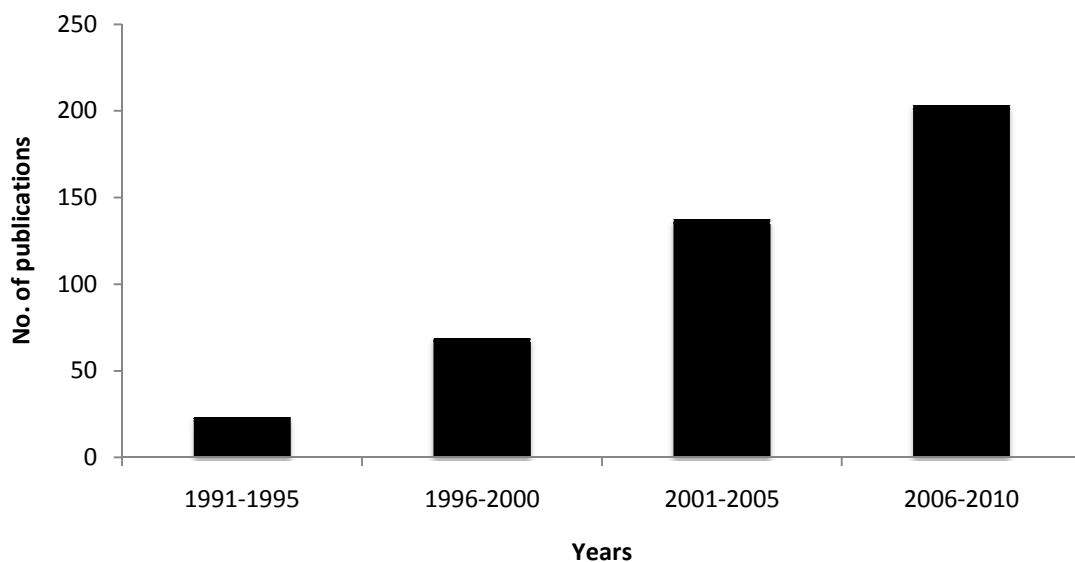
Kamlet and co-workers developed the linear solvation energy relationship (LSER)[15]. They demonstrated that the interactions between the analyte and the solvent are responsible for many chemical properties such as octanol water partition ( $\log P$ ), aqueous solubility and HPLC capacity factors [10]. According to this model these chemical properties depend on the energy required to surround the analyte with the solvent molecules, energy required to stabilize the solvent cavity and the energy lost or gained due to the formation of hydrogen bonds or electrostatic interactions. These analyte solvent interactions in turn depend on the molecular structure of the analytes [11,12]. The disadvantage of LSER is that the solvatochromic parameters are empirical in nature and are available for limited number of compounds.

## 3. QSRR based on quantum chemical indices and/or structural descriptors

The third type of QSRR relates the retention factor to numerous quantum chemical indices and/or structural descriptors from calculation chemistry. For this type of QSRR study one needs: retention data for large of analytes and molecular descriptors that reflects the structural properties of the analytes. Using statistical techniques (multiple regression analysis) the retention parameters are characterized in terms of numerous combinations of molecular descriptors of the analytes [8]. If the results are significant, then the molecular descriptors can be used to predict the retention time of a new analyte and gain insight of the retention mechanism. Some of the molecular descriptors that are commonly used in QSRR studies are: carbon number, polarizability, dipole moment, octanol-water partition coefficient, solvation energies and

electronegativity. The advantage of this QSRR model over the LSER is that there are no restrictions in determining the structural descriptors as they are based simply on the structure of the compounds.

QSRR based on quantum chemical indices and/or structural descriptors was used in this study. An extensive search of the literature revealed that there is currently no reported QSRR study on the lipid biomarkers of interest (metabolites of arachidonic Acid, docosahexaenoic acid and eicosapentaenoic acid).



**Figure 6.1. Number of papers published from 1990 to 2011 in the field of QSRR. (Data adapted from Scifinder Scholar 2007)**

## 6.3 Experimental

### 6.3.1 Chemicals and Materials

The prostanoids (PGE<sub>2</sub>, PGF<sub>2α</sub>, PGD<sub>2</sub>, 6-keto-PGF<sub>1α</sub> and 11-dehydro-TXB<sub>2</sub>, PGJ<sub>2</sub>, Δ<sup>12</sup> PGJ<sub>2</sub>, 15-deoxy Δ<sup>12,14</sup> PGJ<sub>2</sub>, 8-Isoprostane), leukotrienes (LTB<sub>4</sub>, LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub>), lipoxins (LXA<sub>4</sub>, 15-epi LXA<sub>4</sub>) HETEs (5-HETE, 9-HETE, 12-HETE, 15-HETE and 20-HETE), EETs (5,6-EET, 8,9- EET, 11,12-EET, 14 and 15-EET), DHETEs (5,6-DHETE, 8,9-DHETE, 11,12-DHETE and 14,15-DHETE), Arachidonic Acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), anadamide, 2- Arachidonyl glycerol, resolvins (RvD1 and RvD2) and deuterated eicosanoids used as internal standards (3,3,4,4-2H<sub>4</sub>-PGE<sub>2</sub>, 5,6,8,9,11,12,14,15-2 H<sub>8</sub> -15-HETE, 6,7,14,15-2 H<sub>4</sub> - LTB<sub>4</sub> and 5,6,8,9,11,12,14,15-2H<sub>8</sub>-AA) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Acetonitrile, methanol, and formic acid were purchased from Fisher Scientific (Waltham, MA, USA). Resolvin D5, deuterated Resolvin D6 and 2,3-dinor-5, 6,dihydro-ent-15 epi f2t-isop were donated by Dr. Bernd Spur (Department of Cell Biology, UMDNJ, New Jersey, USA).

### 6.3.2 LC-MS method and conditions

The HPLC used was an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA) with a binary pump, in-line degasser, and a Rheodyne manual injector. The HPLC was coupled to an Agilent G1946 B mass selective detector (MSD). The separation was performed on a Symmetry C18 4.6 mm × 250 mm, 5μm particle size column (Waters Corp., Milford, MA, USA). The mobile phase used was a mixture of acetonitrile with 0.1% formic acid (v/v) (B) and water with 0.1% formic acid (v/v) (A)

and the flow rate was set at 1 mL/min. Gradient elution was employed and was used as follows: 40% B for 10 min, 40–60% B from 10 to 25 min, hold 60% B from 25 to 50min, 65–90% B from 50 to 60min, hold 90% B from 60 to 70 min, 5 min re-equilibration at 40% B from 70 to 75 min. The injection volume was 20  $\mu$ L. Electrospray ionization (ESI) in the negative ion and positive ion mode was used as the ionization source. Nitrogen was used as the nebulizer gas and was maintained at a flow of 12.0 L/min with a nebulizer pressure of 35 psi. The gas temperature was set at 350°C and the capillary voltage was 3000 V. The fragmentor voltage was set at 120 V and the gain was 2.0. Chromatograms were obtained using SIM (selective ion monitoring).

### **6.3.3 Sample preparation procedure**

Standard solutions of inflammatory lipid molecule such as AA, DHA, EPA and their metabolites were prepared in methanol and 20  $\mu$ L of each solution was injected into the LC-MS. To correct for slight variations in the retention times, they were referenced to that of 2,3-dinor-5, 6,dihydro-ent-15 epi f2t –isop, which was used as an internal standard.

### **6.3.4 QSRR analysis and molecular descriptors**

The geometry optimization was done with Spartan’06 ES V3.2.2 using the semi-empirical AM1 (Austin model 1) mode. Molecular descriptors including dipole moment, solvation energies and HOMO and LUMO energies were then calculated using the semi-empirical AM1 mode. Clog P (Calculated log P) was calculated using ACD ChemSketch software.

### 6.3.5 Statistical Analysis

To investigate possible correlations between the retention time and the molecular descriptors, regression analysis was performed. Multiple regression equations were derived using the statistical software Medcalc®. Statistical evaluation was performed from the correlation coefficient (r). Correlation coefficient (r) value  $\geq 0.90$  was considered as good correlation. The agreement between two methods was compared using the Bland-Altman method [13] (plotting the difference in RRT between the two methods against the mean RRT for each analyte). The criterion for agreement is that the differences within the accuracy fall within the 95% confidence limit of the mean of the differences.

### 6.3.6 Identification of unknown analyte

One of the goals of the QSRR study was to identify an unknown compound in kidney tissue at RRT (31.6 min) and  $m/z = 319$ . The extraction of the kidney tissue was done as explained by Blewett *et. al.*[14]. The molecular descriptors of the suspected candidates were calculated using semi-empirical AM1 mode as done for the lipid biomarkers. The QSRR equation was then used to determine the predicted RRT. The observed and the predicted RRT's were compared using the Bland-Altman analysis.

## 6.4 Results And Discussion

Molecular properties can be computed by various methods including ab-initio and semi-empirical methods [15]. Semi-empirical methods are much faster as most of the variables are parameterized [15]. They are therefore less expensive than ab-initio, especially in case of large molecules. Austin Model 1 (AM1) and Parameterization 3

(PM3) are the two semi-empirical methods that are widely used in computational chemistry [16]. AM1 developed by Dewar in 1985 is parameterized for H, B, C, N, O, F, Al, Si, P, S, Cl, Zn, Ge, Br, Hg, and I [17]. PM3, developed by Stewart is a re-parameterization of AM1 [17]. Although PM3 is parameterized for greater number of elements than AM1, AM1 was used for this study, as it is known to represent the differences between compounds more reliably than PM3 [17]. This is important as the lipid biomarkers under study are closely related with many of them have similar structures.

The main goal of the QSRR study was to determine the retention mechanism in the given chromatographic system. In case of reverse phase HPLC, three retention mechanisms have been proposed; the partition theory, the adsorption theory and the hydrophobic theory [18]. Retention can be due to one or more of these mechanisms. In the current study, we have investigated all the three retention theories.

**Partition theory** assumes that the analyte distributes between the aqueous mobile phase and the stationary phase [18].  $\log P$  known as distribution/partition coefficient is a property that can depict this distribution of the analyte between the mobile phase (water) and stationary phase (C18 column). In this study Clog P was calculated, which is a fragment-based prediction model of the partition coefficient. According to this model,  $\log p$  of a compound is the sum of the hydrophobicity value from each molecular fragment [19]. Clog P for all the lipid biomarkers were calculated using ChemSketch (Table 6.1). We found that there exist a good correlation ( $r= 0.95$ ) between Clog P and RRT (min) (Figure 6.2) indicating that partition plays a major role in the retention of the lipid biomarkers in the given chromatographic system.

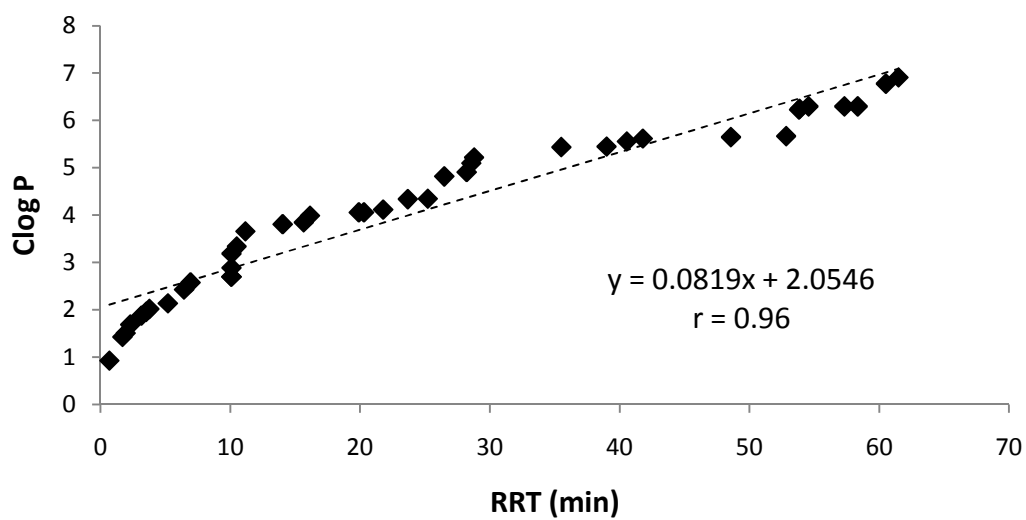
**Adsorption theory** assumes that the analyte remains on the surface and does not penetrate into the pores of the chromatographic column [18]. Adsorption of the analyte on the column surface occurs primarily due to dipole-dipole interactions [18]. Dipole moment and polarizability (derivative of dipole moment in a given electric field) were calculated to determine the role of adsorption in the retention of the analytes (Table 6.3). Figure 6.3 show that there exists a very poor correlation ( $r= 0.22$ ) between the dipole moment and RRT (min) of the analytes. Furthermore, there is no correlation between the polarizability of the analyte molecules (Figure 6.4) and the RRT (min). These results indicate that adsorption may have a very small role to play in the retention mechanism.

**Hydrophobic theory** presumes that the hydrophobic portion of the analyte molecules aggregates together, thus forming cavities in the solvents [18]. The polar groups on the analyte molecules interact with the polar mobile phase. This theory also assumes that the stationary phase has no role in the retention mechanism. To study this we computed the solvation energies of the analyte molecules (Table 6.3). We can see from Figure 6.5, that as the RRT increases the solvation energy increases, indicating that more energy is required to solvate the non-polar analyte molecules. There exists a moderate correlation between the RRT and the solvation energies of the analytes ( $r=0.82$ ). Hydrophobic interactions are hence thought to play a significant role in the retention of these lipid biomarkers.

**Table 6.1. Partition theory: Clog P for all the lipid biomarkers (continued on next page)**

<b>Compounds</b>	<b>RT (min)</b>	<b>Observed RRT (min)</b>	<b>Clog P</b>
6-keto PGF <sub>1a</sub>	4.09	0.69	0.93
LTD4	5.09	1.69	1.43
PGF <sub>3</sub>	5.34	1.94	1.51
8-isoprostane	5.70	2.30	1.69
PGF <sub>2</sub>	6.56	3.16	1.88
PGE <sub>3</sub>	6.92	3.52	1.96
11-dehydro TXB <sub>3</sub>	7.17	3.77	2.02
PGE <sub>2</sub>	8.59	5.19	2.14
11-dehydro TXB <sub>2</sub>	9.84	6.44	2.43
PGD <sub>2</sub>	10.34	6.94	2.58
LXA4	13.49	10.09	2.70
15-epi LXA4	13.49	10.09	2.70
RvD1	13.50	10.10	2.89
RvD2	13.50	10.10	3.19
LTE4	13.90	10.50	3.34
LTC4	14.57	11.17	3.66
RvE2	17.44	14.04	3.81
PGJ <sub>2</sub>	19.06	15.66	3.85
$\Delta^{12}$ PGJ <sub>2</sub>	19.55	16.15	3.99
RVD5	23.31	19.91	4.06
LTB4	23.70	20.30	4.06
RvD6	25.18	21.78	4.12
14,15 DHETE	27.08	23.68	4.34
11,12 DHETE	28.62	25.22	4.35
8,9 DHETE	29.89	26.49	4.82
5,6 DHETE	31.62	28.22	4.91
20-HETE	31.98	28.58	5.10
15-deoxy $\Delta^{12,14}$ PGJ <sub>2</sub>	32.19	28.79	5.22
15-HETE	38.91	35.51	5.44
12-HETE	42.40	39.00	5.45
9-HETE	43.96	40.56	5.56
5-HETE	45.19	41.79	5.62
14,15 EET	51.98	48.58	5.65
11,12 EET	56.24	52.84	5.67
8,9 EET	57.23	53.83	6.23

Anadamide	57.28	53.88	6.25
5,6 EET	57.96	54.56	6.30
Arachidonyl glycerol	60.72	57.32	6.30
EPA	61.75	58.35	6.30
DHA	63.92	60.52	6.78
AA	64.89	61.49	6.91



**Figure 6.2. Partition theory: Clog P vs. RRT (min)**

**Table 6.2. Adsorption theory: Dipole moment and polarizability of all lipid biomarkers (continued on next page)**

<b>Compounds</b>	<b>RT (min)</b>	<b>Observed RRT (min)</b>	<b>Polarizability</b>	<b>Dipole moment (Debye)</b>
6-keto PGF <sub>1a</sub>	4.09	0.69	71.41	2.46
LTD4	5.09	1.69	82.3	1.97
PGF <sub>3</sub>	5.34	1.94	70.50	1.52
8-isoprostane	5.70	2.30	70.73	3.38
PGF <sub>2</sub>	6.56	3.16	70.74	4.45
PGE <sub>3</sub>	6.92	3.52	70.08	1.42
11-dehydro TXB <sub>3</sub>	7.17	3.77	70.70	3.02
PGE <sub>2</sub>	8.59	5.19	70.35	3.32
11-dehydro TXB <sub>2</sub>	9.84	6.44	70.95	3.08
PGD <sub>2</sub>	10.34	6.94	70.36	6.58
LXA4	13.49	10.09	71.76	2.81
15-epi LXA4	13.49	10.09	71.78	2.62
RvD1	13.50	10.10	73.61	4.27
RvD2	13.50	10.10	73.64	2.17
LTE4	13.90	10.50	78.14	2.79
LTC4	14.57	11.17	39.47	1.42
RvE2	17.44	14.04	70.40	4.62
PGJ <sub>2</sub>	19.06	15.66	69.75	2.09
D <sup>12</sup> PGJ <sub>2</sub>	19.55	16.15	69.64	3.04
RVD5	23.31	19.91	72.86	2.15
LTB4	23.70	20.30	71.05	1.93
RvD6	25.18	21.78	73.01	3.49
14,15 DHETE	27.08	23.68	70.90	3.00
11,12 DHETE	28.62	25.22	70.88	1.21
8,9 DHETE	29.89	26.49	70.83	3.22
5,6 DHETE	31.62	28.22	70.74	2.35
20-HETE	31.98	28.58	70.16	2.65
15-deoxy D <sup>12,14</sup> PGJ <sub>2</sub>	32.19	28.79	68.93	3.94
15-HETE	38.91	35.51	70.17	1.56
12-HETE	42.40	39.00	69.93	1.05

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9-HETE	43.96	40.56	69.94	5.42
5-HETE	45.19	41.79	70.18	2.68
14,15 EET	51.98	48.58	69.84	3.31
11,12 EET	56.24	52.84	69.82	2.85
8,9 EET	57.23	53.83	69.79	0.75
Anadamide	57.28	53.88	73.38	3.80
5,6 EET	57.96	54.56	69.70	1.12
Arachidonyl glycerol	60.72	57.32	75.14	2.60
EPA	61.75	58.35	68.97	1.47
DHA	63.92	60.52	71.58	2.07
AA	64.89	61.49	69.25	1.97

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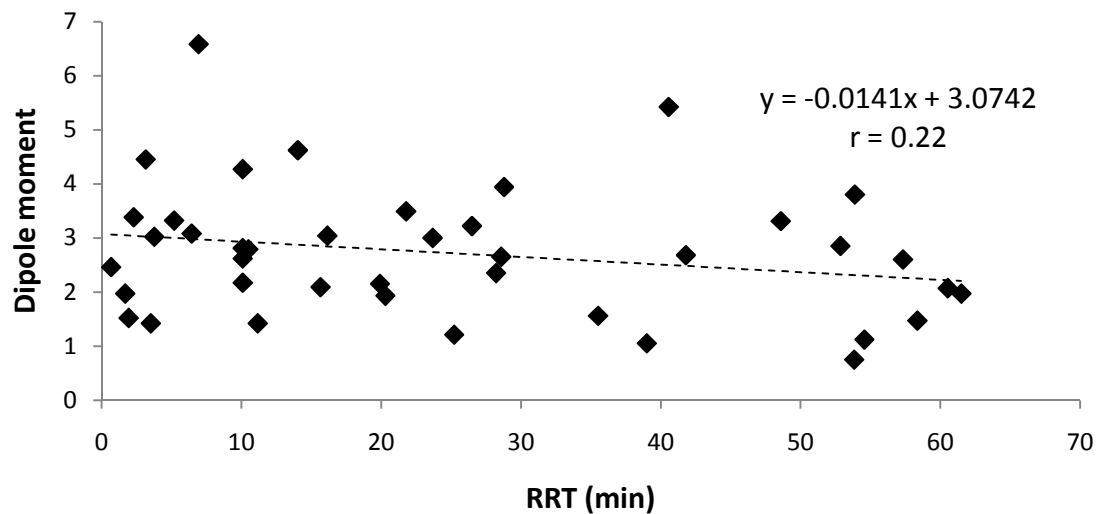


Figure 6.3. Adsorption theory: Dipole moment vs. RRT (min)

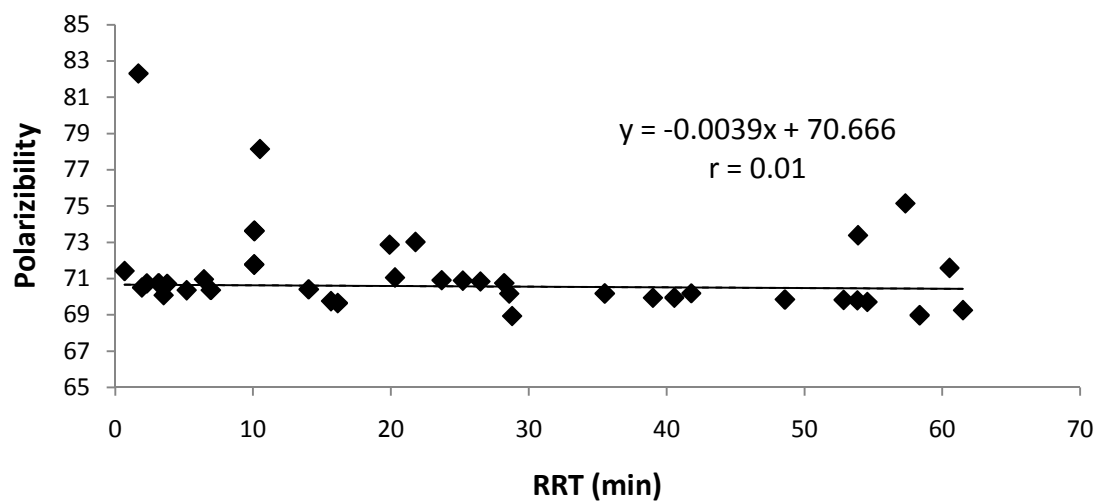


Figure 6.4. Adsorption theory: Polarizability vs. RRT (min)

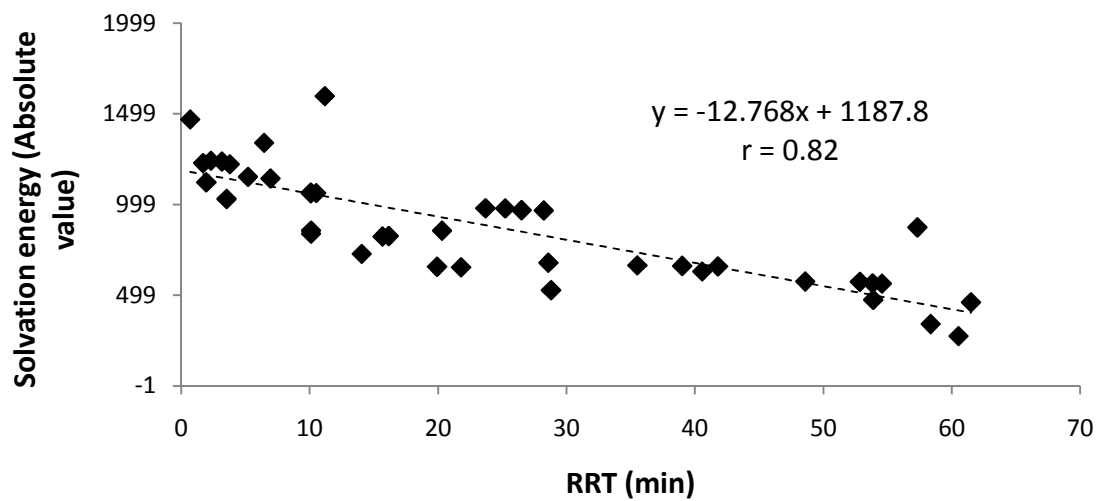
Therefore, we can say that the retention of the lipid biomarkers in the given chromatographic system is primarily due to partition of the analyte between mobile and stationary phases, followed by interaction of the analytes with mobile phase and lastly due to adsorption of the analyte on the stationary phase by weak dipole-dipole interactions. QSRR equations were obtained by performing multiple regression analysis using numerous combinations of molecular descriptors of the analytes; Clog P, dipole moment and solvation energies. Polarizability was not used in the calculation, as it showed no significant relationship to RRT. The QSRR equation thus obtained was then used to calculate RRT (min), which was then compared to the observed RRT (min) (Figure 6.6). There exists a good correlation ( $r=0.96$ ) between the observed and calculated RRT. However, this model does not completely explain the retention mechanism of the analytes.

To investigate the retention mechanism further, we decided to look into the electron pair donor- electron pair acceptor interactions, which are thought to play a significant role in the retention of analytes [20]. Molecular descriptors that took into account chemical reactivity of the analytes; the lowest unoccupied molecular orbital (LUMO) and the highest unoccupied molecular orbital (HOMO) were computed. We found that there exists a moderate correlation between the RRT (min) and the LUMO energies (kcal/mol) (Figure .6.7).

**Table 6.3. Hydrophobic theory: Solvation energies of all lipid biomarkers  
(continued on next page)**

<b>Compounds</b>	<b>RT (min)</b>	<b>Observed RRT (min)</b>	<b>E(aq) (kJ/mol)</b>
6-keto PGF <sub>1a</sub>	4.09	0.69	-1468.9
LTD4	5.09	1.69	-1228.21
PGF <sub>3</sub>	5.34	1.94	-1121.79
8-isoprostane	5.70	2.30	-1241.28
PGF <sub>2</sub>	6.56	3.16	-1236.09
PGE <sub>3</sub>	6.92	3.52	-1030.19
11-dehydro TXB <sub>3</sub>	7.17	3.77	-1221.3
PGE <sub>2</sub>	8.59	5.19	-1152.29
11-dehydro TXB <sub>2</sub>	9.84	6.44	-1340.15
PGD <sub>2</sub>	10.34	6.94	-1143.04
LXA4	13.49	10.09	-1061.24
15-epi LXA4	13.49	10.09	-1065.33
RvD1	13.50	10.10	-838.37
RvD2	13.50	10.10	-856.28
LTE4	13.90	10.50	-1063.09
LTC4	14.57	11.17	-1597.26
RvE2	17.44	14.04	-727.43
PGJ <sub>2</sub>	19.06	15.66	-823.17
$\Delta^{12}$ PGJ <sub>2</sub>	19.55	16.15	-825.80
RVD5	23.31	19.91	-656.26
LTB4	23.70	20.30	-855.45
RvD6	25.18	21.78	-653.55
14,15 DHETE	27.08	23.68	-978.94
11,12 DHETE	28.62	25.22	-977.91
8,9 DHETE	29.89	26.49	-968.42
5,6 DHETE	31.62	28.22	-966.44
20-HETE	31.98	28.58	-678.48
15-deoxy $\Delta^{12,14}$ PGJ <sub>2</sub>	32.19	28.79	-526.90
15-HETE	38.91	35.51	-663.62
12-HETE	42.40	39.00	-660.89
9-HETE	43.96	40.56	-630.35
5-HETE	45.19	41.79	-658.03
14,15 EET	51.98	48.58	-574.95
11,12 EET	56.24	52.84	-573.41
8,9 EET	57.23	53.83	-564.95
Anadamide	57.28	53.88	-474.01
5,6 EET	57.96	54.56	-563.03

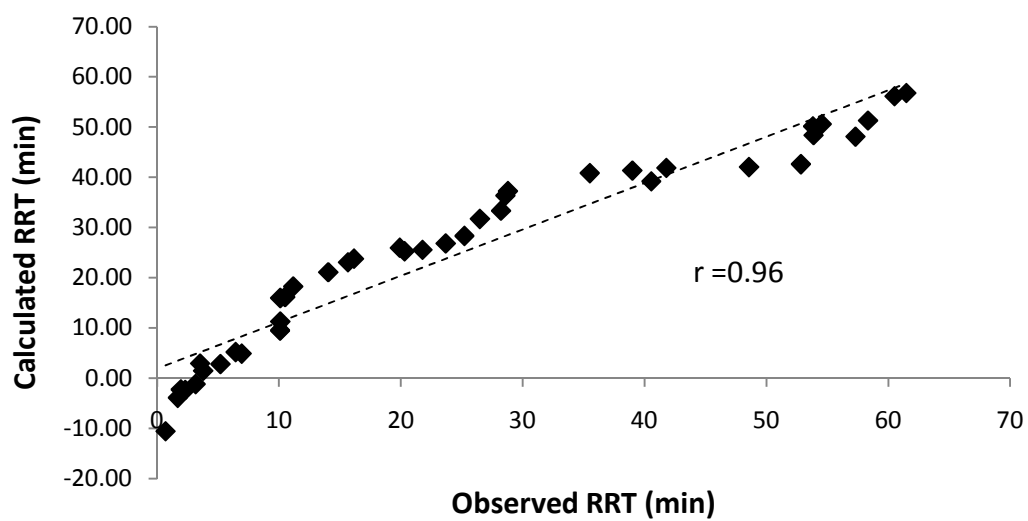
Arachidonyl glycerol	60.72	57.32	-873.34
EPA	61.75	58.35	-340.29
DHA	63.92	60.52	-274.05
AA	64.89	61.49	-460.03



**Figure 6.5. Hydrophobic theory: Solvation energy vs. RRT (min)**

*QSRR equation*

$$\text{Calculated RRT} = -12.005 + 10.461 (\text{Clog } p) - 0.7837 (\text{dipole moment}) + 0.0043 (\text{solvation energy})$$



**Figure 6.6. Chromatographically determined RRT (min) vs. theoretically determined RRT (min) for 41 lipid biomarkers**

On the other hand, the HOMO energies of the analytes were found to be around the same for all the analytes, hence was neglected (Table 6.4). Using MLR, a QSRR equation was obtained using Clog P, dipole moment, solvation energies and LUMO. The RRT calculated using this QSRR equation was plotted against the observed RRT (Figure 6.8). The addition of the LUMO term to the QSRR equation improved the correlation coefficient (0.96 to 0.97). LUMO energy is considered to be a good approximation of the molecular affinity [21]. This suggests that the analyte molecules are involved in electron pair acceptor- electron pair donor interactions with either the mobile phase or stationary phase. The QSRR model imply that the retention of lipid biomarkers in the given chromatographic system is due to various mechanisms in the following order (Figure 6.8) partitioning, adsorption, electron pair donor acceptor interactions and finally hydrophobic interactions involving the analytes and the mobile and/or stationary phases.

To determine whether the retention mechanism follows the same order for all the different sets of lipid biomarkers, we divided them into four groups: Group I- compounds with  $RRT < 10$  min, Group II- compounds with  $RRT (10-25 \text{ min})$ , Group III- compounds with  $RRT (25-70 \text{ min})$  and Group IV- compounds with  $RRT (50-70 \text{ min})$ . The division was done to match the mobile phase changes in the system (Figure 6.9). The whole system can be divided into five regions based on changes in the mobile phase composition. Region IV and V were combined as there are few compounds beyond 60 mins. Using MLR, QSRR equations were obtained using Clog P, dipole moment, solvation energies and LUMO for each group. The RRT calculated using these QSRR equations were plotted against the observed RRT (Figure 6.10, 6.11, 6.12 and 6.13). It was observed that the RRTs calculated using the individual QSRR models were much

closer to the observed RRTs for all the groups. This is especially true for the first group of compounds whose calculated RRTs changed from negative numbers to positive numbers when the individual QSRR model was used. It was also observed that though the coefficients of the individual molecular descriptors had different values, the order of the mechanism remained the same for the first group which being partitioning, electron pair donor acceptor interactions, adsorption and finally hydrophobic interactions. The last group saw a reversal in the order of the mechanism with the adsorption and electron pair donor acceptor interactions. The intercept also differs for each group of compounds (Table 6.5). We believe that this is because the intercept is not simply a random number of fit but is a value that accounts for the mobile phase differences in the system. This may be true especially since these retention models take into account only the properties of the analytes and not that of the mobile and stationary phases.

The accuracies of the calculated RRT obtained by using just the individual molecular descriptors were compared to the QSRR models as well as to the QSRR model based on groups. The % accuracy was calculated using the formula  $[(\text{calculated RRT}/\text{observed RRT}) * 100]$ . It was observed that the % accuracies for all the lipid biomarkers was better when the QSRR model based on groups, was used (Table 6.6 and 6.7). We believe that dividing it into groups gives a better predictive model, as the mobile phase conditions remain more or less constant for all the analytes in a specific group (Figure 6.9).

The observed and the predicted RRT's were compared using the Bland-Altman method to determine if they are comparable [13]. Bland-Altman analysis for analytes

was performed, by plotting the difference in the RRT's vs. the mean of the RRT's. All the measurements are within 95% confidence interval ( $d \pm s$ ).

QSRR was also used in the identification of an unknown compound. The RRT and the  $m/z$  of the unknown compound suggest that it is likely to be a HETE. Though the major HETEs i.e. 20-, 15-, 12-, 9- and 5-HETEs are used for this study, there are few other HETE's that are found *in vivo* and have some biological activity (e.g. 19-, 18-, 17-, 16- HETE). The presence of a quadratic relationship between the retention times of HETEs and the position of the OH substitution has been described by Blewett *et.al* [14]. A similar quadratic relationship exists between the retention times of HETEs and the position of the OH substitution for the instrumental condition described above (Figure 6.14). Using this quadratic equation, it was determined that the position of the OH substitution is likely to be on Carbon 19. The molecular descriptors for 19-, 18-17-, 16-HETE were calculated using semi-empirical AM1 mode as done for the lipid biomarkers. The QSRR equation obtained for Group III was used to determine the predicted RRT (Table 6.8). The % accuracy was calculated for all the HETEs using the formula  $[(\text{calculated RRT}/\text{observed RRT}) * 100]$  and compared to the average accuracy obtained using the QSRR model. Though the accuracy of 19-HETE is much better than the others, the % accuracy of all the HETEs lie outside the standard deviation of the model (Table 6.8). We can conclude that either the unknown compound is not an HETE as predicted or that the obtained QSRR model is not a good predictive model. We believe that the latter is more likely to be true as this model takes in account only the properties of the analytes and not of the mobile and stationary phases which can be considered to be the biggest weakness of this model.

As mentioned earlier, further studies need to be performed to determine the contribution of mobile phase in the retention mechanism. This is important as electrostatic interactions especially dipole-dipole interactions can occur between analyte and mobile phase with high dielectric constant. We know that as the organic content of the mobile phase increases, the dielectric constant decreases (Figure 6.9). This suggests that the electrostatic interactions between analytes and mobile phase will also vary with time. Hence, we firmly believe that this variation needs to be accounted for in the QSRR model.

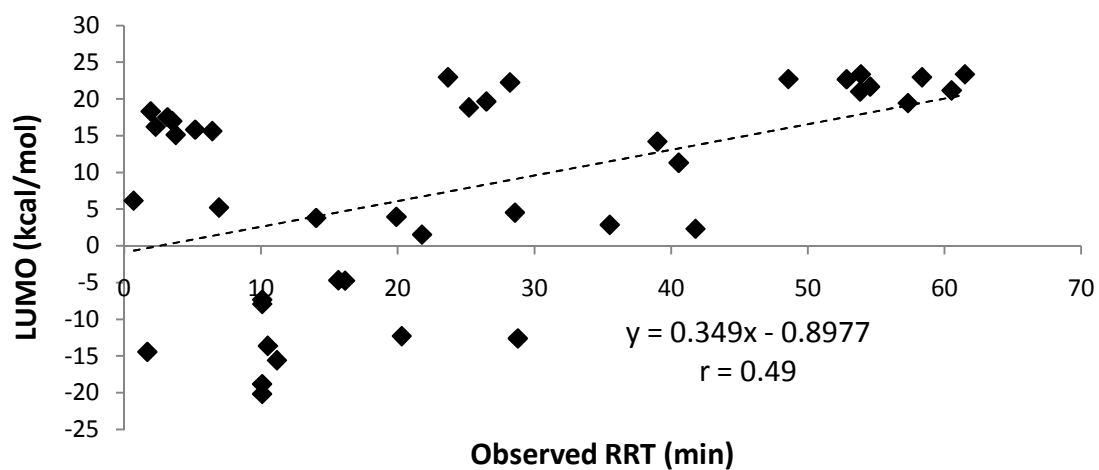
## **6.5 Conclusion**

Quantitative structure- (chromatographic) retention relationship (QSRR) has been used to develop a predictive retention model for fatty acid metabolites. In this chapter, retention behavior of 41 lipid biomarkers has been characterized by application of QSRR analysis utilizing AM1 semi-empirical computations. The retention data of these fatty acids were obtained from an RP-HPLC method utilizing a Symmetry C18 column under gradient elution. Four solute descriptors (Clog P, dipole moment, solvation energies and LUMO energies) showed significance. Values of Log P were calculated using “fragment based prediction”, (Clog P). The dipole moment, LUMO energies and solvation energies, were computed from the optimized semi-empirical AM1 (Austin Model 1) solute geometries. Results from the QSRR analysis showed that, retention of solutes was predicted with Clog P, dipole moment, solvation energies, and LUMO energies. The QSRR equation obtained in this study was applied to predict the molecular character of an unknown analytes in the kidney tissue of hypertensive rat model.

**Table 6.4. Chemical Reactivity: HOMO and LUMO energies of all lipid biomarkers  
(continued on next page)**

Compounds	RT (min)	Observed RRT (min)	HOMO (kcal/mol)	LUMO (kcal/mol)
6-keto PGF <sub>1a</sub>	4.09	0.69	-236.15	6.15
LTD4	5.09	1.69	-203.07	-14.44
PGF <sub>3</sub>	5.34	1.94	-229.44	18.30
8-isoprostane	5.70	2.30	-233.32	16.21
PGF <sub>2</sub>	6.56	3.16	-234.62	17.51
PGE <sub>3</sub>	6.92	3.52	-226.28	16.97
11-dehydro TXB <sub>3</sub>	7.17	3.77	-228.68	15.10
PGE <sub>2</sub>	8.59	5.19	-232.93	15.81
11-dehydro TXB <sub>2</sub>	9.84	6.44	-236.57	15.62
PGD <sub>2</sub>	10.34	6.94	-238.62	5.20
LXA4	13.49	10.09	-205.11	-20.16
15-epi LXA4	13.49	10.09	-204.14	-18.83
RvD1	13.50	10.10	-209.14	-7.34
RvD2	13.50	10.10	-205.15	-7.93
LTE4	13.90	10.50	-203.30	-13.62
LTC4	14.57	11.17	-205.73	-15.58
RvE2	17.44	14.04	-214.45	3.79
PGJ <sub>2</sub>	19.06	15.66	-233.97	-4.69
$\Delta^{12}$ PGJ <sub>2</sub>	19.55	16.15	-230.31	-4.75
RVD5	23.31	19.91	-219.62	3.94
LTB4	23.70	20.30	-210.02	-12.30
RvD6	25.18	21.78	-217.35	1.52
14,15 DHETE	27.08	23.68	-226.07	22.94
11,12 DHETE	28.62	25.22	-229.01	18.83
8,9 DHETE	29.89	26.49	-227.77	19.64
5,6 DHETE	31.62	28.22	-228.40	22.24
20-HETE	31.98	28.58	-213.83	4.52
15-deoxy $\Delta^{12,14}$ PGJ <sub>2</sub>	32.19	28.79	-220.73	-12.61
15-HETE	38.91	35.51	-215.08	2.85
12-HETE	42.40	39.00	-226.27	14.19
9-HETE	43.96	40.56	-214.13	11.30
5-HETE	45.19	41.79	-217.64	2.31
14,15 EET	51.98	48.58	-226.09	22.69
11,12 EET	56.24	52.84	-227.91	22.66
8,9 EET	57.23	53.83	-224.45	20.99
Anadamide	57.28	53.88	-221.42	23.34

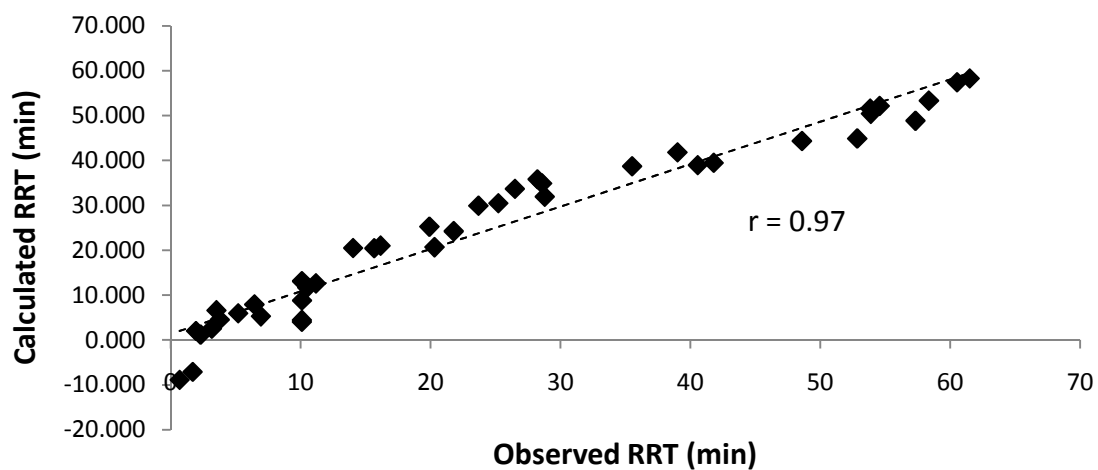
5,6 EET	57.96	54.56	-227.65	21.66
Arachidonyl glycerol	60.72	57.32	-224.67	19.42
EPA	61.75	58.35	-219.59	22.94
DHA	63.92	60.52	-220.9	21.15
AA	64.89	61.49	-225.64	23.34



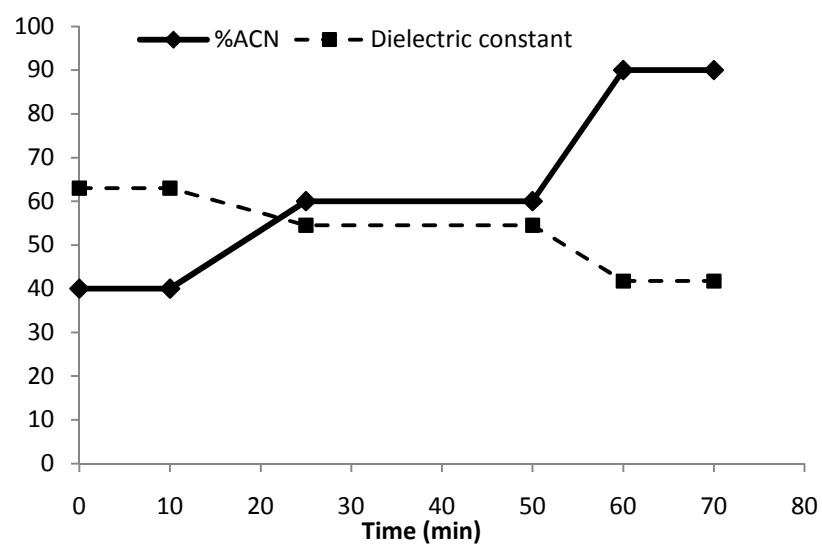
**Figure 6.7. Chemical reactivity: LUMO energies vs. RRT (min)**

*QSRR equation*

$$\text{Calculated RRT} = -9.595 + 9.625 (\text{Clog } p) - 0.7813 (\text{dipole moment}) + 0.0052 (\text{solvation energy}) + 0.2275 (\text{LUMO})$$



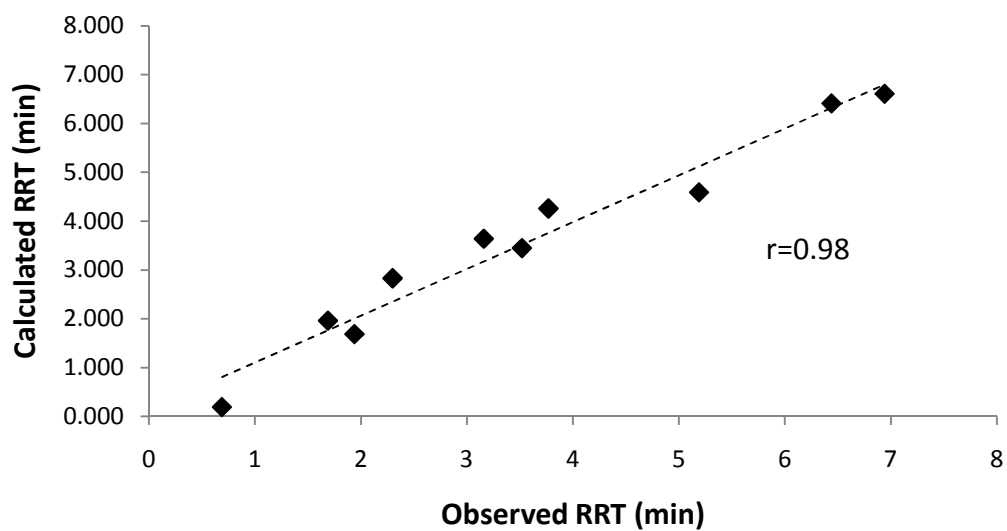
**Figure 6.8. Chromatographically determined RRT (min) vs. theoretically determined RRT (min) for 41 lipid biomarkers**



**Figure 6.9. Change in % acetonitrile (ACN) and dielectric constant with time (min)**

*QSRR equation*

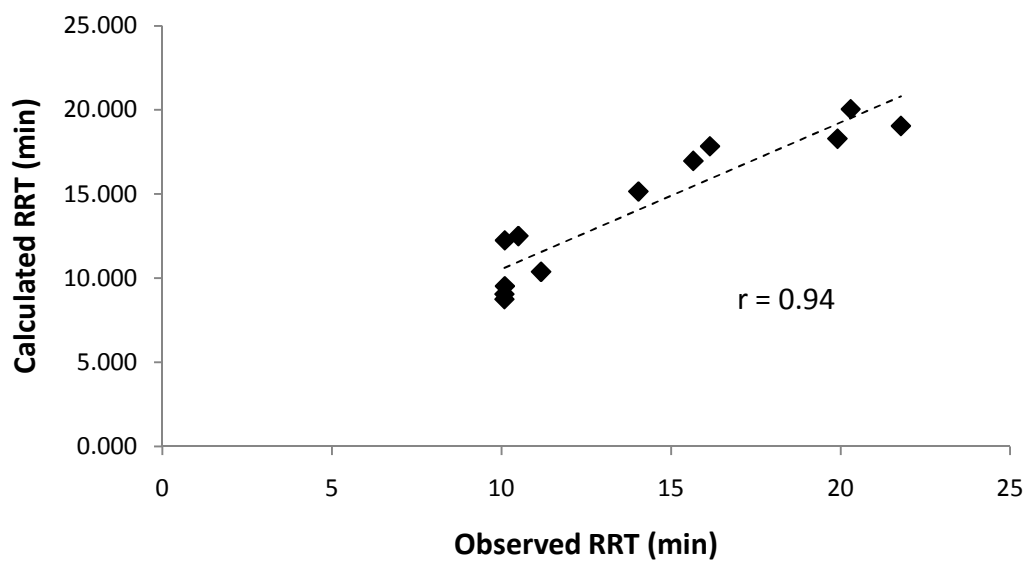
*Calculated RRT = -7.99 + 4.45 (ClogP) - 0.008 (dipole moment) - 0.002 (solvation energy) - 0.01 (LUMO)*



**Figure 6.10. Chromatographically determined RRT (min) vs. theoretically determined RRT (min) for compounds with RRT < 10 min**

*QSRR equation*

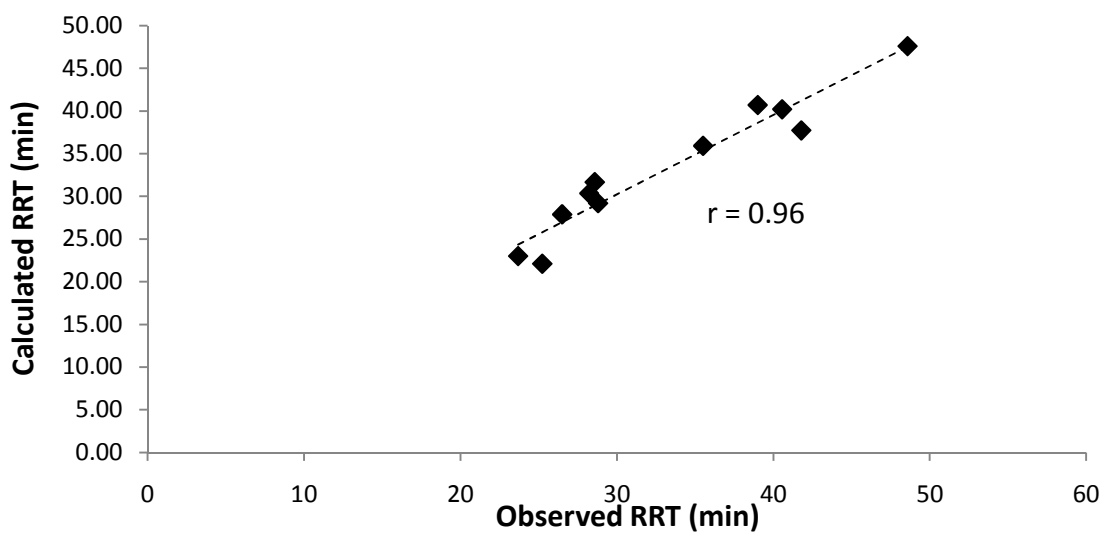
*Calculated RRT= -5.21 + 7.735 (ClogP) -0.211 (dipole moment) + 0.010 (solvation energy) -0.227 (LUMO)*



**Figure 6.11. Chromatographically determined RRT (min) vs. theoretically determined RRT (min) for compounds with RRT (10-25 min)**

*QSRR equation*

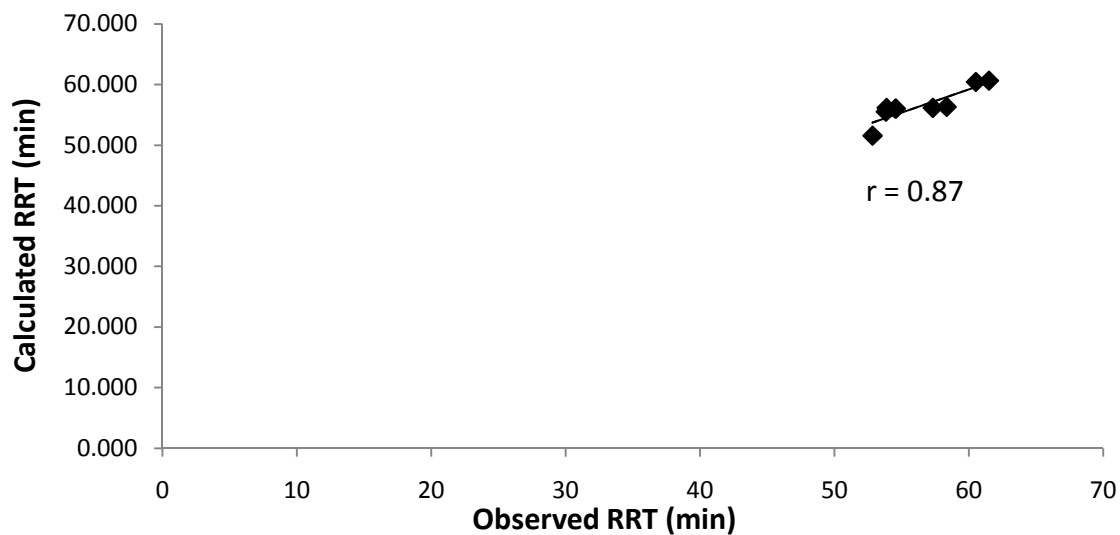
*Calculated RRT = - 19.21 + 12.54 (ClogP) - 0.314 (dipole moment) + 0.021 (solvation energy) + 0.39 (LUMO)*



**Figure 6.12. Chromatographically determined RRT (min) vs. theoretically determined RRT (min) for compounds with RRT (25-50 min)**

*QSRR equation*

*Calculated RRT = 13.91 + 7.39 (ClogP) + 0.230 (dipole moment) + 0.002 (solvation energy) - 0.170 (LUMO)*



**Figure 6.13. Chromatographically determined RRT (min) vs. theoretically determined RRT (min) for compounds with RRT (50-70 min)**

**Table 6.5. Coefficients of the molecular descriptors**

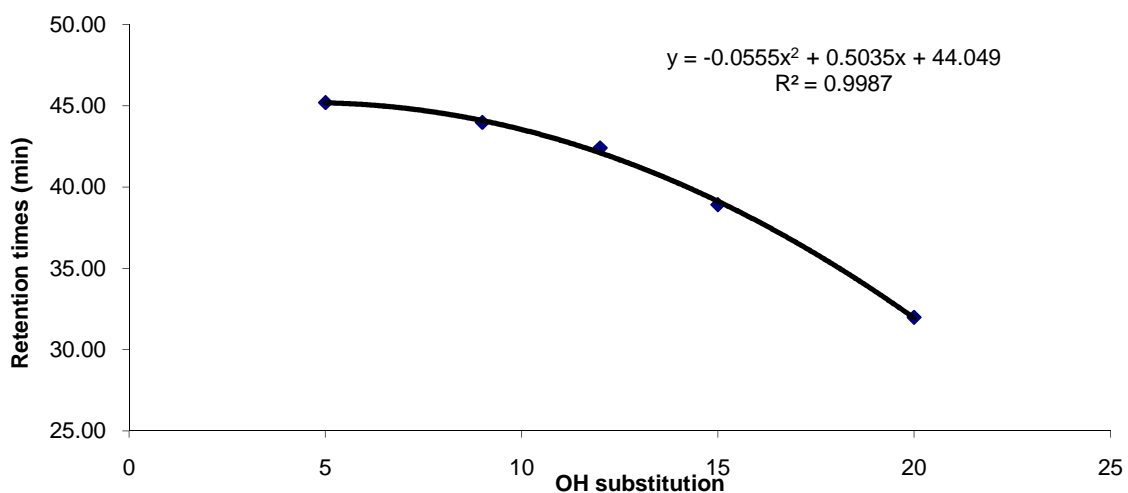
<b>Groups</b>	<b>Clog P</b>	<b>Dipole moment</b>	<b>Solvation</b>	<b>LUMO</b>	<b>Intercept</b>
0-10 min	4.45	-0.008	-0.002	-0.010	-7.99
10-25 min	7.73	-0.21	0.010	-0.23	-5.21
25-50 min	12.54	-0.314	0.021	0.39	-19.21
50-70 min	7.39	0.23	0.002	-0.17	13.91

**Table 6.6: % Accuracy using individual molecular descriptors for all the lipid biomarker**

Compounds	Clog P	Dipole moment	Polarizability	Solvation energies	LUMO
6-keto PGF <sub>1a</sub>	-1990.06	6269.91	-27870.68	-3192.72	2926.66
LTD4	-451.26	4616.22	-176604.46	-187.39	-2296.04
PGF <sub>3</sub>	-342.76	5666.45	2114.72	266.66	2835.45
8-isoprostane	-193.56	-955.91	-780.38	-182.23	2131.27
PGF <sub>2</sub>	-67.46	-3097.23	-649.14	-119.76	1669.12
PGE <sub>3</sub>	-32.81	3324.47	4224.94	350.91	1454.46
11-dehydro TXB <sub>3</sub>	-11.21	94.06	-272.05	-69.64	1215.88
PGE <sub>2</sub>	20.09	-341.63	1531.54	53.62	922.41
11-dehydro TXB <sub>2</sub>	71.17	-11.01	-1154.64	-185.40	734.92
PGD <sub>2</sub>	92.44	-3586.98	1108.40	50.55	251.76
LXA4	78.10	182.75	-2795.35	98.30	-547.01
15-epi LXA4	78.10	316.30	-2846.18	95.12	-509.24
RvD1	100.99	-842.64	-7489.21	271.14	-182.77
RvD2	137.26	631.98	-7565.37	257.24	-199.50
LTE4	149.47	189.13	-18266.18	93.08	-347.18
LTC4	175.49	1047.64	71597.46	-287.28	-376.63
RvE2	152.66	-782.97	474.83	256.97	95.67
PGJ <sub>2</sub>	139.99	443.83	1490.00	182.48	-69.39
D <sup>12</sup> PGJ <sub>2</sub>	146.32	13.17	1619.43	175.67	-68.35
RVD5	122.98	327.72	-2833.26	209.23	69.62
LTB4	120.62	398.28	-492.61	128.31	-160.94
RvD6	115.79	-136.76	-2766.59	192.24	31.81
14,15 DHETE	117.84	20.97	-259.88	69.12	288.44
11,12 DHETE	111.13	523.06	-223.67	65.22	224.13
8,9 DHETE	127.47	-40.16	-164.55	64.90	222.15
5,6 DHETE	123.55	180.95	-72.69	61.47	234.93
20-HETE	130.11	104.22	448.58	139.66	54.32
15-deoxy D <sup>12,14</sup> PGJ <sub>2</sub>	134.25	-214.32	1540.78	179.91	-116.57
15-HETE	116.41	301.58	353.82	115.69	30.24
12-HETE	106.30	367.34	479.95	105.88	110.85
9-HETE	105.53	-410.91	455.17	107.71	86.17
5-HETE	104.17	66.19	294.51	99.35	21.99
14,15 EET	90.37	-35.04	432.80	98.87	139.12
11,12 EET	83.54	29.53	407.62	91.12	127.75
8,9 EET	94.71	305.66	414.41	90.68	116.51
Anadamide	95.07	-96.09	-1294.42	103.82	128.90
5,6 EET	95.01	253.48	451.16	89.74	118.47
Arachidonyl glycerol	90.43	58.15	-2004.04	42.99	101.56
EPA	88.84	194.47	742.64	113.83	117.06
DHA	95.34	117.19	-389.78	118.33	104.39
AA	96.41	126.87	587.96	92.76	112.94
Average	15.09	380.97	-4049.38	7.52	287.93
Stdev	344.50	1773.45	30390.32	528.85	897.24

**Table 6.7. % Accuracy for all the lipid biomarkers (Model I: QSRR using Clog P, dipole moment and solvation energies and Model II: QSRR using CLOG P, dipole moment, LUMO and solvation energies, Group I-IV explained in text)**

Compounds	% Accuracy						Group IV (50-70)
	Model I	Model II	Group I	Group II	Group III(25-70)	Group III (25-50)	
6-keto PGF <sub>1α</sub>	-1533.10	-1281.13	28.31	-	-	-	-
LTB <sub>4</sub>	-231.99	-418.42	116.18	-	-	-	-
PGF <sub>1α</sub>	-116.99	105.92	86.97	-	-	-	-
8-isoprostane	-132.65	53.66	121.10	-	-	-	-
PGF <sub>2α</sub>	-37.64	80.69	115.23	-	-	-	-
PGF <sub>2α</sub>	82.83	189.64	97.99	-	-	-	-
11-dehydro TXB <sub>2</sub>	38.73	129.52	112.98	-	-	-	-
PGF <sub>2α</sub>	53.56	115.34	88.45	-	-	-	-
11-dehydro TXB <sub>2</sub>	80.54	123.30	99.59	-	-	-	-
PGI <sub>2</sub>	70.15	76.49	95.22	-	-	-	-
LXA <sub>4</sub>	93.49	40.31	-	89.86	-	-	-
15-epi LXA <sub>4</sub>	94.79	44.57	-	86.85	-	-	-
RvD1	111.32	87.49	-	94.43	-	-	-
RvD2	157.92	139.97	-	121.36	-	-	-
LTE <sub>4</sub>	153.68	111.63	-	119.20	-	-	-
LTC <sub>4</sub>	163.29	113.12	-	93.01	-	-	-
RvE2	150.11	148.22	-	107.96	-	-	-
PGI <sub>2</sub>	147.26	139.67	-	109.39	-	-	-
D <sup>2</sup> PGJ <sub>2</sub>	147.18	139.28	-	113.49	-	-	-
RVD5	130.26	126.93	-	91.87	-	-	-
LTB <sub>4</sub>	124.35	102.91	-	98.73	-	-	-
RvD6	117.19	111.41	-	87.46	-	-	-
14,15 DHETE	113.17	126.43	-	-	101.56	97.21	-
11,12 DHETE	112.25	129.96	-	-	90.43	87.71	-
8,9 DHETE	119.64	127.19	-	-	114.47	105.31	-
5,6 DHETE	118.99	127.00	-	-	114.78	107.57	-
20-HETE	127.11	122.14	-	-	109.97	119.79	-
15-oxo D <sup>12,14</sup> PGJ <sub>2</sub>	129.31	119.97	-	-	103.46	101.42	-
15-HETE	114.90	109.97	-	-	101.31	101.17	-
12-HETE	105.95	107.23	-	-	101.33	104.36	-
9-HETE	96.59	96.07	-	-	103.68	99.13	-
5-HETE	109.10	94.59	-	-	92.46	90.39	-
14,15 EET	86.48	91.31	-	-	94.12	97.97	-
11,12 EET	80.60	85.00	-	-	87.02	-	97.60
8,9 EET	93.13	95.87	-	-	99.82	-	103.16
Anandamide	89.72	93.59	-	-	102.97	-	104.21
5,6 EET	92.71	95.59	-	-	103.96	-	102.58
Amchidonyl glycerol	83.87	85.26	-	-	92.26	-	97.97
EPA	87.87	91.41	-	-	96.99	-	96.49
DHA	92.72	94.89	-	-	105.16	-	99.84
AA	92.28	94.79	-	-	106.17	-	98.61
Average	44.66	58.76	103.97	103.90	103.67	103.27	103.37
Stdev	264.08	231.97	13.15	12.22	7.52	6.94	2.90



**Figure 6.14. Relationship between retention time and position of OH substitution for selective HETEs**

**Table 6.8. Values of molecular descriptors for possible candidates of an unknown**

**HETE (RRT =31.58 min)**

Unknown compound	Clog P	Dipole moment (Deb)	E (aq) (kJ/mol)	LUMO (kcal/mol)	Calculated RRT	%Accuracy
19-HETE	4.79	1.62	-667.32	23.27	35.66	112.92
18-HETE	4.84	1.51	-659.59	23.05	36.40	115.25
17-HETE	4.91	3.22	-665.59	23.74	36.88	116.79
16-HETE	5.13	2.16	-659.3	22.70	39.70	125.70

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## CHAPTER 7

### **7. COMPARISON OF AN ULTRA HIGH PRESSURE LIQUID CHROMATOGRAPHY-UV METHOD WITH A CONVENTIONAL HPLC-UV METHOD FOR THE SIMULTANEOUS DETERMINATION OF PROSTANOIDS “Are the methods equivalent in terms of their accuracy and precision?”**

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#### **7.1 Introduction**

As new methods are developed to increase efficiency and higher analytical performance, it is necessary to evaluate their quality in comparison to standard methods. To understand how the analytical performance changes between methods, it is common to compare the validation parameters; sensitivity, linearity, accuracy and precision. In this chapter, we compare an UHPLC-UV method to the HPLC-UV method (reference method) for the simultaneous determination of seven prostanoids. Though the basic chromatography theory is the same for HPLC (High Performance Liquid Chromatography) and UHPLC (Ultra High Performance Liquid Chromatography), the instrumentation has been modified to accommodate higher pressures, lower flow rates and smaller sample size. The differences in analytical instrumentation and procedures can give rise to method in-equivalencies. Our approach evaluates the UHPLC and HPLC methods and poses the question: Are the methods equivalent? To answer this question a statistical comparison of the analytical performance and method parameters is necessary. High performance liquid chromatography (HPLC) is a reliable and well-established

separation technique, but lacks the efficiency of both time and materials. The demand for high throughput assay methods with reduced costs has led to supercritical fluid chromatography and ultrahigh pressure chromatography. Both the techniques allow a larger number of analyses to be done in a shorter period of time, thus improving the productivity [1]. Supercritical fluid chromatography represents a significant change in the nature of the separation and instrumentation, while UHPLC shares separation characteristics of traditional HPLC, with similar stationary and mobile phases.

Though the basic chromatography theory is the same for HPLC and UHPLC, there are significant operational differences in the analytical procedures that give rise to method in- equivalencies. UHPLC systems need to compensate for solvent compressibility (a function of high pressure). High pressure can compress the solvent thus decreasing its volume and in turn this will affect the mobile phase ratio. Faster run-times and higher chromatographic efficiencies give narrow peaks, which necessitate rapid sampling for the detector to prevent loss of resolution [2]. The UHPLC detectors have low cell volume (2-3 $\mu$ l) [3]. Hence, the detector time constant needs to be fast (< 100 ms), as the peak width is very small in UHPLC. The detector sampling rate also needs to be high enough to capture sufficient data points across the peak so that the peak shape and symmetry is reproducible and can be accurately integrated [2]. To understand whether these operational differences change the analytical performance between the two chromatographic techniques, we have compared validation parameters: sensitivity, linearity, accuracy and precision of the UHPLC-UV method to the HPLC-UV method (reference method) for the simultaneous determination of prostanoids.

Although the HPLC and UHPLC methods have individually fulfilled assay validation criteria, they cannot be assumed equivalent. This is because testing for method validation and testing for method equivalencies are different. The objective of method validation is to demonstrate that it is suitable for its intended purposes according to FDA guidance to the industry: Bioanalytical validation. Two methods can be equivalent, commutable or incompatible [4]. To be equivalent, the two methods must be interchangeable without loss of accuracy. Methods are commutable if they give results within an acceptable tolerance level. Incompatible methods give differences beyond the tolerance limit. The test for interchangeability normally involves regression analyses, which include Ordinary Linear Regression, the Deming Regression or the Passing-Bablok regression. Tolerances are evaluated by analysis of scatter plots or through comparison of residual differences as described by Bland and Altman.

In this work we compare a HPLC-UV method for seven prostaglandins with an UHPLC-UV method. The seven prostaglandins were chosen because they are important in evaluating tissue inflammation in musculoskeletal disorders (MSDs) [5] and rheumatoid arthritis [6]. In addition, 8-isoprostane, 6-keto PGF<sub>1 $\alpha$</sub>  and 11-dehydro TXB<sub>2</sub> are implicated in hypertension disorders [7-9]. This selection of analytes and the analyses described finds application in many clinical laboratories.

## **7.2 Experimental**

### **7.2.1 Materials**

The prostaglandins (6-keto PGF<sub>1 $\alpha$</sub> , PGF<sub>2 $\alpha$</sub> , PGE<sub>2</sub>, 11-dehydro TXB<sub>2</sub> and PGD<sub>2</sub>, 15-deoxy  $\Delta^{12,14}$  PGJ<sub>2</sub>) were purchased from Biomol International (Plymouth Meeting, PA,

USA). 8-isoprostane was purchased from Cayman Chemicals (Ann Arbor, MI, USA). HPLC water and acetonitrile were purchased from Fisher Scientific (Waltham, MA, USA).

### **7.2.2 Instrumentation**

HPLC-UV: The Jasco HPLC system consisting of the following parts was used; Jasco pumps (PU-980), a Jasco UV-VIS detector (UV-975) (Jasco Incorporated Easton, MD, USA) and a Rheodyne manual injector (Rheodyne LLC, Rohnert Park, CA, USA). Jasco-Borwin software (version 3.3.5) was used for data collection.

UHPLC-UV: Jasco XLC system consisting of the following parts was used; Jasco pumps (3185 PU), a Jasco auto sampler (3159 AS) and a Jasco UV-VIS detector (3075 UV). EZ chrome software was used for data collection.

### **7.2.3 Chromatographic conditions**

HPLC-UV: The analysis was performed on a Symmetry Shield RP18 4.6 x 250 mm column with 5  $\mu\text{m}$  particle size (Waters Corporation, Milford, MA, USA) at ambient temperature. The method employed a gradient elution of 17 mM phosphoric acid (pH =2.11) (A) and acetonitrile (B) as follows: 35% B for 16.4 min, 35-80% B from 16.4 min to 16.5 min, hold 80% B from 16.5 min to 23.0 min, 80-35% B from 23.0 min to 23.1 min, re-equilibration from 23.1 min to 25.0 min. The flow rate was set to 1.3 ml/min, wavelength of detection was 196 nm and the injection volume was 100  $\mu\text{l}$ .

UHPLC-UV: The analysis was performed on a Restek Pinnacle DB C<sub>18</sub> 2.1mm x 50 mm column with 1.9  $\mu\text{m}$  particle size (Bellefonte, PA) at ambient temperature. This method

used a mixture of 0.1% formic acid (pH =2.66) (A) and acetonitrile (B) in a gradient elution as follows: 35% B for 4.0 min, 35-90% B from 4.0 min to 6.0 min, 90-35% B from 6.5 min to 6.6 min, re-equilibration from 6.6 min to 7.0 min. The flow rate was set to 0.2 ml/min, wavelength of detection was 196 nm and the injection volume was 5  $\mu$ l.

#### **7.2.4 Standard Solutions**

8-isoprostane was supplied in methanol and 15-deoxy  $\Delta^{12,14}$  PGJ<sub>2</sub> was supplied in methyl acetate with stock mass concentrations of 1 mg/ml. 11-dehydro TXB<sub>2</sub> was dissolved in methanol such that its stock mass concentration was 10  $\mu$ g/ $\mu$ l. All the other prostanoids were also dissolved in methanol to obtain the stock mass concentrations of 1 mg/ml. Working standards for all the analytes were prepared by serial dilution using 17mM phosphoric acid and acetonitrile in the ratio of 1:1 and 0.1 % formic acid and acetonitrile in the ratio of 1:1 for the HPLC and UHPLC respectively. Calibration standards for the prostanoids were prepared at mass concentrations of 0.1, 0.2, 0.3, 0.5, 1, 2, 3, 5, 10, 20, 40, 60,100, 160 and ng/ $\mu$ l and from the respective stock solutions. Quality control (QC) samples were prepared in triplicate for all the analytes representing low, medium and high concentrations in linearity curve. All the solutions were stored at – 80°C when not in use.

#### **7.2.5 Validation**

The methods were validated according to FDA guidance to the industry: Bioanalytical validation [10]. The linearity for all the analytes was established by injecting in triplicate; standard solutions with mass concentration ranging from LOQ to 10 ng/ $\mu$ l for the HPLC method and LOQ to 200 ng/ $\mu$ l for the UHPLC method. The

linearity curve was obtained by plotting the peak areas of the prostanoids with the respective theoretical column loading (ng). Five solutions of each mass concentration of the QC samples were prepared and injected in triplicate to determine the accuracy (n = 15). The calculated concentrations for each of the samples were then compared to the theoretical concentration to determine the % accuracy for each of the compounds. Intra-assay precision were determined by injecting in triplicate; five replicate preparations of three mass concentration (low, medium and high) of each analyte spiked in urine for a single day. The robustness and the ruggedness of the methods were also tested as per the validation guidelines.

#### **7.2.6 Matrix Effect**

To study the matrix effect urine samples from control subjects were used. The matrix effect was examined by calculating the recovery of the analytes at three concentrations (low, medium and high) spiked in the control urine. Obtained concentrations, were calculated from the linearity curve of the standard solutions. The concentrations thus obtained, were corrected for the presence of endogenous prostanoids. The recoveries were then calculated by comparing the corrected concentrations with the spiked theoretical concentrations.

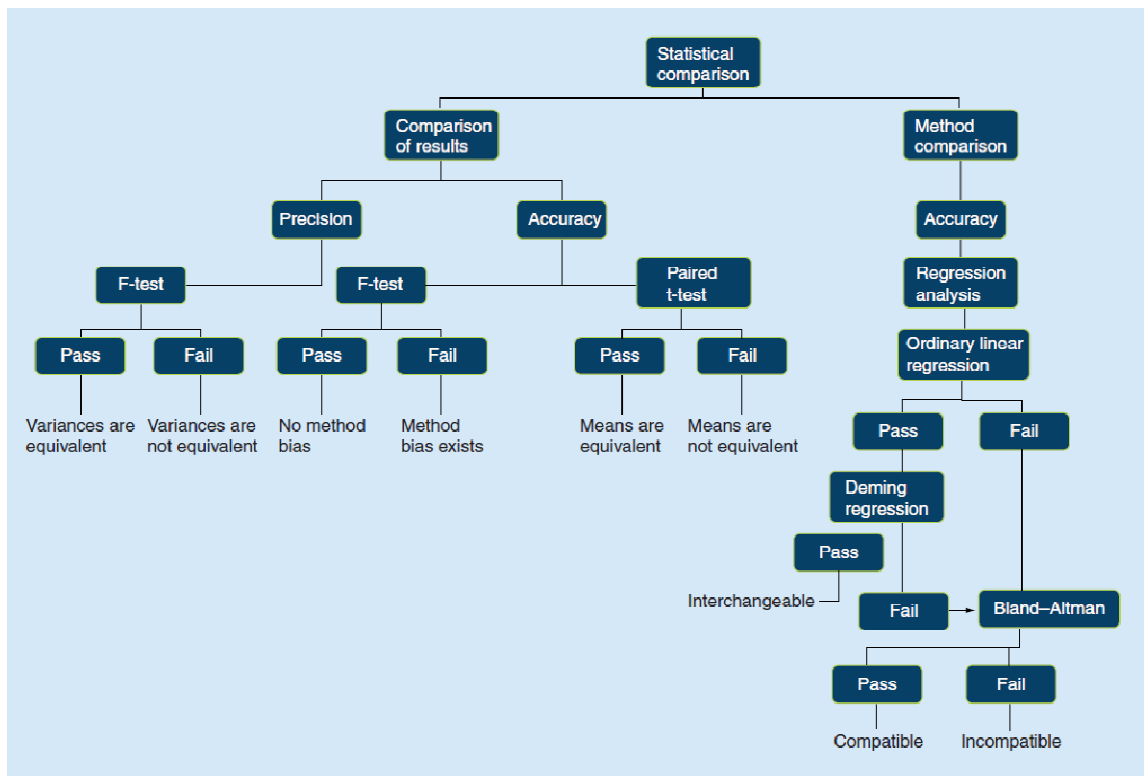
#### **7.2.7 Statistical methods**

The F-Test, T-Test and the Bland-Altman analysis were performed using Microsoft Excel. The regression analysis was done using MedCalc version 11.1. Figure 7.1 shows a flow chart/decision tree of the statistical analysis that was performed.

## **7.3 Results**

### **7.3.1 Validation**

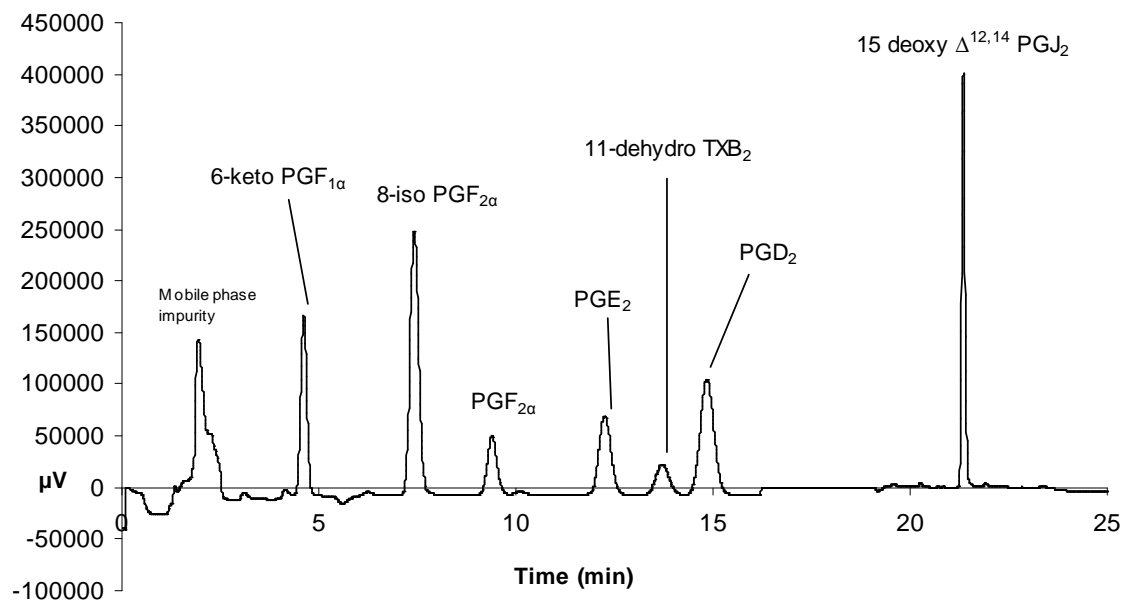
Figures 7.2 and 7.3 are representative HPLC and UHPLC chromatogram for all the seven prostanoids. The resolution and the asymmetry for all the prostanoids are within the acceptable limits [10]. Peak purity was tested and all the peaks meet the peak purity standard of 99.99%. The average peak width at  $\frac{1}{2}$  peak height for the prostanoids in the UHPLC method (Figure 7.3) is approximately 0.15 min (detector time constant = 100 Hz), which is a little larger than the average peak width's reported in literature (0.07-0.10 min). But, there is no established peak width criterion in the validation guidelines of either the ICH or the FDA.



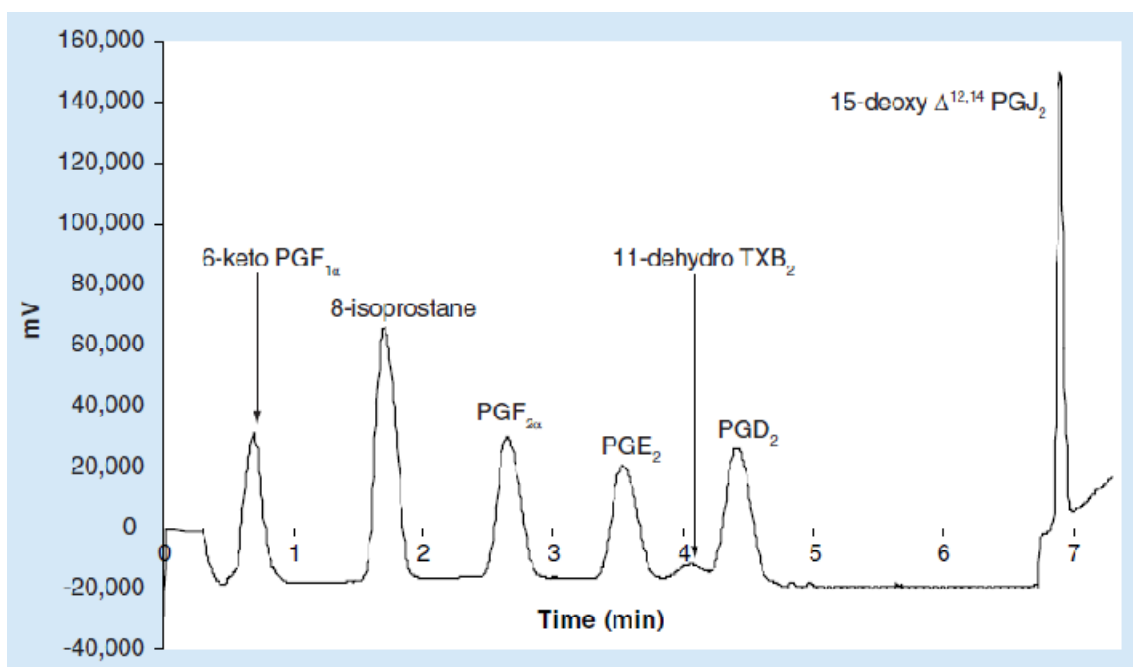
**Figure 7.1. Flow chart/ decision tree representation of the statistical analysis performed**

The sensitivity, linearity and accuracy for the prostanoids by both the methods are within the acceptable limits for bioanalytical method validation as can be seen from the Tables 7.1 and 7.2. The methods are linear over three orders of magnitude. The data for each compound and each method was tested for homoscedascity. Several compounds failed the test of homoscedascity for one or both methods. Table 7.1 shows the regression line for each compound for the HPLC and UHPLC with the weighting factor applied to reduce the residuals. No matrix bias was identified in this study using mobile phase, ACN, methanol, buffer or urine. Our results are presented for samples prepared and recovered from mobile phase matrix.

The sensitivity of both the methods (LOQ) was found to be statistically similar on comparison by the paired T-test. The limit of quantification (LOQ) was calculated as the lowest concentrations that give the signal to noise ratio of 10:1.



**Figure 7.2. Representative HPLC chromatogram of all the prostanooids**



**Figure 7.3. Representative UHPLC chromatogram of all the prostanoids**

**Table 7.1. Method Validation Results: Sensitivity and Linearity- Eq. of the line with the weighting factors (Amount in ng refers to column loading)**

Compound	Linearity ( $R^2$ )		Linearity		LOQ (ng)	
	UHPLC	HPLC	UHPLC	HPLC	UHPLC	HPLC
6-keto PGF <sub>1</sub>	0.998	0.999	$y = 1537.5x - 31039.2$	$y = 907.6x - 7699$	25	20
8-iso PGF <sub>2</sub>	0.999	0.999	$y = 4587.5x + 590.7 (1/x)$	$y = 1584.0x + 1899.5$	20	27
PGF <sub>2</sub>	0.999	1.000	$y = 3588.7x + 3586.1 (1/y)$	$y = 2189.6x - 4975.1$	15	7.5
PGE <sub>2</sub>	0.998	1.000	$y = 1957.8x + 19207.9 (1/y)$	$y = 848.9x + 9.7$	20	25
11-dehydro TXB <sub>2</sub>	0.998	0.999	$y = 2515.3x + 8690.9$	$y = 639.1x - 6099.4 (1/y^2)$	30	30
PGD <sub>2</sub>	0.999	0.999	$y = 4180.6x + 11364$	$y = 1624.5x - 1880.9 (1/x^2)$	22	13
15-deoxy $\Delta^{12,14}$ PGJ <sub>2</sub>	0.999	0.999	$y = 2114.1x + 23626.9 (1/y)$	$y = 2370.8x - 79.2$	4	9

**Table 7.2. Method Validation Results: Recovery Accuracy - Level I (50 ng-75 ng), Level II (100 ng-200 ng) and Level III (500 ng-800 ng) represents the low, medium and high concentrations of the linearity range.**

Compounds	% Accuracy (standard solutions)			% Accuracy (standard solutions)		
	UHPLC			HPLC		
	I	II	III	I	II	III
6-keto PGF <sub>1</sub>	104.6	98.5	90.7	97.9	99.6	102.6
8-iso PGF <sub>2</sub>	97.1	104.4	107.1	104.5	105.8	100.4
PGF <sub>2</sub>	115.1	109.0	101.6	97.2	98.8	98.9
PGE <sub>2</sub>	105.4	99.8	101.2	97.3	104.0	100.3
11-dehydro TXB <sub>2</sub>	102.7	102.8	105.2	100.6	97.9	90.4
PGD <sub>2</sub>	115.0	110.5	104.2	97.4	106.4	112.7
15-deoxy $\Delta^{12,14}$ PGJ <sub>2</sub>	115.0	109.7	105.3	100.9	100.7	98.8

### 7.3.2 Matrix effect

The average recoveries for all the three concentrations for both the methods lie within 90-115% (Table 7.3). They are found to be within the acceptable limits of recovery. There is no specific acceptable limit for the matrix effect in biological samples [10]. Also, the p-values obtained from the paired T-test for all the analytes in the matrix compared with the solvent were found to be greater than 0.05 indicating that the differences are not statistically significant. The % RSD for all the analytes in matrix is <15 %, which meets validation requirements. Previous work in various matrices such as brain, heart, kidney, and blood establishes that assays of these biomarkers, using our protocol do not show matrix bias [11, 12].

### 7.3.3 Comparison Studies

#### 7.3.3.1 Comparison of results

Because there was no matrix bias for the series of biomarkers of study, the precision, accuracy and regression analyses results for standards in solvent are discussed. Results for urine matrix are statistically similar and thus are not provided here.

##### *a. Precision*

The % RSD for all the analytes in solvent is <15 %, which is an acceptable limit for bioanalytical validation. The precision was also compared statistically using the F-Test.  $F_{\text{calculated}} > F_{\text{tabulated}}$  and the 2-tailed p-values are less than 0.05 for the majority of the prostanoids (Table 7.4), thus suggesting a statistically disagreement.

**Table 7.3 Matrix effect- Level I (50 ng-75 ng), Level II (100 ng-200 ng) and Level III (500 ng-800 ng) represents the low, medium and high concentrations of the linearity range**

Compounds	% Accuracy (urine matrix) UHPLC			% Accuracy (urine matrix) HPLC		
	I	II	III	I	II	III
	6-keto PGF <sub>1</sub>	99.5	107.1	104.3	107.1	105.3
8-iso PGF <sub>2</sub>	114.2	109.8	104.2	102.3	98.8	101.3
PGF <sub>2</sub>	111.4	114.4	104.6	100.9	106.5	95.8
PGE <sub>2</sub>	94.3	106.4	110.7	107.4	101.1	107.7
11-dehydro TXB <sub>2</sub>	110.5	104.6	92.4	106.9	107.7	100.9
PGD <sub>2</sub>	92.0	100.1	97.7	108.3	104.6	106.9
15-deoxy $\Delta^{12,14}$ PGJ <sub>2</sub>	113.6	110.8	95.6	100.9	100.7	98.1

**Table 7.4. Comparison of Precision: p values (F-test) (2-tailed)**

<b>Compounds</b>	<b>p value (2-tailed)</b>		
	<b>I</b>	<b>II</b>	<b>III</b>
6-keto PGF <sub>1</sub>	0.56	0.02	<0.001
8-iso PGF <sub>2</sub>	0.02	<0.001	<0.001
PGF <sub>2</sub>	0.20	0.08	0.05
PGE <sub>2</sub>	<0.001	0.001	0.001
11-dehydro TXB <sub>2</sub>	<0.001	0.01	<0.001
PGD <sub>2</sub>	<0.001	<0.001	<0.001
15-deoxy $\Delta^{12,14}$ PGJ <sub>2</sub>	0.27	0.99	0.11

We can conclude that, though the % RSD is within acceptable limits of method validation, the amount of variation (i.e. the precision of the two methods) is statistically different

*b. Accuracy (Direct Determination)*

The accuracy of the two methods was evaluated by comparing the obtained mean concentrations of all the analytes in solvent statistically using the F-Test and paired T-Test. The F-Test fails only for 6-keto PGF<sub>1 $\alpha$</sub> , indicating the variances are statistically different for this compound. Since,  $t_{\text{calculated}} < t_{\text{tabulated}}$  for all the compounds within 95% confidence and all the 2-tailed p-values are greater than 0.05 (Table 7.5) the means of the two methods are statistically similar.

7.3.3.2 Comparison of methods

*Regression analysis*

Regression analysis is done to determine whether there is a constant and/or proportional and/or differences in random error between the two methods and to what degree the methods are correlated. A constant bias is identified by a shift in the intercept of the regression plot. A proportional bias is identified by a change in the slope. Both Ordinary Linear Regression (OLR) and Deming regression models are used for the comparison. The Passing-Bablok regression was not used; as it is a non-parametric test that it does not require that the data fit a normal distribution.

### Classic Linear Regression Model or Ordinary Linear regression (OLR)

The accuracy of the methods were compared using the classic linear regression model which assumes that the nominal concentrations of the standards are the independent variable (x) and contribute no uncertainty to the determined value for the dependent variable (y) [13,14]. The regression line is calculated by minimizing the squared residuals by an iterative adjustment of the slope and residual. For the OLR, all of the data being compared met the test of homoscedascity. The slope, intercept and the correlation coefficients for all the prostanoids using the least squares linear regression model are given in Table 7.6. Ideal comparison would result in the exact same assay values from both methods, thus resulting in a line with slope = 1 and intercept = 0. The correlation coefficient,  $r$ , is a good measure of the linear relationship between the two variables. However, the standard deviation of the residuals is a better parameter, as it does not depend on the data range. It takes into account the random errors associated with the variables and is influenced by two factors; total imprecision and sample related effects. The calculated standard deviation ( $s_{y/x}$ ) of the residuals was compared to the theoretical value ( $s_{a\ tot}$ ) (Table 7.7). The theoretical standard deviation of the residuals was calculated by taking the root of the sum of the square of the residuals of each individual method [15, 16]. Calculated standard deviation of the residuals ( $s_{y/x}$ ) for all the seven prostanoids was smaller than the theoretical value ( $s_{a\ tot}$ ). From these results, we conclude that the methods are statistically similar according to OLR.

**Table 7.5. Comparison of Accuracy: p values (T-test) (2-tailed)**

<b>Compounds</b>	<b>p value (2-tailed)</b>		
	<b>I</b>	<b>II</b>	<b>III</b>
6-keto PGF <sub>1</sub>	0.87	0.10	0.08
8-iso PGF <sub>2</sub>	0.83	0.76	0.06
PGF <sub>2</sub>	0.41	0.18	0.97
PGE <sub>2</sub>	0.25	0.32	0.89
11-dehydro TXB <sub>2</sub>	0.25	0.91	0.16
PGD <sub>2</sub>	0.20	0.83	0.43
15-deoxy $\Delta^{12,14}$ PGJ <sub>2</sub>	0.31	0.19	0.93

**Table 7.6. Values for slope, intercept, 95% CI and r obtained from Ordinary Linear Regression analysis, \*Quality of comparison [15]**

Compound	Slope	95% CI	Intercept	95% CI	r	Comment*
6-keto PGF <sub>1</sub>	0.858	-0.435 to 2.150	6.204	-61.788 to 74.196	0.879	Poor
8-isoprostane	1.068	1.040 to 1.095	-14.068	-29.207 to 1.069	0.999	Good
PGF <sub>2</sub>	0.899	0.867 to 0.931	7.014	-0.925 to 14.953	0.999	Good
PGE <sub>2</sub>	0.948	0.366 to 1.529	5.195	-57.338 to 67.728	0.957	Borderline
11-dehydro TXB <sub>2</sub>	1.132	1.108 to 1.156	-9.940	-16.670 to -3.210	0.999	Good
PGD <sub>2</sub>	0.916	0.893 to 0.939	-1.999	-6.124 to 2.127	0.999	Good
15-deoxy $\Delta^{12,14}$ PGJ <sub>2</sub>	1.121	0.997 to 1.245	-6.006	-15.648 to 3.637	0.993	Good

**Table 7.7. Values for  $S_{y/x}$  and  $S_{a\ tot}$  from Ordinary linear regression analysis (Note:  $S^2_{a,x}$  = variance of residuals (HPLC) and  $S^2_{a,y}$  = variance of residuals (UHPLC) )**

<b>Compound</b>	<b><math>S_{y/x}</math></b>	<b><math>S_{a\ total} = \sqrt{S^2_{a,x} + S^2_{a,y}}</math></b>
6-keto PGF <sub>1</sub>	2.719	4.483
8-isoprostane	21.050	33.245
PGF <sub>2</sub>	10.957	12.270
PGE <sub>2</sub>	6.167	9.342
11-dehydro TXB <sub>2</sub>	10.824	23.798
PGD <sub>2</sub>	14.861	46.883
15-deoxy $\Delta^{12,14}$ PGJ <sub>2</sub>	5.296	14.800

### Deming Regression (orthogonal regression)

The Deming regression model in contrast to the OLR, takes into account the random error in both the dependent (x) and independent (y) variables (Reference and the test methods) [15,17]. In this model, the sum of the squares of the residuals is minimized from both the axes [15, 17]. In this case, the two methods are statistically identical if the 95% CI (Confidence Interval) of the slope includes 1.00 and the 95% CI of the intercept includes 0.0. The slopes and intercepts for all the prostanoids using the Deming regression model are given in Table 7.8. The Deming regression fails for many prostanoids by not meeting at least one of the criteria. 11-dehydro TXB<sub>2</sub> does not meet either the slope or intercept criteria whereas 6-keto PGF<sub>1 $\alpha$</sub>  and PGE<sub>2</sub> meet both the criteria. 8-isoprostane, PGF<sub>2 $\alpha$</sub> , PGD<sub>2</sub>, and 15-deoxy  $\Delta^{12,14}$  PGJ<sub>2</sub> fails the slope criteria, indicating the presence of a proportional bias.

### *Bland-Altman Analysis*

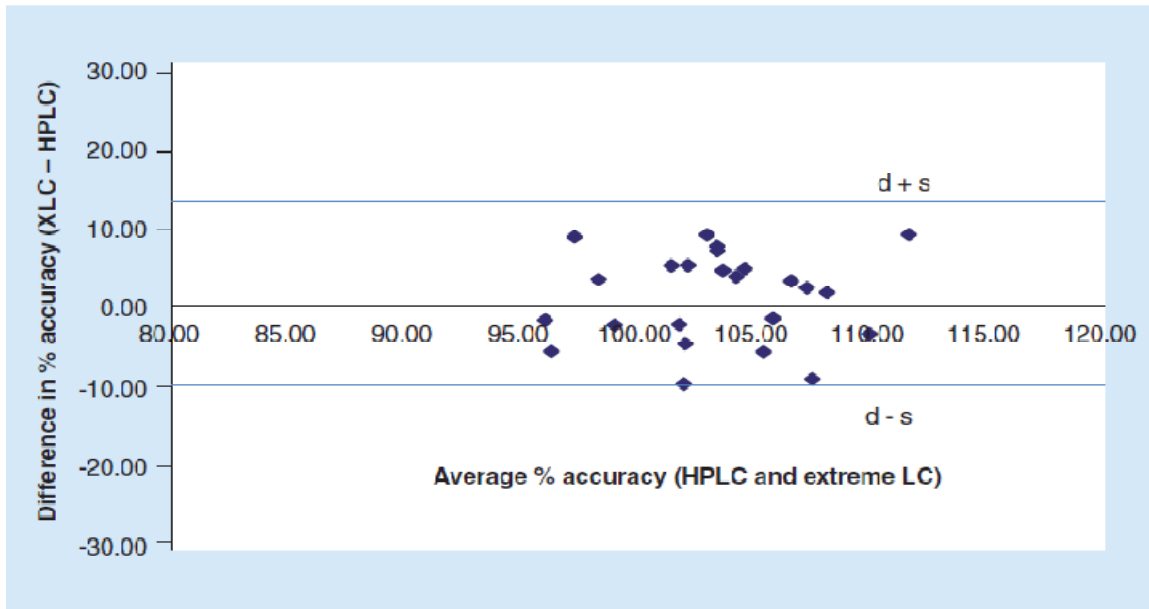
The accuracy of the two methods was also compared using the Bland-Altman method [18] to determine whether the old established HPLC method is commutable with the new UHPLC method. According to Bland and Altman, the regression models suggest the degree of correlation between the methods, and not the agreement between the results of the methods. A Bland-Altman analysis was performed for all the analytes by plotting the difference in accuracy between the two methods against the mean accuracy for each concentration. The criterion for agreement is that the differences within the accuracy fall within the 95% confidence limit of the mean of the differences. A representative Bland-Altman plot is shown for 8-isoprostane (Figure 7.4). 8-isoprostane is the compound with

the greatest dispersion in accuracy measurements ( $s_{y/x}$ ) (see Table 7.7). The effect of the matrix on accuracy and precision was statistically compared by Bland-Altman, as the two methods are not interchangeable according to the regression studies on standard solutions. The measurements, obtained by the Bland-Altman Analysis are within 95% confidence ( $d \pm s$ ), thus fulfilling the criteria of agreement. According to the Bland and Altman, the measurements are within 95% confidence ( $d \pm s$ ) for all the prostanoids in both the solvent and urine matrix, thus fulfilling the criteria of agreement

**Table 7.8. Values for slope and intercept obtained from Deeming regression analysis**

(Note: + and – indicates the presence and the absence of the bias respectively)

<b>Compound</b>	<b>Slope</b>	<b>95% CI</b>	<b>Intercept</b>	<b>95% CI</b>	<b>Proportional Bias</b>	<b>Constant Bias</b>
6-keto PGF <sub>1</sub>	0.949	-0.355 to 2.252	1.423	-61.134 to 69.981	-	-
8-isoprostane	1.070	1.042 to 1.097	-14.888	-30.035 to 0.258	+	-
PGF <sub>2</sub>	0.900	0.868 to 0.932	6.858	-1.082 to 14.799	+	-
PGE <sub>2</sub>	1.039	0.444 to 1.634	-4.632	-68.641 to 59.376	-	-
11-dehydro TXB <sub>2</sub>	1.133	1.109 to 1.157	-10.110	-16.841 to -3.379	+	+
PGD <sub>2</sub>	0.916	0.893 to 0.939	-2.021	-6.147 to 2.104	+	-
15-deoxy $\Delta^{12,14}$ PGJ <sub>2</sub>	1.127	1.002 to 1.251	-6.419	-16.066 to 3.228	+	-



d= mean of the difference between the two methods

s = 2\*standard deviation

**Figure 7.4. Bland Altman plot for 8-Isoprostane**

## 7.4 Conclusion

The UHPLC-UV and the HPLC-UV methods for the simultaneous profiling of the prostanoids are sensitive, robust, precise and linear over three orders of magnitude. No matrix bias was observed in urine samples. Statistical comparison of the results, suggested that the precision (amount of variability) is different for the HPLC and UHPLC methods, whereas the accuracy (method bias and the means) is similar for 8-isoprostane, 11-dehydro TXB<sub>2</sub>, PGE<sub>2</sub>, PGF<sub>2 $\alpha$</sub> , PGD<sub>2</sub>, and 15-deoxy  $\Delta^{12,14}$  PGJ<sub>2</sub>. A summary of the statistical results is given in Table 7.9.

Method comparison by the Ordinary Linear Regression (OLR) shows that the methods are well-correlated for all seven compounds. The Deming regression, which assumes error in both the methods, suggests the existence of a proportional and constant bias for 11-dehydro TXB<sub>2</sub> and only proportional bias for 8-isoprostane, PGF<sub>2 $\alpha$</sub> , PGD<sub>2</sub>, and 15-deoxy  $\Delta^{12,14}$  PGJ<sub>2</sub>. According to Deming regression, the two methods are statistically similar for 6-keto PGF<sub>1 $\alpha$</sub>  and PGE<sub>2</sub>. We conclude that the two methods are not interchangeable. The Bland-Altman analyses for the seven compounds show good agreement between the UHPLC and the HPLC method. These data are consistent with the conclusions from the T-Test. The Bland-Altman analyses indicate the two methods are commutable.

**Table 7.9. Summary of the statistical analysis (Note: P = passes F = fails)**

Compounds	Comparison of results			Comparison of methods			
	Precision	Accuracy		Accuracy			Matrix effect
	F-test	F-test	T-test	OLR	Deming	Bland Altman	Bland Altman
6-keto PGF <sub>1</sub>	F	F	P	P	P	P	P
8-isoprostane	F	P	P	P	F	P	P
PGF <sub>2</sub>	P	P	P	P	F	P	P
PGE <sub>2</sub>	F	P	P	P	P	P	P
11-dehydro TXB <sub>2</sub>	F	P	P	P	F	P	P
PGD <sub>2</sub>	F	P	P	P	F	P	P
15-deoxy $\Delta^{12,14}$ PGJ <sub>2</sub>	P	P	P	P	F	P	P

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## CHAPTER 8

### 8. DETERMINING THE ROLE OF URINARY PROSTANOIDS IN HYPERTENSIVE MIDDLE-AGED AFRICAN AMERICAN POPULATION FOLLOWING ONE BOUT OF EXERCISE

#### 8.1 Introduction

Hypertension is a common primary condition among African Americans. According to CDC (Centers for disease control and prevention) by the age of 45 - 54, 36.2% of American men and women have hypertension and this increase to 64.1% and 70.8% by the age of 65-74 [1]. Hypertension and its related complications account for 20% of the African American deaths in the United States - twice the percentage of deaths from hypertension among Caucasian Americans [2]. National survey also shows that majority of middle-aged, urban African Americans engage in little or no leisure time physical activity [3]. This inactivity contributes to the disproportionate number of hypertension and coronary heart disease in African Americans [4-5]. There is much research focused on understanding the mechanism and treatment of hypertensive disorder under clinical conditions.

Emerging evidences suggests that endothelial dysfunction and oxidative stress play an important role in the molecular processes leading to hypertension [2,6-7]. Endothelium is the lining of cells, covering the internal surface of blood vessels. It is an organ that produces large number of vasodilating and vasoconstrictive substances [8]. Equilibrium between the vasodilating and vasoconstrictive substances is necessary to maintain vascular homeostasis. Endothelium is involved in a number of physiological

functions such as leukocyte adherence, platelet activation, coagulation and mitogenesis [9]. An impaired function of endothelium known as endothelial dysfunction causes thrombosis, molecular oxidation, vascular inflammation and atherosclerosis [9]. A healthy endothelium is therefore necessary to maintain homeostasis and in the local regulation of vascular tone and structure [8]. The other important participant in hypertension is oxidative stress. Oxidative stress is a condition in which there is an imbalance between the production of reactive oxygen species and the biological system ability to detoxify them. The exact role of oxidative stress in the development of hypertension is still not clear. But, various animal studies have observed an increased formation of reactive oxygen species from the vascular walls in hypertension [10]. Also, a relationship was observed between high blood pressure and vascular oxidative stress in patients diagnosed with hypertension [11,12]. The increased production of ROS causes a decrease in the bioavailability of nitric oxide, a vasodilator and promotes vascular cell proliferation and migration, inflammation and apoptosis. All these processes contribute to the development of hypertension [13].

Metabolites of polyunsaturated fatty acids such as Arachidonic Acid (AA) are known to participate in these vascular effects. Prostanoids binds to receptors on target cell plasma membrane and causes biological effects such as vasoconstriction, vasodilation, platelet aggregation and bronchoconstriction [14]. Prostanoids includes prostaglandins (PGE<sub>2</sub>, PGF<sub>2</sub>, PGD<sub>2</sub>), prostacyclin (PGI<sub>2</sub>), thromboxane (TXA<sub>2</sub>) and isoprostanes (8-iso PGF<sub>2α</sub>). 8-iso PGF<sub>2</sub>, the most abundant form of the F<sub>2</sub> isoprostane is considered to be an indicator of *in vivo* oxidative stress [15]. Measurement of 8-iso PGF<sub>2</sub> in urine is known to be the best non-invasive approach in the determination of oxidative

stress [16]. PGE<sub>2</sub> and PGI<sub>2</sub> predominantly behave as pro-inflammatory markers. They increase edema formation and leukocyte infiltration [14]. PGI<sub>2</sub> also increases the vascular permeability [14]. In addition to that, both these prostanoids are known to regulate systemic blood pressure [17]. PGF<sub>2</sub> and TXA<sub>2</sub> are potent vasoconstrictors though PGF<sub>2</sub> is primarily a constrictor of the pulmonary arteries and veins and TXA<sub>2</sub> of the whole body [14]. Equilibrium needs to be maintained between PGI<sub>2</sub>, which inhibits platelet aggregation, and TXA<sub>2</sub>, a platelet activator in order to maintain vascular homeostasis [18]. Both of these lipid molecules are modulated by inflammation and oxidative stress, both of which are implicated in the disease pathway [14]. Reduction in the urinary output of PGE<sub>2</sub> and PGI<sub>2</sub> while maintaining a normal output of TXA<sub>2</sub> has been reported in hypertension [19]. This creates an imbalance between PGI<sub>2</sub> and TXA<sub>2</sub>, favoring TXA<sub>2</sub>, which acts as a vasoconstrictor. It is not yet clear whether this imbalance is the cause or the response of hypertension.

Lifestyle affects clinical markers of hypertension. Exercise is a physiological intervention that is widely prescribed and recommended for lowering blood pressure and inflammation in hypertension [20]. It is well documented that physical training has beneficial effects on reducing cardiovascular risk factors such as high blood pressure and glucose tolerance [21, 22]. Acute exercise is known to enhance the generation of reactive oxygen species (ROS) such as superoxide, hydroxyl and nitric oxide ions. This increase in ROS after exercise results in the release of cytokines along with production of prostanoids [21]. It is well documented in the literature that exercise increases serum levels of vasodilator prostacyclin (PGI<sub>2</sub>) [22-24]. Zoladaz *et al.* recently reported an increase in prostacyclin production following exercise in hypertensive patients with

coronary artery disease when compared to healthy control [22]. However, there are contradictory reports on exercise-induced production of TXA<sub>2</sub>. There are few studies that indicate an increase while there are others that have reported no change in the TXA<sub>2</sub> levels post exercise [25-28].

The purpose of this study was to determine the effect of acute exercise on hypertensive African Americans, toward establishing a therapeutic exercise regime that can and will be accommodated by patient populations.

## **8.2 Protocol**

### **8.2.1 Participants**

Participants for this study were from Baltimore, Maryland and Washington DC. Ten volunteers were used for this study. These participants were sedentary (< 2 sessions /wk of regular aerobic exercise and <20 min/session in terms of occupation) African Americans who have been diagnosed having pre-hypertension (pre-HTN) or stage I hypertension (systolic blood pressure: 143±7 and diastolic pressure 87±7 mm Hg). The study consisted of 5 men and 5 women with the following characteristics: Age – 57±3 yrs; BMI (Body Mass Index) – 30.46± 3.50.; VO<sub>2</sub> (oxygen consumption or aerobic capacity) (mL/kg) – 22.587 ±3.724. All subjects have C - reactive protein (CRP) levels greater than 3.5.

None of the participants were on anti-hypertensive medications. BP level was determined by taking the average of three casual BP readings from three different days (according to the standards established by the Joint National Committee of prevention,

detection and treatment of High Blood Pressure 7 guidelines). Each participant then underwent a physical examination by the study physician. Individuals with abnormal blood chemistries were excluded from the study. Smoking, a body mass index (BMI) > 35, alcohol intake of more than 3 drinks per day, diabetes (fasting glucose level > 126 mg/dl), total cholesterol >141mg/dl, evidence of renal or cardiovascular disease were the exclusion criteria.

### **8.2.2 Urine collection**

Urine samples were collected for a 24 hour period for baseline (B), a week before the exercise session began. Post exercise (PE) samples were also collected for a 24-hour period following their last period of exercise. Urine collection for both the baseline and the post exercise study was done after the first void in the mornings and in five time periods (8am-12pm, 12pm-4pm, 4pm-8pm, 8pm-12am, and 12am-8am). The participants collected urine in provided labeled containers for each of the time points and the samples were taken from the pooled urine from those collection periods. The collected urine samples were then stored in a -80°C till use.

### **8.2.3 Acute Exercise Session**

The acute exercise session began with a 10 minute warm up which consisted of walking and stretching exercises. This was followed by 30-minute walk on the treadmill, followed by 5 minutes rest and then 20 minutes of walking on the treadmill or cycle ergometry for 50 minutes of exercise. The exercise was done at 65 %  $VO_{2max}$  for each participant.

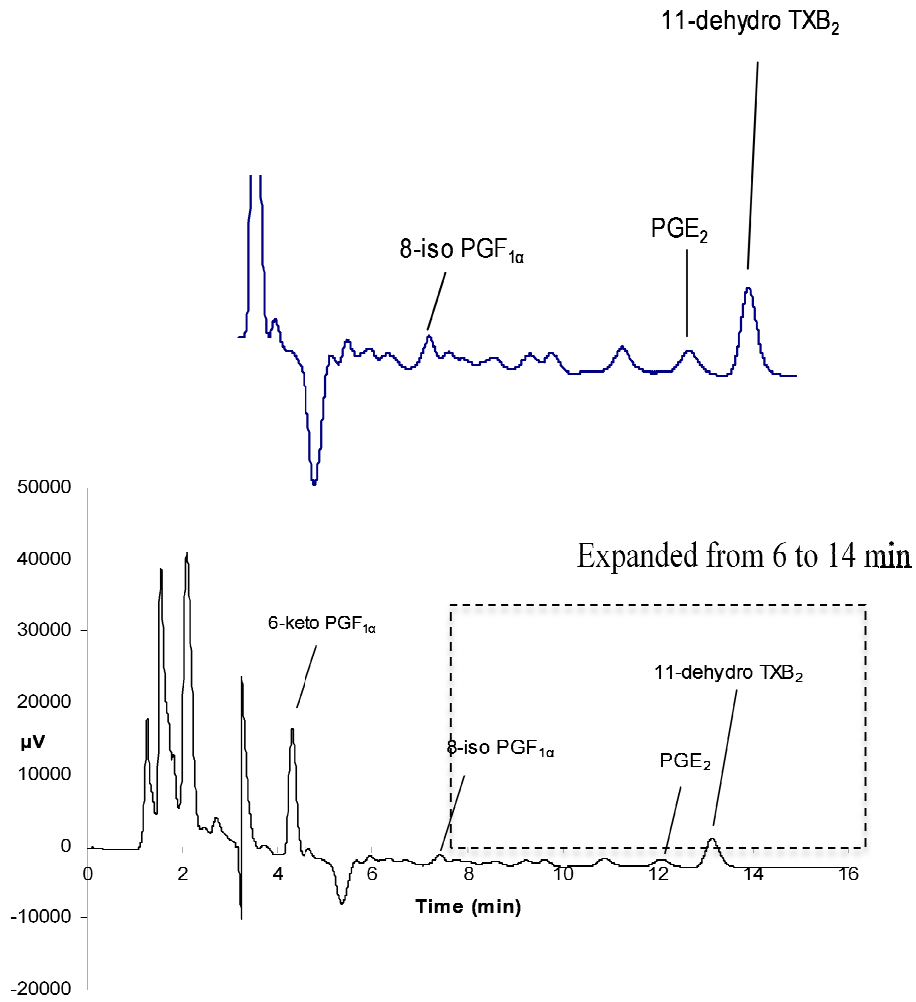
## 8.2.4 Statistics

Urinary prostanoid levels were compared before and after a single bout of acute exercise by ANOVA (Analysis of variance). A value of  $P \leq 0.05$  is considered statistically significant [30].

## 8.3 RESULTS

After analyzing the urine sample using HPLC-UV method and quantitation (Chapter 3), the levels of prostanoids before and after exercise in the hypertensive African American population were compared. Figure 8.1 is a representative HPLC-UV chromatogram of prostanoids in control urine. Because of hydration variability and therefore urine concentration, the data obtained for the biomarkers of this study were normalized with urinary creatinine. This is a standard method used to control for hydration effects [30]. Urinary creatinine was analyzed by the standard ELISA assay. The most sensitive markers were 6-keto  $\text{PGF}_{1\alpha}$ , 11-dehydro  $\text{TXB}_2$ , and 8-iso  $\text{PGF}_{2\alpha}$ . Average values of 6-keto  $\text{PGF}_{1\alpha}$ , 11-dehydro  $\text{TXB}_2$ , and 8-iso  $\text{PGF}_{2\alpha}$  normalized to urinary creatinine ( $\text{ng}/\mu\text{g}$ ) at baseline and post exercise for all the subjects.

Over the 24-hour period, the 6-keto  $\text{PGF}_{1\alpha}$  levels was found to be elevated for 8 out of 10 subjects, whereas 11-dehydro  $\text{TXB}_2$  levels decreased for 6 out of 10 subjects post-exercise (Table 8.1). On the other hand, 8-iso  $\text{PGF}_{2\alpha}$  levels showed an increase for 4 subjects post exercise remains constant for 1 decrease for 3 and is undetected for 2 of the 10 subjects (Table 8.2).



**Figure 8.1. Representative HPLC-UV chromatogram of prostanooids in control urine**

**Table 8.1. Average urinary levels of 6-keto PGF<sub>1α</sub> and 11-dehydro TXB<sub>2</sub> in terms of ng/μg of creatinine (B= baseline and PE = Post exercise)**

Subject No.	6-keto PGF <sub>1α</sub> /creatinine			11-dehydro TXB <sub>2</sub> /creatinine		
	Baseline	Post-Exercise	Change	Baseline	Post-Exercise	Change
1	16.59	47.89	+	67.14	73.75	No change
2	78.84	104.53	+	25.66	55.57	+
3	5.84	135.31	+	154.24	259.84	+
4	50.45	70.23	+	9.65	18.73	+
5	54.12	73.16	+	130.15	74.18	-
6	37.83	88.29	+	15.94	10.12	-
7	41.78	67.44	+	1.78	1.95	No change
8	120.31	54.57	-	8.33	42.24	+
9	18.09	15.09	No change	3.02	1.64	-
10	24.76	91.87	+	13.07	12.58	No change

**Table 8.2. Average urinary levels of 8-iso PGF<sub>2α</sub> in terms of ng/μg of creatinine**

**(B= baseline and PE = Post exercise)**

<b>Subject No.</b>	<b>8-iso PGF<sub>2α</sub>/creatinine</b>		
	<b>Baseline</b>	<b>Post-Exercise</b>	<b>Change</b>
1	0.04	1.22	+
2	1.75	0.32	-
3	Not Detected	0.68	Not detected
4	Not Detected	0.02	Not detected
5	3.70	3.36	-
6	3.89	1.66	-
7	0.25	1.31	+
8	0.04	0.18	+
9	0.29	0.44	+
10	1.11	1.07	No change

## 8.4 Discussion

Exercise increases vascular shear stress and activates as well as mobilizes cytokines [8]. This leads to the release of AA from the cell membranes, which is then converted into biologically active prostanoids by the COX-2 enzyme. The present study shows that a 50-minute exercise session increased the urinary excretion of 6-keto PGF<sub>1α</sub> in sedentary urban hypertensive African Americans. In contrast, exercise decreased excretion of 11-dehydro TXB<sub>2</sub> indicating inhibition of platelet aggregation. 6-keto PGF<sub>1α</sub> and 11-dehydro TXB<sub>2</sub> are formed in the epithelial cells and platelets' respectively. One widely accepted reason for the increase in 6-keto PGF<sub>1α</sub> and the decrease in 11-dehydro TXB<sub>2</sub> is the shift of cyclic endoperoxides from the platelets to endothelium, thus providing a substrate for endothelial prostacyclin synthase [30]. Our study shows a 2-fold increase in prostacyclin which is similar to the results observed by Wennmalm and FitzGerlad [31], who found a three-fold increase and Lemne *et al.* [32], who found a 76-160% increase in the excretion of urinary metabolite of prostacyclin. Williamson *et al.* observed that the participants with higher VO<sub>2max</sub> (marker of physical fitness) produced higher levels of 6-keto PGF<sub>1α</sub> in the morning (AM) following the exercise session [8]. In addition, they found a significant relationship between 6-keto PGF<sub>1α</sub> and VO<sub>2max</sub> (r= 0.67) [8]. This exercise induced increase in prostacyclin have been reported previously by Koivisto *et al.* [33], Wennmalm *et al.* [34], Rasmanis *et al.* [35], Ronni- Sivula *et al.* [36], Boger *et al.* [37] in urine, Ritter *et al.* [38], Barrow *et al.* [39] in blood or in the interstitial fluid of muscles reported by Frandsen *et al.* [40], Karamouzis *et al.* [41]. It has been suggested that this may be due to the anti-platelet activity of prostacyclin, which increases during exercise [25]. These results indicated that poor physical capacity

decreases the production of prostacyclin, which has antiplatelet, vasculoprotective, cardioprotective and antiatherogenic activity [25].

On the other hand, the reports on the expression of 11-dehydro TXB<sub>2</sub> are contradictory [26, 42]. Wennmalm and FitzGerlad [31] found no change whereas Lemne *et al.* [32] found an increase in the expression of urinary metabolite of thromboxane post exercise. In our study, 11-dehydro TXB<sub>2</sub> levels decreased after a single bout of exercise. The source of this variability may be the difference in the exercise intensity in various studies. According to Todd *et al.*, exercise intensities below 70% % VO<sub>2max</sub> may not be sufficient to stimulate the production of 11-dehydro TXB<sub>2</sub> [25]. Crespo *et al.* similarly found that higher levels of exercise intensity was required for the production of 11-dehydro TXB<sub>2</sub> whereas lower levels of intensities have stronger effects on 6-keto PGF<sub>1α</sub> expression. [3]. The ratio of urinary 6-keto PGF<sub>1α</sub> to 11-dehydro TXB<sub>2</sub> reflects the in vivo vascular homeostasis. This ratio (Table 10.3) increased post exercise in the current study. This may be due to shifting in favor of the production of the vasodilator and cardio-protective prostacyclin.

Generation of ROS is a very natural process and under normal physiological conditions antioxidant enzymes, proteins and vitamins remove them from the body.

**Table 8.3. Ratio of urinary levels of 6-keto PGF<sub>1α</sub> /creatinine and 11-dehydro TXB<sub>2</sub> /creatinine (B= baseline and PE = Post exercise)**

Subjects	Ratio of 6-keto PGF <sub>1α</sub> to 11-dehydro TXB <sub>2</sub>	
	B	PE
1	0.25	0.65
2	3.07	1.88
3	0.04	0.52
4	5.23	3.75
5	0.42	0.99
6	2.37	8.72
7	23.47	34.58
8	14.44	1.29
9	5.99	9.20
10	1.89	7.30

During hypertension, the vascular production of reactive oxygen species especially superoxide ( $O_2^-$ ) radical increases. The superoxide radical reacts with endothelium derived NO (nitric oxide), which acts as a vasodilator to produce peroxynitrite ( $ONOO^-$ ), a powerful oxidant [43]. In other words, oxidative stress is a condition, which involves an increase, a decrease in the bioavailability of vasodilators decreases and a decrease in antioxidant enzymes in hypertensive subjects [11, 12]. Edwards *et al.* [44] found that endurance exercise training resulted in decreased oxidative stress in patients with coronary artery disease. This may be because of the fact that exercise induces the activity of anti-oxidant enzymes (superoxide dismutase) and attenuates the activity of nicotinamide adenine dinucleotide (NADH/ NADPH) oxidase, an enzyme responsible for the formation of peroxynitrite [45]. However, in our study, we observed that urinary excretion of 8-iso  $PGF_{2\alpha}$  increases for 4, decreases for 2 and remains unchanged for 2 subjects, post exercise. This inconsistency may partly be because of the several factors that influence oxidative stress such as age, dietary intake and intensity and duration of exercise.  $PGE_2$ ,  $PGD_2$  and  $PGF_2$  were observed in the urine samples for very few subjects. Hence they are not reported.

## **8.5 Conclusion**

In the current study, we found that the prostacyclin production increases by 2-fold after a single bout of exercise in hypertensive middle-aged African American population. In contrast, exercise decreased excretion of 11-dehydro  $TXB_2$  indicating inhibition of platelet aggregation. This increase in 6-keto  $PGF_{1\alpha}$  and the decrease in 11-dehydro  $TXB_2$  may be due to the shift of cyclic endoperoxides from the platelets to endothelium, thus providing a substrate for endothelial prostacyclin synthase. In addition, the ratio of

urinary 6-keto  $\text{PGF}_{1\alpha}$  to 11-dehydro  $\text{TXB}_2$ , which reflects the in vivo vascular homeostasis, increases post exercise. These results indicate that physical activity increases the activity of 6-keto  $\text{PGF}_{1\alpha}$ , a vasodilator. Extending the study to a larger population should yield statistically significant results. Future studies involve, evaluating the role of sex hormones in hypertension, against the suppressive effect of exercise and also, studying the effect of long-term exercise therapy.

## **8.6 Acknowledgment**

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## CHAPTER 9

### 9. EVALUATION OF THE ROLE OF LOX METABOLITES OF ARACHIDONIC ACID IN THE PROGRESSION OF RHEUMATOID ARTHRITIS

#### 9.1 Introduction

Rheumatoid Arthritis (RA) is an inflammatory disease that affects about 0.5%-1% of the world population [1, 2]. It is found to occur more frequently in women than in men [3]. It is an autoimmune disease prevalent in the synovial tissues and is characterized by systemic and local inflammation, resulting in cartilage and bone destruction [2, 3]. Moreover, it is thought to be a multi-factorial disease except its etiology is still not completely known. Though genetics, environment, smoking and infectious agents are thought to play a role in RA, their exact contribution is not yet defined [4-7]. RA like any inflammatory disease has defined phases of initiation, progression and resolution. It has been widely accepted that both the initiation and resolution phases of inflammation consist of processes that are actively coordinated by protein and lipid molecules [8]. The initiation phase of an inflammatory process sees an influx of pro-inflammatory molecules, innate immune cells and tissue destruction. On the other hand, the resolution phase of inflammation is characterized by an influx of anti-inflammatory/pro-resolution lipid molecules and macrophages, and tissue repair and remodeling [8]. Arachidonic Acid derived metabolites are said to be involved in the three phases of inflammation [8].

AA is metabolized by one of the three-enzymatic pathway (Chapter 1) and released from the cell membrane by numerous cellular stresses. Depending on the cell type, either COX or LOX enzymes predominates [9]. However, in some cases both pathways are present. Various experimental models have demonstrated the importance of COX-2 in RA. It is expressed in the synovial tissues of patients with RA and is induced in inflammatory cells by various cytokines such as IL-1 and TNF $\alpha$  [10, 11]. PGE<sub>2</sub>, a COX -2 metabolite of AA, is most noted for its pro-inflammatory role in RA [12, 13]. Inhibition of PGE<sub>2</sub> is the typical mode of action of non-steroidal anti-inflammatory drugs (NSAIDs) [14-15]. Moreover, NSAIDs have been demonstrated to reduce symptoms in collagen induced arthritis [9, 16-19]. It has been suggested that these drugs not only inhibit the production of PGE<sub>2</sub> but also interferes with the production of other lipid mediators by the same or other enzymatic pathways [19].

The LOX pathway has also been recognized in having deleterious effects in RA [20]. AA metabolism via the LOX enzymes (5-LOX, 12-LOX and 15-LOX) gives leukotrienes (5-LOX pathway- LTB<sub>4</sub>, LTC<sub>4</sub>, LTD<sub>4</sub>, LTE<sub>4</sub>), HETEs (5-LOX pathway: 5-HETE, 12-LOX pathway: 12-HETE and 15-LOX pathway: 15-HETE) and lipoxins (15-LOX pathway: LXA<sub>4</sub> and LXB<sub>4</sub>). 5-LOX, 12-LOX and 15-LOX enzymes are expressed in the RA synovial fibroblast. The role of 5-LOX enzyme in RA is the most studied and documented [20]. LTB<sub>4</sub>, a 5-LOX metabolite of AA, is one of the most powerful pro-inflammatory molecules that have been implicated in joint inflammation in RA [20,21]. The 15-LOX product, 15-HETE, exhibits anti-inflammatory properties [22]. It has been shown to inhibit the production of LTB<sub>4</sub> and TNF $\alpha$ , a key cytokine in the pathogenesis of RA [23]. Furthermore, it has been shown to inhibit the activity of 5-LOX and 12-LOX

enzymes [22-24]. 12-HETE on the other hand, is considered to be a pro-inflammatory biomarker. IL-1 and TNF $\alpha$  enhance the production of 12-HETE [25]. Lipoxins (LXA4 and LXB4) are novel eicosanoid mediators that have anti-inflammatory properties. They assist resolution in animal models of inflammation [9]. Recent studies have shown that LXA4 inhibits the production of IL-6 in human synovial fibroblasts [9]. Currently there is very little research on the role of LXs in RA. Though the roles of many AA metabolites have been well understood, the exact role and significance of these molecules in the initiation, progression and resolution phase of inflammation in RA is not fully elucidated.

The initial stages of inflammatory response sees AA metabolized to prostaglandins and leukotrienes, by COX-2 and 5-LOX enzymes respectively. These two groups of lipid mediators are primarily responsible for edema formation and leukocyte influx [26]. There is evidence that inhibition of COX-2 and 5-LOX enzymes ameliorate the progression of inflammatory diseases such as RA and atherosclerosis [9, 27-30], suggesting that LOX and COX pathways may cross talk. Marcouiller *et al.* [31] reported that inhibition of COX enzyme increased the expression of LTB<sub>4</sub>, thus suggesting the shunting of AA metabolism from the COX-pathway to LOX pathway. The exact mechanism of this shunting is not fully understood. The aim of this project was to investigate the role of 12 LOX and 15 LOX metabolites of AA in rheumatoid arthritis and the impact of NSAID on the expression of these biomarkers.

## **9.2 Protocol**

### **9.2.1 Animal studies and Induction of Arthritis [32]**

Arthritis was induced in 6- to 8-wk-old, male DBA/1 (Dilute Brown Non-Agouti) mice (The Jackson Laboratory, Bar Harbor, ME) with intra-dermal injection of chicken collagen II (Chondrex, Redmond, WA).

#### Time point study

In this study, mice (n=37) were sacrificed at various time points related to the three phases of arthrogenesis. Their limbs were collected, weighed; snap frozen and stored at -80°C till analysis.

#### Inhibition Study

In the inhibition study, a COX-2 inhibitor is fed to the collagen injected mice (n=31). They were sacrificed at time intervals related to the three phases of inflammation. The lipids were extracted from the limb tissues by the extraction protocol.

### **9.2.2 Sample preparation**

Mouse footpads (weights ranging from 128-300 mg) were ground in liquid nitrogen until fine powder. It was then homogenized in 600 uL of PBS (Phosphate Buffered Saline). The homogenate was centrifuged at 10,000 rpm for 1 minute and then re-suspended and adjusted to pH approximately 3.5 with 2 M HCl. It was then allowed to sit for 15 min at 4 °C. It was again centrifuged at 10,000 rpm for 2 minutes. A C18 extraction column was sequentially preconditioned by running 4 mL of 15% Ethanol

followed by 4mL de-ionized water. The supernatant was loaded on the column and washed with 4mL deionized water, 4mL 15% ethanol and 4mL hexane. The eicosanoids were eluted with 250 uL of ethyl acetate. Evaporated samples were reconstituted to 300 uL and 20 uL was injected into the LC-MS system. The operating conditions described in Chapter 6 were used for this analysis.

### **9.2.3 Statistics**

Simple regression analyses were performed with each LOX metabolite as the independent variable and a COX metabolite (PGJ<sub>2</sub>) as the dependent variables. Correlation Coefficient ( $r \geq 0.5$ ) was considered to be significant for animal and human studies [33].

## **9.3 RESULTS**

Chan *et al* [32] in their laboratory found that following the induction of the collagen, all the limbs of the mice are asymptomatic from 0-30 days indicating that this was the initiation phase of inflammation. The footpads become progressively swollen and inflamed from days 30-55 and the swelling subsides from days 55-70. This indicates that the progression of inflammation in RA occurs between days 30-55 followed by the resolution phase (days 55-70). In our experiment, we compared the eicosanoid levels (pg/mg of tissue) in the three phases of inflammation as determined by Chan *et al*.

### Time point study

In the time point study, the expression of 15 HETE increases from day 7 to 35 and then it decreases (Figure 9.1). This corresponds to the initiation and progression of

inflammation in RA. In contrast, 12-HETE has a downward trend throughout the time point study and has maximum expression in the initiation phase of arthritis, indicating that it has pro-inflammatory properties (Figure 9.2).

#### Inhibition study

In the inhibition study, the total expression of 12-HETE decreases when compared to the time point study indicating that COX-2 inhibitor suppresses the expression of 12-HETE in RA (Figure 9.3). In contrast, the total expression of 15-HETE increases and shows two maxima, one at day 35 and other at day 55. These correspond to the progression and the resolution phase of inflammation in RA (Figure 9.4). Expression of LXA4 was low in both the studies and showed no particular trend (Figure 9.1 and 9.4).

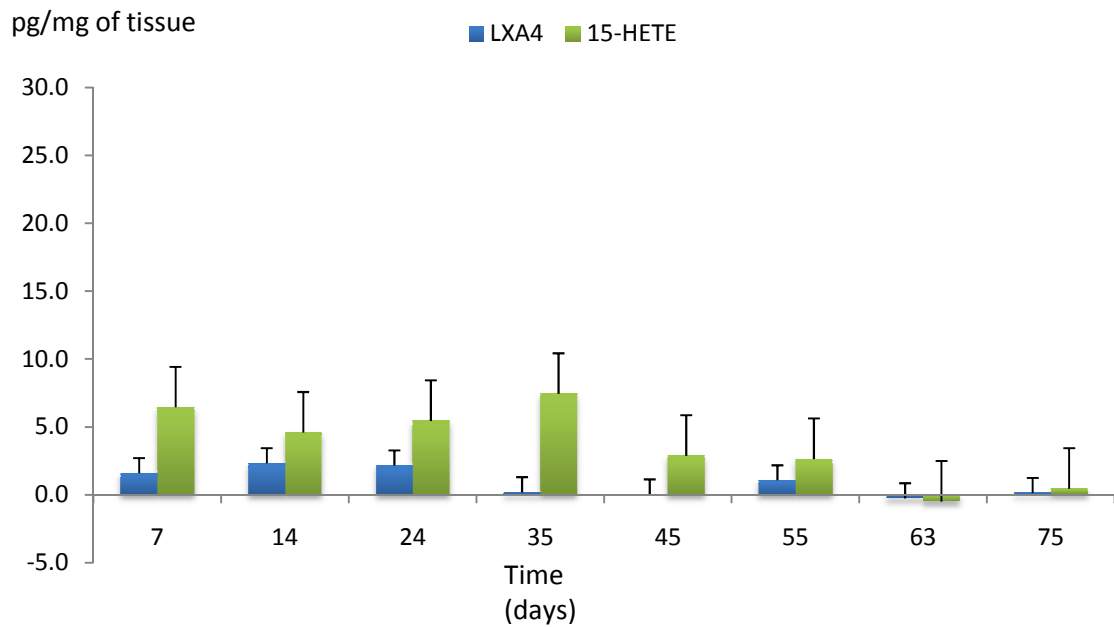
### **9.4 Discussion**

Collagen induced arthritis is obtained by immunizing the mouse with heterologous type II collagen [34]. The immune response against this antigen leads to chronic arthritis that has numerous features similar to RA [34]. The disease is produced only in certain strains of mice one of which is the DBA/1 [34].

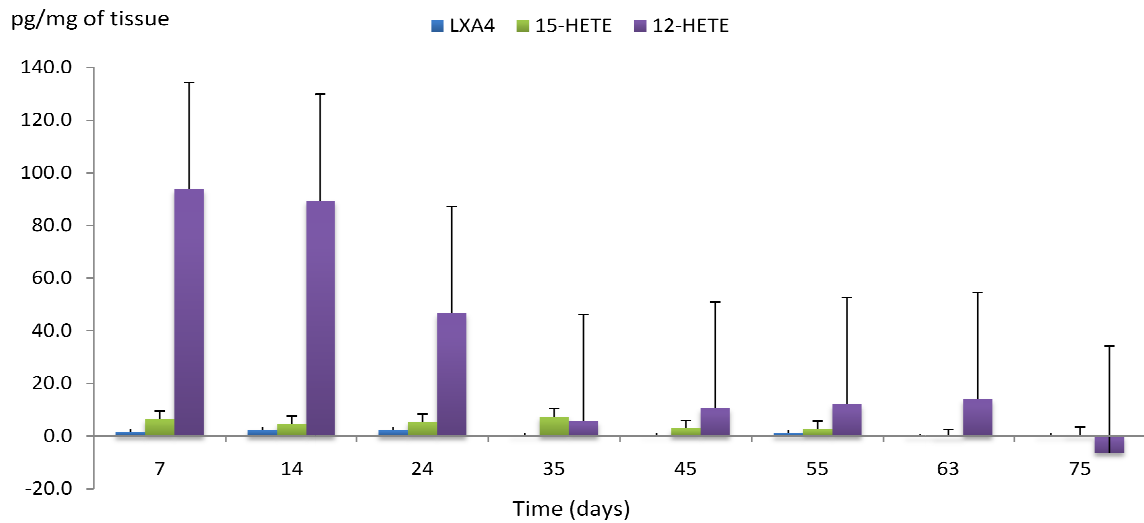
There are very few studies investigating the role of 12 LOX and 15 LOX in various phases of inflammatory diseases like RA, although, it has been demonstrated that 12-HETE and 15-HETE play an important role in immune regulation [35-39]. Kronke *et al.* were the first to investigate the involvement of 12/15 LOX in the pathogenesis of arthritis in 2009 [40]. The authors found that 12 HETE, 15 HETE and LXA4 act as anti-inflammatory markers in RA by counteracting the exacerbation of inflammation and associated tissue damage [40]. The role of 15-HETE in literature is contradictory. There

are studies that suggest that 15-HETE has no pro-inflammatory capacity and exhibits anti-inflammatory properties through decreased production of LTB<sub>4</sub> in arthritis [23-25]. There are also results that indicate that 15-LOX metabolites have pro-inflammatory action by enhancing the expression of TNF $\alpha$  [41]. In the present study, we showed that 15-HETE is expressed in the initiation and progression phases of inflammation in the normal course of the disease suggesting a pro-inflammatory role. Moreover, our results indicate 12-LOX to be the predominant enzyme in the initiation phase of inflammation. The expression decreases indicating that 12-LOX is active only in the initial phase of an inflammatory response. 12-HETE (12-LOX) is hence thought to generate a pro-inflammatory response in RA.

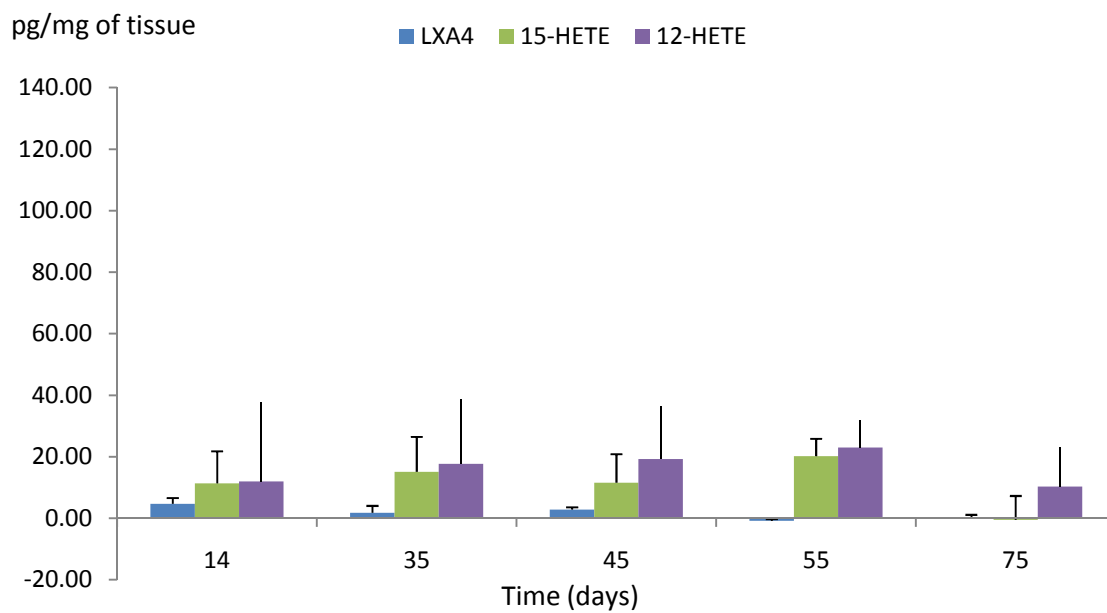
As mentioned earlier COX-2 inhibitors are the first line of treatment for RA. These are widely used to manage pain, swelling and stiffness in RA. COX-2 inhibitors such as NS-398 are known to inhibit carrageen-induced acute pleurisy during inflammation but it also interferes with the resolution [32,42]. Chan *et. al.* observed that the inhibitor NS-398, interferes with the resolution, when given after the disease is symptomatic [32].



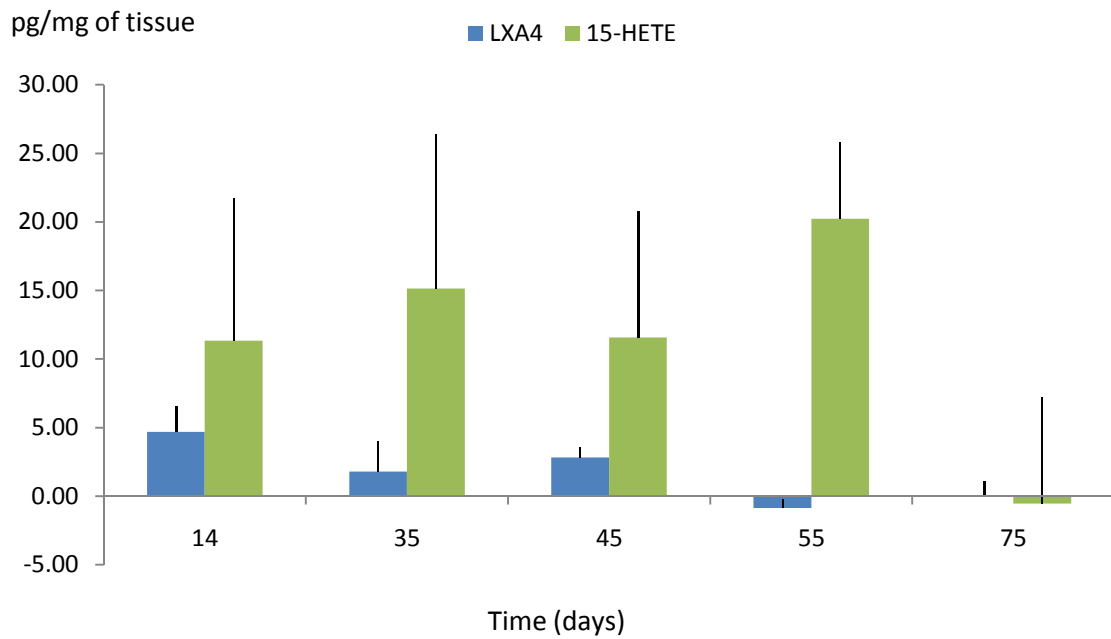
**Figure 9.1. Time point study: Amount of 15-HETE and LXA4 in terms of pg/mg of tissue in the initiation, progression and resolution phase of inflammation**



**Figure 9.2. Time point study: Amount of 12-HETE, 15-HETE and LXA4 in terms of pg/mg of tissue in the initiation, progression and resolution phase of inflammation**



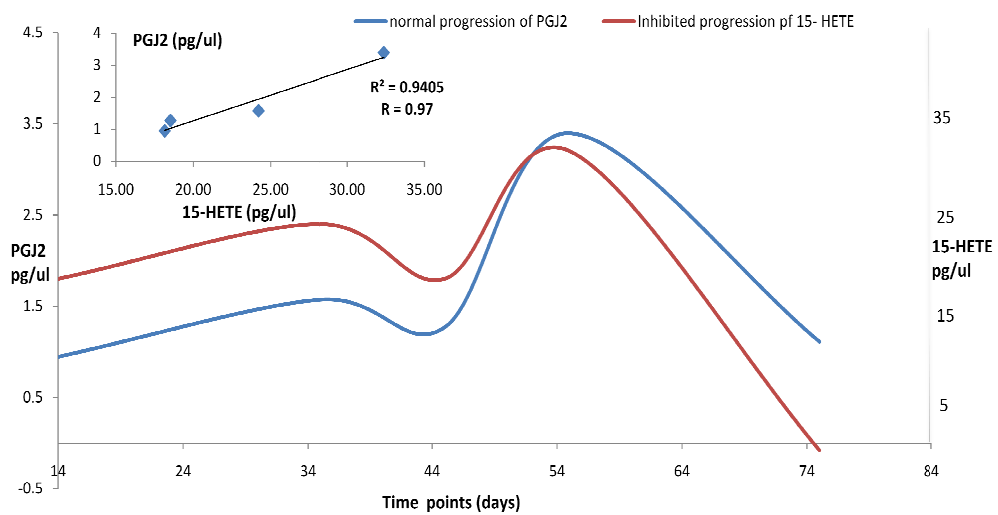
**Figure 9.3. Inhibition study: Amount of 12-HETE, 15-HETE and LXA4 in terms of pg/mg of tissue in the initiation, progression and resolution phase of inflammation**



**Figure 9.4. Inhibition study: Amount of 15-HETE and LXA4 in terms of pg/mg of tissue in the initiation, progression and resolution phase of inflammation**

In contrast, when given earlier it just delays the onset of the disease. This suggests that COX- inhibitors do not halt the progression of the disease but just helps in reducing the symptoms. COX-2 inhibitors act by acetylating the active site of COX-2 [43]. This suppresses the production of prostaglandins [43].

In the inhibition study, we found that the expression of 15 HETE increases as compared to the time point study. Furthermore, it is expressed in the progression and the resolution phases of inflammation indicating that it is a molecule with dual properties. On the other hand, the total expression of 12 HETE decreases compared to the time point study. We believe that in the presence of a COX-2 inhibitor, AA metabolism is shunted from the COX to the 15 LOX pathway. This increases the overall expression of 15 HETE. 15-HETE is also known to inhibit the production of 12 HETE. We think this is the reason for the decrease in 12 HETE levels in the inhibition study when compared to the time point study. From these studies we can conclude that 12 HETE is a pro-inflammatory molecule involved in the initiation phase of inflammation in RA. On the other hand, 15 HETE exhibits dual inflammatory properties in RA.



**Figure 9.5. Comparison of PGJ<sub>2</sub> (Time point study) with 15-HETE (Inhibition study)**

Simple regression analyses were performed with each LOX metabolite as the independent variable and a COX metabolite (PGJ<sub>2</sub>) as the dependent variables. An ELISA assay was used to obtain PGJ<sub>2</sub> levels in the mouse feet pad. PGJ<sub>2</sub> has been shown to participate in the resolution of inflammation in RA [44]. On comparison, it was found that the expression of 15-HETE in the inhibition study followed the same trend as PGJ<sub>2</sub> expression in the time point study (Figure 9.5). Simple regression analysis showed that there existed a good correlation ( $r=0.97$ ) between the expression of 15 HETE and PGJ<sub>2</sub> (Figure 9.5). The common feature of 15 HETE and PGJ<sub>2</sub> other than the fact that they are metabolites of AA is that they are naturally occurring ligands of peroxisome proliferator-activated receptors (PPAR $\gamma$ ) [45-47]. PPAR $\gamma$  belong to the steroid hormone receptor super-family and are involved in ligand-inducible lipid metabolism. Activation of this receptor is known to reduce pro-inflammatory reactions by reducing cell migration and inhibiting TNF $\alpha$  and cytokines [47]. Studies have shown the administration of PPAR $\gamma$  agonist PGJ<sub>2</sub> reduced the cell infiltration in arthritis and also when PPAR $\gamma$  antagonist GW9662 was administered inhibited the expression of 15-HETE, indicating that their effects are mediated by PPAR $\gamma$  [48,49]. We believe this may be the reason that PGJ<sub>2</sub> and 15-HETE are strongly correlated. In the last decade, there has been increasing number of studies to define the role of PPAR $\gamma$  in the pathogenesis of diseases. Investigating the role of PPAR $\gamma$  in RA is well beyond the scope of our study.

## **9.5 Conclusion**

In the current study, we show that 12 HETE is a pro-inflammatory molecule involved in the initiation phase of inflammation in RA. On the other hand, 15 HETE

exhibits dual inflammatory properties. It participates in the initiation and progression phase of inflammation in the normal course of the disease. Furthermore, in the presence of a COX-inhibitor, 15 HETE is involved in the resolution of inflammation. We also found that 15-HETE in the inhibition study followed the same trend as PGJ<sub>2</sub> expression in the time point study. On performing simple regression analysis we found that there exists a good correlation between the two molecules ( $r=0.97$ ). We believe this is because of the fact that PPAR $\gamma$  is known to mediate the effects of 15 HETE and PGJ<sub>2</sub>, which are its naturally occurring ligands.

## **9.6 Acknowledgment**

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## GLOSSARY OF TERMS

- Apoptosis –programmed cell death
- Atherogenic – promoting atherosclerosis
- Atherosclerosis – condition in which the arterial walls thicken due to fatty disposition leading to luminal narrowing.
- Autocrine – related to a substance secreted by a cell acting on the surface receptor of the same cell
- Basophils – type of white blood cell
- Bradykinin – peptide released during inflammation (vasodilator)
- Bronchoconstrictor - constriction (narrowing) of airway
- C-Reactive protein – protein released during inflammation
- Carregeenan – common food additive used a thickener that comes from seaweed
- Chemoattractant – a chemical agent that causes the cells (leukocyte) or organism to travel towards it
- Chemotaxis – movement of cells or organisms in presence of a chemical stimulus
- Cytokines – signaling protein molecule
- Edema – collection of fluid in the dependent portion of the body
- Eicosanoids – signaling molecules produced by oxidation of carbon 20 essential fatty acids
- Endopeptidases – enzymes that break peptide bonds of non-terminal amino acids
- Eosinophils - type of white blood cell
- Fibrin – a protein involved in the clotting process

- Fibroblast – type of cell found in connective tissue
- Fibrinogen – plasma glycoprotein that is synthesized by the liver and which is converted to fibrin.
- Glomerular filtration – first step of urine formation in which the waste materials, chemicals and water are filtered out of the blood. It takes place in the glomeruli
- Histamine – amine involved in the immune response
- Homoestasis – ability to maintain internal equilibrium
- Hyperalgesia – abnormal perception of pain from a non-painful stimulus
- Idiopathic – arising spontaneously from an unknown cause/source
- Interleukin – type of cytokine
- Leukocyte –white blood cell
- Lipoprotein – a complex of lipid and protein
- Macrophage – a type of white blood cell
- Mast cells – a type of cell found in connective tissue
- Mitogenesis – induction of mitosis
- Mitosis – a process of cell division that produces two daughter cells from a single parent cell
- Matrix metalloproteinases – group of endopeptidases that are responsible for hydrolyzing extracellular proteins
- Monocytes – a type of white blood cell
- Neutrophil - a type of white blood cell
- Paracrine - related to a substance secreted by a cell acting on adjacent cells
- Periodontitis – inflammatory disease that affects the tissue around the teeth

- Phagocytosis – a process in which certain cells (macrophages and neutrophils) ingest other cells/particles
- Phospholipase – enzyme that hydrolyzes phospholipids into fatty acids and other lipophilic substances
- Platelets – cellular component of blood involved in clotting
- Peroxisome Proliferator-Activated Receptors – group of nuclear receptor protein that regulates the expression of genes
- Reticulocyte – immature form of red blood cell
- Rhinitis – inflammation of mucous lining of nose
- Serum Amyloid A – protein that is expressed in response to an inflammatory stimuli
- Serotonin – monoamine neurotransmitter
- T cells – group of white blood cells known as lymphocytes
- Tumor Necrosis Factor - type of cytokine

## ABBREVIATIONS

- AA - Arachidonic Acid
- ALA – Alpha Linoelic Acid
- AM – Austin Model
- BMI – Body mass Index
- COX -Cyclooxygenase
- CRP – C-Reactive Protein
- CYP – Cytochrome
- DBA - Dilute Brown Non-Agouti
- DHA – Docosahexaenoic Acid
- DHETE –Dihydroxyeicosatrienoic Acid
- EET – Epoxyeicosatetraenoic Acid
- ELISA – Enzyme-Linked Immunosorbent Assay
- EPA – Eicosapentaenoic Acid
- FDA – Food Drug Administration
- HDL –High Density Lipoprotein
- HETE – Hydroxyeicosatrienoic Acid
- HODE - Hydroxyoctadecadienoic
- HOMO – Highest Occupied Molecular Orbital
- HPLC – High Pressure Liquid Chromatography
- IL - Interleukin
- LA – Linoeleic Acid

- LC-MS – Liquid Chromatography Mass Spectrometry
- LDL – Low Density Lipoprotein
- LLE – Liquid Liquid Extraction
- LOD – Limit of Detection
- LOQ – Limit of Quantification
- LOX - Lipoxygenase
- LT - Leukotriene
- LUMO – Lowest Unoccupied Molecular Orbital
- LX - Lipoxin
- NADH - Nicotinamide Adenine Dinucleotide
- NADPH - Nicotinamide Adenine Dinucleotide Phosphate
- OLR – Ordinary Linear Regression
- PG - Prostaglandin
- PPAR - Peroxisome Proliferator-Activated Receptors
- PPT – Protein Precipitation
- PUFA – Polyunsaturated Fatty Acid
- QC – Quality Control
- QSAR - Quantitative Structure Affinity Relationship
- QSRR – Quantitative Structure Retention Relationship
- RPHPLC – Reverse Phase High Pressure Liquid Chromatography
- RRT – Relative Retention time
- RvD – Resolvin of D series
- RvE – Resolvin of E series

- SAA – Serum Amyloid A
- SPE – Solid Phase Extraction
- TNF – Tumor Necrosis Factor
- UHPLC – Ultra High Pressure Liquid Chromatography
- UV – UltraViolet
- VLDL – Very Low Density Lipoprotein

## APPENDICES

### APPENDIX A DEVELOPMENT OF AN HPLC-UV METHOD FOR THE DETERMINATION OF SEX HORMONES IN BIOLOGICAL MATRICES

#### A.1 Introduction

The differences between the sexes in diseases have been getting more attention than ever before. In the recent years, scientists have discovered that estrogens and the other sex hormones regulate the development of diseases in women differently than in men. A 5-10 year lag period in cardiovascular disease (CVD) incidences in women when compared to men have been related to the differences in the endogenous sex hormones especially the estrogens [1]. Idiopathic pulmonary fibrosis is more prevalent in men whereas idiopathic pulmonary arterial hypertension predominately affects women [1]. Also, according to the National center of health statistics, asthma is more prevalent in boys before puberty whereas after puberty the females with asthma outnumber the males by a factor of 2 [1]. It is still unclear whether these differences are directly due to or modified by the sex hormones. These findings may provide answers to how diseases can be treated more effectively.

The parent compound for all the steroid hormones is cholesterol and hence they all have similar structure (Figure A.1). Estrogens are steroid compounds that function primarily as female sex hormones. They are named as such for their importance in the estrous cycle. The three major naturally occurring estrogens in women are estrone (E1), estradiol (E2) and estriol (E3). Estrone is the least abundant of the three estrogens in pre-

menopausal women whereas it is present in higher quantities post menopause. Estradiol (17 $\beta$ -estradiol) is the predominate estrogen in women. It is also produced in men, as it is a metabolic product of testosterone. Estriol is produced primarily during pregnancy as is made by the placenta from 16-hydroxy-dehydro-epiandrosterone sulfate (16-OH DHEAS)[1]. 2-hydroxy estrone, 16 $\alpha$ -hydroxy estrone, 2-methoxy estrone and 4-hydroxy estrone are oxidation products of estrogens. The ratio of 2-hydroxyestrone (2-OHE1) to 16 $\alpha$ -hydroxyestrone (16 $\alpha$ -OHE1) is considered to be an index of breast cancer risk [2]. Progesterone is involved in the female menstrual cycle and pregnancy. Progesterone levels are lower in post-menopausal women and men compared to the pre-menopausal women. Testosterone is a steroid hormone from the androgen group, which is present at much higher concentration in men than in women. It functions primarily as a male sex hormone.

Common laboratory conditions for the analysis of the sex hormones and their metabolites in water, tissue or plasma, require multiple ELISA assays and/or application of variety of chromatographic methods. The analytical methods used for the analysis of the sex hormones are immunoassays [3-5], HPLC-UV [6], HPLC with fluorescent detection [7], GC-MS [8,9], LC-MS [10] and LC-MS/MS [11,12]. Though immunoassays can be sensitive, they generally suffer from poor specificity and reproducibility due to cross-reactivity and variation of antibodies in different lots [13]. The GC/MS methods are specific, sensitive and accurate but they require numerous steps of extractions (solid or liquid phase) and derivatizations. The LC-mass spectrometry methods are accurate, precise and do not require derivatization. However, they can be expensive. In this chapter, we report a single, sensitive and a straightforward HPLC

assay for the rapid profiling of estriol, estradiol, estrone, progesterone, 2-hydroxy estrone, 16- $\alpha$  hydroxyl estrone, 2- methoxy estrone and 4- hydroxyl estrone.

## **A.2. Experimental**

### **A.2.1. Chemicals and Materials**

Estriol, estradiol, estrone and progesterone were purchased from Sigma Aldrich. 2-hydroxy estrone, 16 $\alpha$ -hydroxyl estrone, 2-methoxy estrone and 4-hydroxy estrone were purchased from Steraloids. HPLC water and acetonitrile were purchased from Fisher Scientific (Waltham, MA, USA).

### **A.2.2. Instruments and operating conditions**

The Jasco HPLC system consisting of the following parts was used; Jasco pumps (PU-980), a Jasco UV-VIS detector (UV-975) (Jasco Incorporated Easton, MD, USA) and a Rheodyne manual injector (Rheodyne LLC, Rohnert Park, CA, USA). Jasco-Borwin software (version 3.3.5) was used for data collection. The analysis was done on a Restek Biphenyl 4.6 x 150 mm column with 5  $\mu$ m particle size. (Restek, USA). The HPLC method employed a gradient elution of water (solvent A) and acetonitrile (solvent B) with a flow rate of 1.0 ml/min. Gradient elution was employed as follows: 50% B for 6.0 min., 50% -60% B from 6.0-6.1 min., hold 60% B from 6.1-8.0 min., 60%-80% B from 8.0-8.1 min., hold 80% B from 8.1-14.0 min., 80% -50%B from 14.0-14.1 min., hold 50% B from 14.1- 16.0 min. The run time for the method was 16.0 min. The analytes were separated at ambient temperature with an injection volume of 100  $\mu$ L. 220 nm was used for the detection.

### **A.2.3. Preparation of standard solutions and quality control (QC) samples**

Working standards for all the sex hormones were prepared from the respective stock solutions by proper dilutions with acetonitrile. Calibration standards for the sex hormones were prepared at mass concentrations of 0.1, 0.2, 0.3, 0.5, 1, 2, 3, 5 and 10 ng/ $\mu$ L from the respective stock solutions. Quality control (QC) samples were prepared in triplicate for all the analytes representing low, medium and high concentrations in linearity curve.

### **A.2.4 Sample preparation- Solid phase extraction**

a. Urine: Prior to analysis the urine sample was allowed to thaw at room temperature. 500  $\mu$ L of the thawed, filtered urine sample was transferred into a 2.0 ml polypropylene micro-centrifuge tube. To the tube, 200  $\mu$ L of methanol with 0.01 M BHT and 50  $\mu$ L of formic acid were added. Prior to the loading of the sample, an Oasis Solid phase extraction cartridge was sequentially preconditioned with 2 ml 0.1 % formic acid (v/v), 2 ml methanol and 2 ml ethyl acetate. The entire urine sample in solvent was then loaded onto the preconditioned column. The column was then washed with 2 ml of 0.1% formic acid (v/v) and 2 ml of 10% methanol with 0.1% formic acid. The sex hormones were then eluted with 1.5 ml of ethyl acetate with 0.01 M BHT and 0.5 ml methanol with 0.2 % formic acid and 0.01 M BHT [14]. Samples were evaporated to dryness under nitrogen and then reconstituted to 450  $\mu$ L with acetonitrile.

b. Tissue: Prior to analysis the tissue was allowed to thaw at room temperature. 100 mg of the thawed tissue was transferred into a 2.0 ml polypropylene micro-centrifuge tube. To the tube, 200  $\mu$ L of methanol with 0.01 M BHT and 50  $\mu$ L of formic acid were added.

The tissue was homogenized with a Tissue Terror homogenizer and then centrifuged at 14000rpm at 0°C for 15min. The supernatant was collected and the extraction using SPE was performed as given in the above paragraph.

### **A.2.5 Method Validation**

The specificity, sensitivity, linear range, accuracy and precision of the HPLC method validation were determined as per the FDA guidance for the Industry: Bioanalytical method validation [15].

#### **A.2.5.1 Selectivity**

Five different urine samples were analyzed by HPLC to determine peak selectivity. Solvent blanks were also analyzed to investigate possible interferences.

#### **A.2.5.2 Linearity and Sensitivity**

The linearity for all the analytes was established by injecting in triplicate; standard solutions with mass concentration ranging from LOQ to 10 ng/μL. The linearity curve was obtained by plotting the peak areas of the prostanoids with the respective theoretical column loading (ng). The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as the lowest concentrations that give the signal to noise ratio as 3:1 and 10:1 respectively.

#### **A.2.5.3 Accuracy and Precision**

Five solutions of each mass concentration of the QC samples were prepared and injected in triplicate to determine the accuracy (n = 15). The calculated concentrations for each of the samples were then compared to the theoretical concentration to determine the

% accuracy for each of the compounds. Inter-assay and intra-assay precision were determined by injecting in triplicate; five replicate preparations of three mass concentration of each analyte spiked in urine for a single day and for four consecutive days respectively.

### **A.3 Results and Discussion**

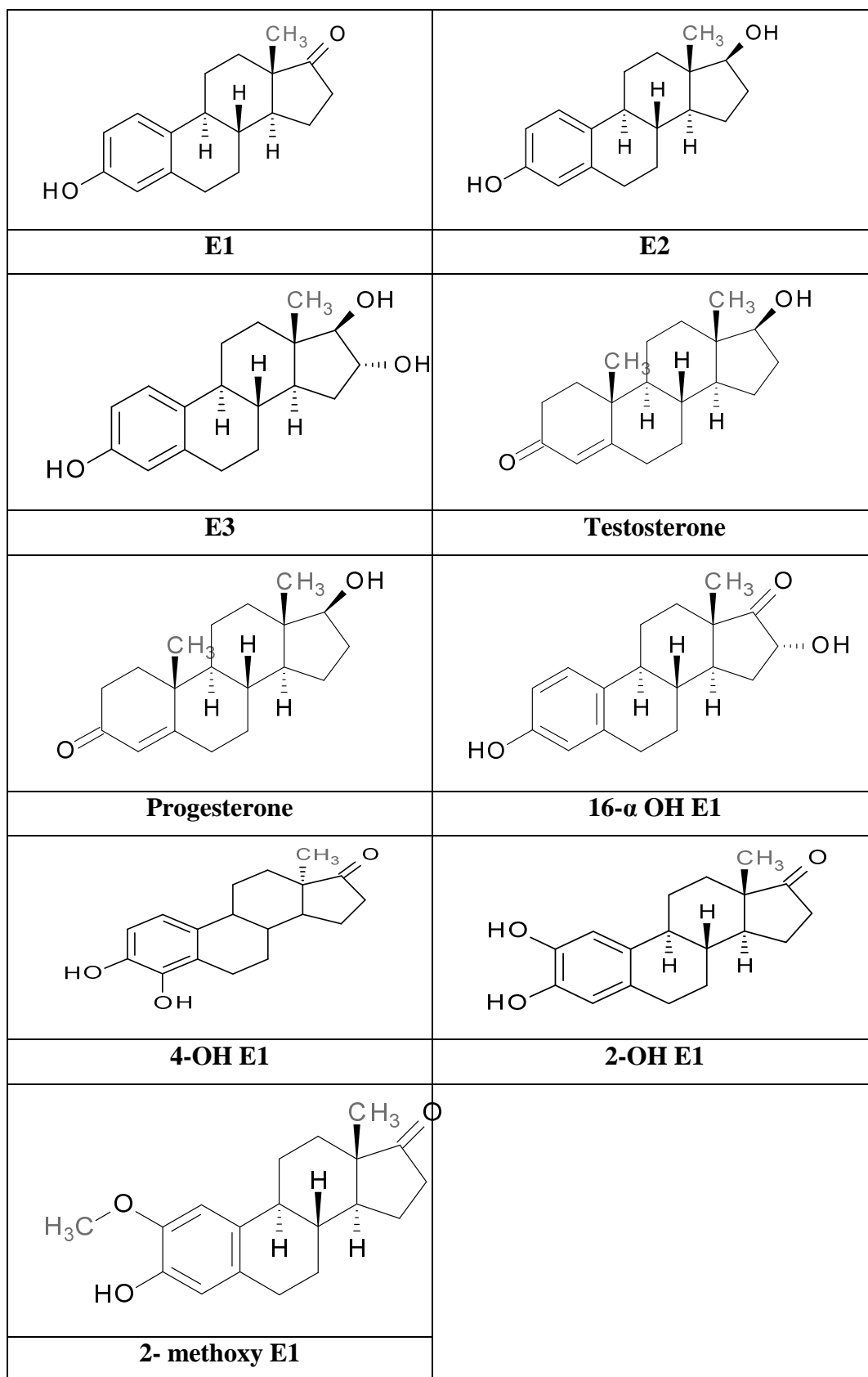
#### **A.3.1 Chromatography**

An Allure biphenyl column was used for the separation of the sex hormones. The stationary phase is unique with two phenyl groups bonded to each other and then bonded to the silica. The separation occurs due to the interaction between the  $\pi$ - $\pi$  electrons on the biphenyl group with the unsaturated aromatic sex hormones. The column is very sensitive in dealing with small differences in the structure, which is necessary as the sex hormones are very similar in structure (Figure A.1).

#### **A.3.2 Method validation**

##### **A.3.2.1 Selectivity**

There are no interfering endogenous compounds in the urine at the retention times of the sex hormones. A representative HPLC chromatogram of standard solution and urine sample of a hypertensive subject are shown in Figure A.2 and A.3 respectively. The resolution, asymmetry and the retention times of each sex hormone in the solvent and the urine are summarized in Table A.1.



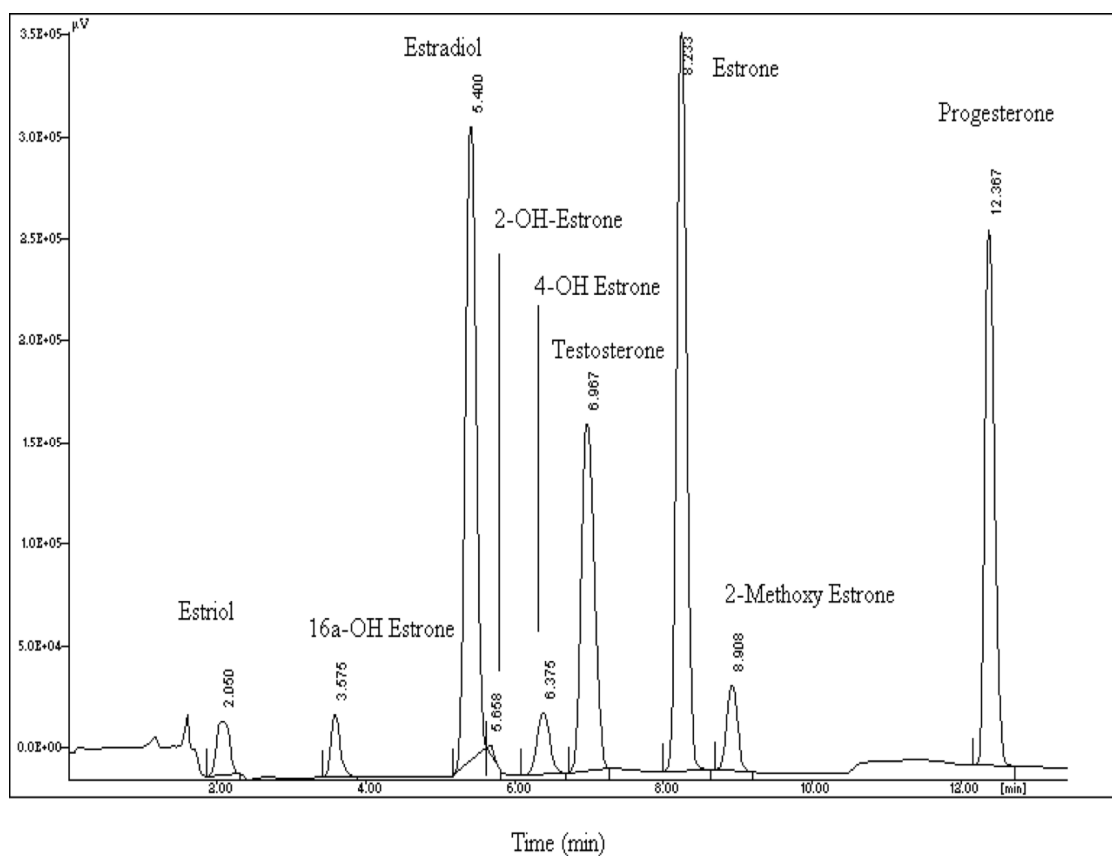
**Figure A.1. Structure of sex hormones**

**Table A.1. Method Validation Results: Selectivity**

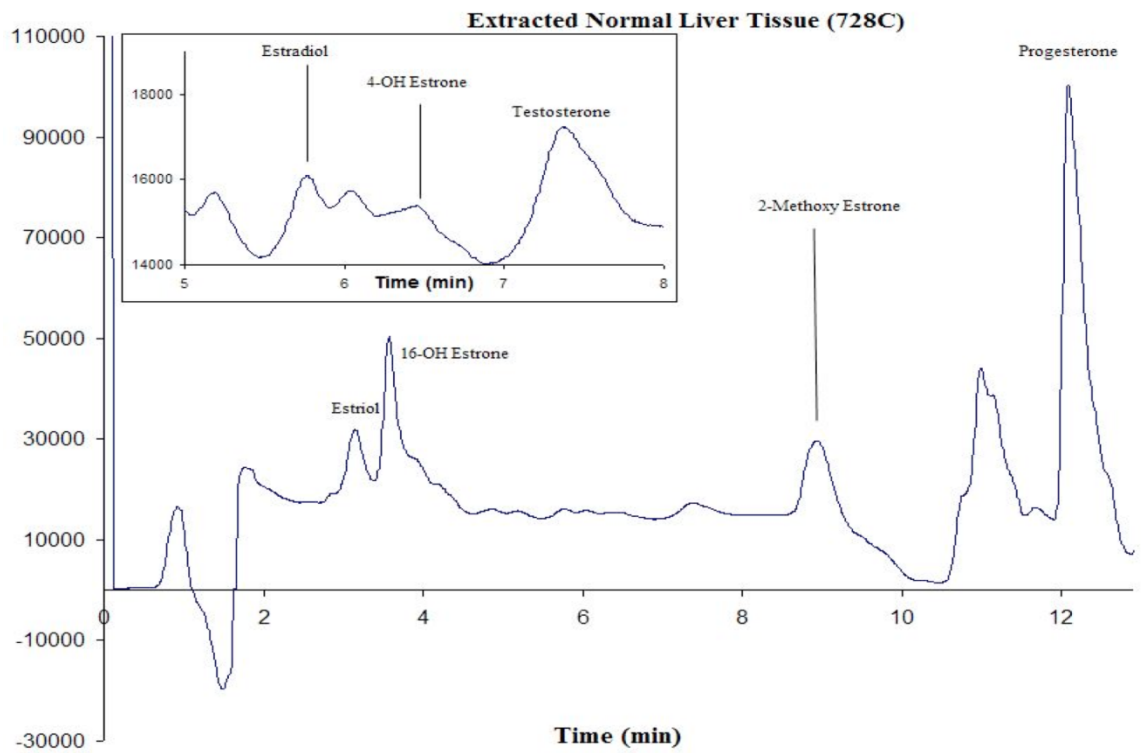
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<b>Compound</b>	<b>RT (min)</b>	<b>Asymmetry (<math>A_s</math>)</b>		<b>Resolution</b>
		$A_s = W_{2/2} / W_{1/2}$	$W_{1/2} = \text{peak first half width}$	
		$W_{2/2} = \text{peak second half width at 10\%}$		
Estriol (E3)	2.05	1.72	-	
16- $\alpha$ OH E1	3.58	1.32	5.75	
Estradiol (E2)	5.40	0.98	8.10	
2-OH E1	5.66	1.78	1.23	
4-OH E1	6.38	1.09	2.98	
Testosterone	6.97	1.20	1.97	
Estrone (E1)	8.23	1.09	5.00	
2-Methoxy E1	8.91	1.10	2.94	
Progesterone	12.3	1.35	15.11	

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**Figure A.2. Representative chromatogram of standard solution of sex hormones**



**Figure A.3. Representative chromatogram of sex hormones in extracted liver tissue**

#### A.3.2.2 Linearity and Sensitivity

All the compounds show a good linear response with  $R^2 > 0.995$  in the linear range as mentioned in Table A.2. The LOD and the LOQ are given in terms of column loading (ng) and concentrations (ng/  $\mu$ L). Column loading was calculated by multiplying concentration (ng/ $\mu$ L) and the injection volume (in this case 100  $\mu$ L). The sensitivity for all the analytes are in the nanogram range demonstrating that this HPLC method can be used to analyze sex hormones as their concentrations are sufficiently high in urine. However, if necessary, urine samples can be concentrated easily, reducing concerns about method sensitivity.

#### A.3.2.3 Accuracy and Precision

The % accuracy for all the sex hormones were between 91.5 - 114.5 % (Table A.3), meeting the requirement as per the FDA guidance for the Industry: Bioanalytical Method Validation [15]. The method meets precision criteria as the % RSD for the system suitability, intra-assay and inter-assay precision (Table A.4) for all analytes lie within 10%

**Table A.2. Method Validation Results: Sensitivity & Linearity**

<b>Compound</b>	<b>LOD</b>	<b>LOQ</b>	<b>Equation of the line</b>	<b>R<sup>2</sup></b>
	<b>Conc.</b> <b>(ng/μL)</b>	<b>Conc.</b> <b>(ng/μL)</b>		
Estriol (E3)	0.27	0.89	$y = 1468.2 x + 33882$	0.999
16-α OH E1	3.00	0.97	$y = 1720x + 16375$	0.997
Estradiol (E2)	0.05	0.16	$y = 1586.1 x + 4488.4$	0.998
2-OH E1	0.71	2.35	$y = 2731.4 x - 20388$	0.999
4-OH E1	2.40	7.90	$y = 1143.1 x - 49339$	0.998
Testosterone	0.10	0.30	$y = 1149.9x + 11684$	0.999
Estrone (E1)	0.04	0.14	$y = 1341.8 x + 82827$	0.998
2-Methoxy E1	0.18	0.58	$y = 1173.6 x - 37165$	0.998
Progesterone	0.06	0.19	$y = 1122.9 x + 10372$	0.999

**Table A.3. Method Validation Results: Accuracy levels I, II &III represents low, medium & high concentration respectively of the linearity range**

Compound	Accuracy Level I		Accuracy Level II		Accuracy Level III	
	Conc. (ng/ μL)	%Accuracy (n=15)	Conc. (ng/ μL)	%Accuracy (n=15)	Conc. (ng/ μL)	%Accuracy (n=15)
Estriol (E3)	0.75	91.5	1.00	104.0	5.00	102.8
16-α OH E1	0.75	110.9	1.00	100.2	5.00	104.8
Estradiol (E2)	0.75	96.7	1.00	98.7	5.00	96.5
2-OH E1	0.40	114.9	0.60	102.4	3.00	99.74
4-OH E1	0.75	100.2	1.00	98.6	5.50	105.7
Testosterone	0.75	97.7	1.00	99.3	5.00	97.0
Estrone (E1)	0.75	113.7	1.00	106.9	5.00	113.5
2-Methoxy E1	0.75	109.1	1.00	105.8	5.00	95.4
Progesterone	0.75	97.8	1.00	97.1	5.00	102.8

**Table A.4. Method Validation Results: Intra assay and inter assay precision**

<b>Compound</b>	<b>Conc. (ng/<math>\mu</math>L)</b>	<b>Intra-Assay precision %RSD</b>	<b>Inter-Assay precision %RSD</b>
Estriol (E3)	0.75	4.59	3.55
	1.00	3.71	4.76
	5.00	4.55	4.33
16- $\alpha$ OH E1	0.75	3.55	6.78
	1.00	6.23	5.66
	5.00	5.01	7.32
Estradiol (E2)	0.75	1.30	4.90
	1.00	5.62	6.78
	5.00	4.75	4.55
2-OH E1	0.40	9.22	8.90
	0.60	7.06	6.75
	3.00	3.60	6.31
4-OH E1	0.75	9.42	7.54
	1.00	7.86	5.43
	5.00	6.62	6.32
Testosterone	0.75	9.15	8.99
	1.00	7.90	7.43
	5.00	7.89	8.12
Estrone (E1)	0.75	3.31	2.64
	1.00	4.25	3.11
	5.00	2.75	4.89
2-Methoxy E1	0.75	0.55	2.62
	1.00	6.81	5.02
	5.00	6.70	4.10
Progesterone	0.75	4.05	2.92
	1.00	4.72	4.55
	5.00	4.28	3.74

#### **A.4 Conclusion**

The HPLC UV method for rapid profiling of estriol, estradiol, estrone, progesterone, 2-hydroxy estrone, 16- $\alpha$  hydroxyl estrone, 2-methoxy estrone and 4-hydroxyl estrone. All the sex hormones can be determined in a single assay in less than 16 minutes using UV detection. The method was found to be specific, sensitive, accurate and precise. The sex hormones were separated on an Allure biphenyl column (150 mm\*4.6 mm) by a gradient elution of water and acetonitrile at a flow rate of 1.0 ml/min. The wavelength used for detection was 220 nm. The method was validated and was found to be having sufficient sensitivity (limit of quantification - 14 ng - 790 ng). The accuracy and precision were within bioanalytical method validation limits (91.5 to 114.5 % and RSD < 10%, respectively) and the method was linear over three orders of magnitude. A solid phase extraction method was developed to measure the hormone levels in biological samples (urine and tissue).

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