EVALUATION OF THE COMBINATION OF PROSTANOIDS AND FEMALE SEX HORMONES AS INFLAMMATION BIOMARKERS AND THEIR IMPLICATION ON HYPERTENSION

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ABSTRACT

Hypertension is a chronic medical condition, and a primary contributor to severe cardiovascular disease. It can increase mortality risk. Studies have found that a low-grade inflammation is involved in the development of hypertension. In addition, men have been found to have a higher risk of hypertension than age-matched women, while women have an increased risk of hypertension after menopause. Female sex hormones are considered to play a protective role in the development and progression of hypertension. My study focuses on investigating the combination of bioactive prostanoids and female sex hormones to find effective biomarkers to study the progression of hypertension.

Prostanoids are short-chain fatty acids derived from arachidonic acid (AA). They are inflammatory mediators, and have important physiological roles on promoting or suppressing inflammation. Female sex hormones are lipids derived from cholesterol, and are considered to play a protective role in the development and progression of the disease. Studying the combined roles of these two biochemical families in disease development will be helpful to understand the balance between a self-protective effect and inflammatory response in hypertension, and find effective biomarkers to predict the progression of hypertension.

In the study, a reversed phase high performance liquid chromatography is used to develop the separation method. A combination of up to nine prostanoids (PGD_2, PGE_2, 6-keto $PGF_{1\alpha}$, $PGF_{2\alpha}$, 11-dehydro TXB_2, 8-iso $PGF_{2\alpha}$, 13,14-dihydro-15-keto PGA_2, 13,14-dihydro-15-keto PGE_2 and 15-deoxy $\Delta^{12,14}$ PGJ_2) and eight female sex hormones (E_1, E_2, E_3, progesterone, 2-OHE_1, 4-OHE_1, 16α-OHE_1, and 2-$MeOE_1$) were chosen as analytes. The separation was performed on a Symmetry C18 4.6 × 250 mm column with 5-μm

particle size. A UV detector and is selected to determine the levels of analytes in a single 25-minute run. A solid phase extraction procedure was used to pretreat urine samples before injection. Then the validated separation method was applied to urine samples collected from African American patients with hypertension stage 1 before and after a special physical training. The role of physical exercise on hypertension was investigated by studying the level of change in urinary prostanoids and female sex hormones before and after exercise.

The level of urinary prostanoids and female sex hormones is usually too low to be detected with HPLC-UV, which encouraged us to develop a more sensitive method to detect the combination in urine. In the study, a LC-ESI-MS technique was chosen to separate and quantify the analytes. An LC-MS method was developed to quantify the above combination of prostanoids and female sex hormones. The same gradient condition of mobile phase as the HPLC-UV method was used in LC-MS method, except the mobile phase modifier Phosphoric acid was replaced with the volatile acid formic acid. In the method, negative ionization mode was used to determine prostanoids, while positive ionization mode for female sex hormones. Compared with the HPLC-UV method, LC-MS provides a lower LOD for analytes.

The LC-MS method was applied to urine samples from hypertensive patients to study the role of physical exercise on hypertension. In the study, it found that exercise changed the level of prostanoids such as 6-keto $PGF_{1\alpha}$, 11-dehydro TXB_2 and 8-iso $PGF_{2\alpha}$. After exercise, a shift from 2-OHE_1 to 4-OHE_1 occurred in women to promote inflammation, however, an opposite shift happened to men to inhibit inflammation. A

larger population is required to be involved in the future study to obtain a statistically significant result.

In addition, a theoretical model was developed to build a link between the retention behavior of analytes and chemical structure at an isocratic condition with HPLC-UV. The model was called quantitative structure-retention relationship (QSRR). The molecular descriptors were calculated in a semi-empirical AM1 mode. Multiple linear regression analysis was applied to the molecular descriptors to find a QSRR equation to predict the retention times of prostanoids and female sex hormones. The study is helpful to interpret the retention mechanism of prostanoids and female sex hormones on a C18 column.

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DEDICATION

To my sons and husband

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CHAPTER 1

INFLAMMATION, BIOMARKERS AND SEX HORMONES

1.1 Inflammation

Inflammation is a defensive immune response to harmful stimuli such as infection and tissue injury. The purpose of inflammation is to remove offending causes and repair tissue, and restore cellular homeostasis. From ancient times, people found that inflammation had symptoms including redness, swelling, heat, pain, and loss of function [1]. Nowadays, inflammation can be divided into acute and chronic inflammation based on the lasting duration of an inflammatory condition. Acute inflammation is an immediate process to remove infectious agents and repair tissue, whereas chronic inflammation is a local long-term inflammatory condition associated with malfunction of tissue [2, 3]. When acute inflammation fails to resolve harmful factors, the inflammatory condition will transition to chronic inflammation.

Inflammation is an important risk factor for many diseases and disorders such as diabetes [4], cardiovascular diseases [5, 6], arthritis [7] and even cancers [8, 9]. It is involved in all stages of disease. Inflammation can be induced by exogenous or endogenous inducers [3]. Exogenous inducers include microbial agents such as pathogen-associated molecular patterns and virulence factors, and non-microbial causes such as foreign bodies and toxic compounds; endogenous inducers are signals generated by stressed, damaged or malfunctioning tissues, products of the breakdown of the extracellular matrix, and endogenous crystals [3]. The role of inducers is to activate the release of inflammatory mediators such as lipid mediators, cytokines, chemokines and proteolytic enzymes [10].

Then the inflammatory mediators work on tissues and organs to change their functions.

1.2 Mechanism of inflammation

Inflammation is a complicated physiological process. The mechanisms of inflammation can be divided into two types of immune response: innate immune response and adaptive immune response [11, 12]. Once tissue is infected or injured, innate immunity quickly responds to tissue damage, signaling macrophages to express various scavenger receptors and Toll-like receptors with ligands containing pathogen-associated molecular patterns (PAMPs) [13]. These receptors can recognize pathogens containing the characteristic molecular patterns and bind them to initiate endocytosis and lysosomal degradation [14]. Meanwhile, Toll-like receptors activate nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways to resolve inflammation by regulating pro-inflammatory cytokine production, leukocyte recruitment, or cell survival [11, 15]. The adaptive immune response also works on inflammation. Compared with the innate immune response, the adaptive immune response is slower and more specific to antigens [11].

In the past, efforts have been made to uncover the mechanisms of acute and chronic inflammation. However, only acute inflammation can be said to be well understood at present. The cause and pathology of chronic inflammation is still unclear.

1.3 Biomarkers of inflammation

A biomarker is a substance present in serum, plasma, blood or tissues, the presence and measured concentration of which can help to predict the stages of progression of a

disease. Many studies have been dedicated to finding useful biomarkers to predict, prevent and treat diseases. The common biomarkers for inflammation include C-reactive protein (CRP), cytokines, soluble CD40 ligand, serum amyloid A, and adhesion molecules [16]. CRP is a biomarker of systemic inflammation, and is released in response to injury, infection, and other inflammatory stimuli [17]. Cytokines are a group of pleiotropic proteins including IL-1, IL-6, IL-10, tumor necrosis factor alpha (TNF-α), and MCP-1 [18]. Elevated IL-6 levels are associated with more severe events. TNF-α has been found to play an important role in myocardial dysfunction after acute coronary events [19]. MCP-1 is a chemokine with the function of activating recruit monocytes to promote atherosclerosis [20]. Soluble CD40 ligand (sCD40L) is a pro-inflammatory marker [21]. Serum amyloid A (SAA) is similar to CRP, an acute phase protein. Cell adhesion molecules play an important role in the interactions between leucocytes, platelets, and vascular endothelium [22].

Prostaglandins (PGs) significantly increase in concentration during inflammation. They are involved in the development of inflammation in diseases such as cardiovascular disease [23]. Prostanoids, which include the prostaglandins, are widely involved in inflammatory reactions. Each of the prostaglandins has its special role during inflammation. In order to understand these roles, it is useful to study their biosynthesis.

1.3.1 Biosynthesis of prostanoids

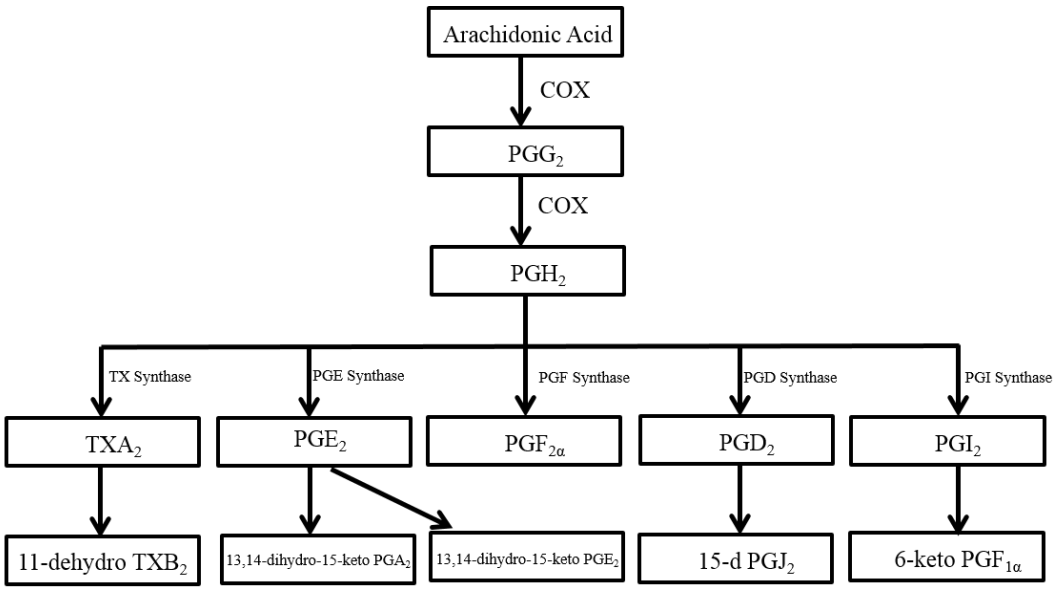

Figure 1.1 Biosynthesis of prostanoids

All prostanoids are short-chain fatty acids with a similar structure, including a cyclic ring, a *trans*-double bond, and a hydroxyl group. Prostanoids consist of prostaglandins (PG) with a cyclopentane ring, and thromboxanes (Txs) with a cyclohexane ring. They are lipid mediators, and have important physiological roles in promoting or suppressing inflammation [24]. They are derived from arachidonic acid (AA), ω6-20-carbon polyunsaturated fatty acid. AA is stored by esterifying to the phospholipids of the cell membrane. The pathway of prostaglandin biosynthesis is shown in Figure 1. When a body is injured or receives harmful stimuli, phospholipase A2 (PLA2) or diacylglycerol lipase on the cell membrane immediately release AA to respond to the offensive actions. Then free AA is metabolized by enzymes cyclooxygenase (COX), lipoxygenease (LOX) and cytochrome P450 [25-27].

In our studies, we focus on the metabolites generated from the COX pathway that plays an important role in inflammation. In the COX pathway, AA is oxygenated to form hydroperoxy endoperoxide (PGG_2) under the action of the enzyme prostaglandin endoperoxide synthase (PGHS) or COX. Then PGG_2 is reduced to form highly unstable hydroxy endoperoxide PGH_2 via a separate peroxidase active site of PGHS [28]. By specific syntheses, the intermediate substrate PGH_2 is transformed by the COX pathway to prostanoids consisting of prostaglandins, thromboxanes and prostacyclins. The main prostanoids include prostaglandin PGD_2, prostaglandin PGE_2, prostaglandin PGF2α, prostaglandin PGI_2 (prostacyclin), or TXA_2 (thromboxane A_2).

Researchers have found that COX has three isomerases including endogenous COX-1, inducible COX-2, and COX-3, a splice variant of COX-1 retaining intron-one [28]. COX-1 is involved in the expression of PGs with preservative roles in physiological functions such as cytoprotection and homeostasis (or regulating platelet aggregation and renal function [29]), whereas COX-2 is responsible for the production of prostanoid during inflammation [27]. COX-3 has a structure and enzymatic function similar to COX-1 and COX-2 but slower enzymatic activity [30]. Once inflammation begins, the level of PGs is increased dramatically in response to infection or harmful stimuli. PGs are mainly produced by the COX-1 and COX-2 pathways. Inflammation can be controlled by inhibiting the COX pathways with anti-inflammatory medicine such as aspirin.

Mast cells mainly produce PGD_2, whereas macrophages produce PGE_2 and TXA_2. Each PG molecule has its own individual physiological effects during inflammation. TxA_2, $PGF_{2\alpha}$, PGE_2, PGI_2, and PGD_2 are main prostaglandins through COX-1 and COX-2 pathways [31].

Prostaglandin D_2 (PGD_2)

PGD_2 is a major eicosanoid generated from activated mast cells by harmful stimuli [32]. PGD_2 has many physiological effects including inducing vasodilatation and bronchoconstriction [33], inhibiting platelet aggregation [34, 35], increasing vascular permeability [36], and acting as a pro-inflammatory mediator. PGD_2 has several receptors such as TP. It can have a complex effect during inflammation. Recent studies have claimed a contrary result: that PGD_2 can also play an anti-inflammatory role at higher concentration by inhibiting the expression of inducible nitric oxide synthase and plasminogen activator inhibitor [37, 38]. PGD_2 exerts pro- and anti-inflammatory effects depending on concentration. At low concentration, it recruits and activates leukocytes to produce pro-inflammatory effects; at micromolar concentration, PGD_2 induces T lymphocyte apoptosis to inhibit inflammation [39]. PGD_2 also plays a role in regulating sleep [40] and pain perception [41].

Prostaglandin E_2 (PGE_2)

PGE_2 is generally considered a pro-inflammatory mediator [42], and is responsible for inflammation symptoms such as redness, swelling, and pain [43]. It has many physiological effects including working as a vasodilator [44], playing a role in the development of fever and hyperalgesia, regulating the production of cytokines such as interleukin 6 [45], and regulating immune responses, blood pressure, gastrointestinal integrity, and fertility. It can also have anti-inflammatory effects [46], and protective effects in asthma and non-asthmatic eosinophilic bronchitis [47]. Dysregulated PGE_2 synthesis or degradation will cause pathological conditions [48].

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)

$PGF_{2\alpha}$ is mainly synthesized in the female reproductive system by COX-1. It regulates luteolysis, ovarian function, luteal maintenance of pregnancy, the induction of interleukin synthesis, and uterine contraction and has an effect on the contraction and relaxation of smooth muscles and controlling of the blood pressure [49]. It is a pro-inflammatory mediator in acute and chronic inflammation [50].

6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$)

6-keto-$PGF_{1\alpha}$ is a stable metabolite of the bioactive lipid prostacyclin (PGI_2) and serves as a marker of PGI_2. PGI_2 regulates cardiovascular homeostasis, inhibits platelet aggregation and leukocyte adhesion [51], and has a vasodilator effect [52]. However, PGI_2 is an unstable compound and is quickly converted to stable $PGF_{2\alpha}$ by non-enzymatic processes. PGI_2 has biphase effects, and can work both as a pro- and an anti-inflammatory mediator [53].

11-dehydro-thromboxane B_2 (11-dehydro-TXB_2)

11-dehydro TXB_2 is a stable metabolite of TXA_2, and urinary 11-dehydro TXB_2 is a marker to evaluate platelet activation [54, 55]. TXA_2 can stimulate platelet aggregation and induce smooth muscle contraction [56]. However, it is unstable and is quickly converted to stable 11-dehydro TXB_2. TXA_2 is a pro-inflammatory mediator, and is involved in the process to promote the initiation and progression of atherogenesis [57].

8-iso-$PGF_{2\alpha}$

8-iso-$PGF_{2\alpha}$ (also called 8-iso-prostaglandin F-2) is a biomarker of oxidative stress in clinical diagnosis and an effective vasoconstrictor [49, 58-60]. It also plays a pro-inflammatory role during inflammation. Studies have been found that oxidative stress can promote a low-grade inflammation [61]; on the other hand, inflammation may induce oxidative stress [62]. Schwedhelm *et al.* found that patients with established coronary artery disease had a higher level of 8-iso-$PGF_{2\alpha}$ than control subjects, and 8-iso-$PGF_{2\alpha}$ could be used as a marker to evaluate the risk of coronary heart disease [63]. Studying the level of 8-iso-$PGF_{2\alpha}$ in diseases will correlate oxidative stress with inflammation.

13,14-dihydro-15-keto-PGA_2

13,14-dihydro-15-keto PGA_2 is a byproduct of PGE_2, appearing in small quantities [64, 65].

13,14-dihydro-15-keto-PGE_2

13,14-dihydro-15-keto PGE_2 is a main stable metabolite of PGE_2 [66], and can be used as a marker to evaluate the biosynthesis of PGE_2 in plasma or urine. Axelrod *et a*l. found that rats with diabetic ketoacidosis had higher plasma levels of 13,14-dihydro-15-keto-PGE_2 than normal subjects [67]. This suggests that the level of 13,14-dihydro-15-keto-PGE_2 may be used to interpret the mechanism of inflammation for diabetic ketoacidosis.

15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2)

15d-PGJ_2 is a derivative of PGD_2 catalyzed by PGD_2 synthase, and a strong endogenous ligand for peroxisome proliferator-activated receptor γ (PPARγ) [68], where

PPARγ regulates adipocyte metabolism [69] and inhibits obesity-associated inflammation [70]. 15d-PGJ_2 has an anti-inflammatory effect by inhibiting the activities of pro-inflammatory transcription factors such as NF-κB, and resolves inflammatory response [71, 72].

1.3.2 Clinical application

By studying inflammatory biomarkers, researchers attempt to understand the role of inflammation in the progression of disease. Li *et al.* found that the level of biomarkers including interleukin (IL)-6, IL-8, IL-10, tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, vascular endothelial growth factor (VEGF), and N-terminal pro-brain (B-type) natriuretic peptide (NTpBNP) were significantly increased in atrial fibrillation patients compared to control subjects [73]. Their studies indicated that inflammation was involved in the development of atrial fibrillation.

Inflammation is also involved in the development of diabetes. Type 2 diabetes (T2D) is a disease caused by insulin resistance. Studies have found that the patients with T2D have elevated circulating levels of inflammatory mediators including C-reactive protein, interleukin-1β (IL-1β) and IL-6 [74]. The release of the inflammatory mediators activates the nuclear factor-kappa B (NF-κB) pathway to contribute to the progress of insulin resistance [75].

Alzheimer's disease (AD) is a neurodegenerative disorder with memory loss. It commonly strikes people at the age of 65 years or older. Researchers have found that neuro-inflammation in the cerebral cortex is involved in Alzheimer's disease (AD) [76, 77]. The inflammation is caused by damaged tissue, highly inert abnormal materials, and

neurodegeneration in the brain of AD patients.

Cardiovascular disease is a disease of the heart and blood vessels. Atherosclerosis is a main cause of cardiovascular diseases. Studies have found that inflammation is involved in the initiation and the development of atherosclerosis [78]. Smoking or medical conditions such as hypertension and obesity activate endothelial cells to produce adhesion molecules such as leukocytes to deposit on the arterial wall, thus narrowing the inner diameter of the vessels. By activating the inflammatory mediator nuclear factor-κB, pro-inflammatory cytokines and oxidized lipids are produced as adhesion molecules. The risk of cardiovascular disease can be evaluated by measuring oxidized lipoproteins and pro-inflammatory cytokines [79].

Cancer is a leading cause of mortality in the United States [80]. Studies have found that the activation of nuclear factor-κB (NF-κB) can promote chronic inflammation to cancer and stimulate tumor progression [81]. Understanding the link between cancer and inflammation will help to more effectively prevent and treat cancer, and reduce mortality.

1.4 Sex hormones and inflammation

Women increase the risk of autoimmune diseases after menopause; before manopause, women have a lower risk compared with age-matched men. The levels of female sex hormones (estrogens) in women decrease a lot after menopause. It indicates that natural female sex hormones may play a protection role on women during disease development and progression. Schmidt *et al.* studied synovial cells from rheumatoid arthritis, and found that androgens are anti-inflammatory, whereas estrogens are pro-inflammatory, and androgen supplementation can benefit in the treatment of rheumatoid

arthritis [85]. Cushman *et al.* found that estrogens therapy in postmenopausal women increased the concentration of the inflammation factor C-reactive protein, which indicates that estrogens are inflammatory, but decreased the concentration of soluble E-selectin, which indicates that estrogens play an anti-inflammatory role. Their results showed that estrogens did not mediate inflammation by the way of C-reactive protein. [102]. Straub reviewed recent research on estrogens and found that estradiol at periovulatory to pregnancy levels plays an anti-inflammatory role by inhibiting iNOS and NO release, and down-regulating expression of proinflammatory cytokines and prostaglandin E_2 production, whereas E_2 stimulates the production of inflammatory mediators such as TNF [103]. Studies of the effect of gender on cancer risk have found that pre-menopausal women have a lower risk of cancer than age-matched men. Female sex hormones are therefore considered to play a protective role in the development and progression of the disease [82].

1.4.1 Sex hormone biosynthesis

The biosynthesis pathway of estrogens gives a direct picture of the relationship of estrogens and other hormones. From Figure 1.2, it can be seen that estrogens are originally derived from cholesterol [83]. Cholesterol is first oxidized by CYP11A, a mitochondrial cytochrome P450 oxidase, to produce pregnenolone. In the second step, pregnenolone is converted to 17-OH-pregnenolone by the CYP17A enzyme. Under the effect of this same enzyme, 17-OH-pregnenolone is continuously converted to dehydroepiandrosterone. In the next step, dehydroepiandrosterone is oxidized by 3-β-HSD enzyme to produce androstenedione. In the final step, androstenedione is reduced by 17-β hydroxysteroid

dehydrogenase to generate testosterone, or by CYP19 to produce estrone. Estrone can be continuously converted into estradiol, then into estriol.

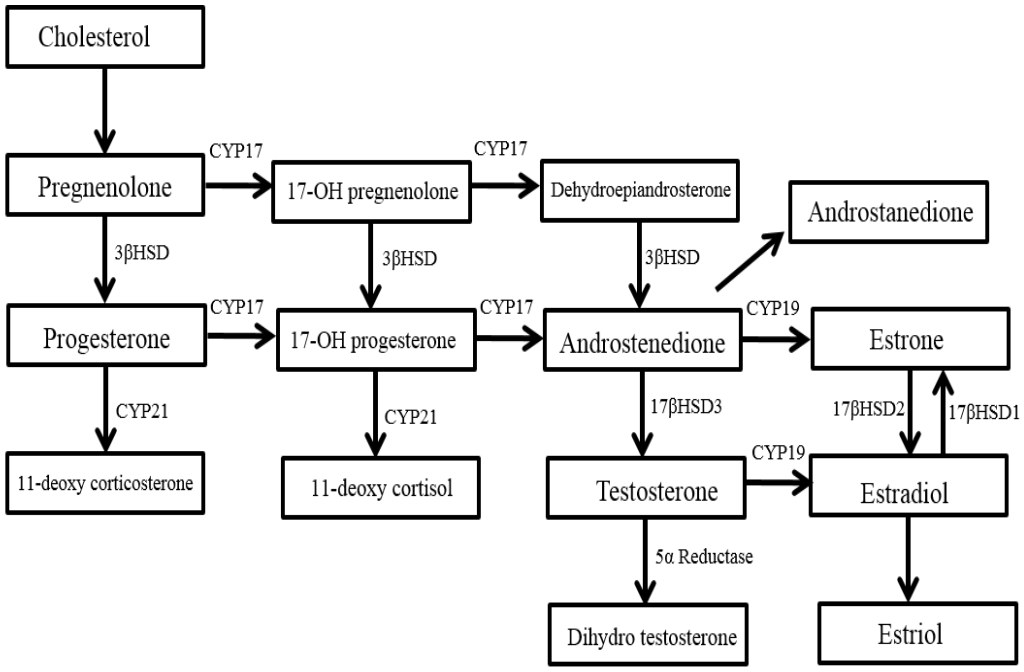

Figure 1.2 Biosynthesis of steroid hormones of cholesterol pathway

Sex hormones are lipids derived from cholesterol, serving as distant chemical messengers in the body. In the current study, we focus on estrone (E_1), estradiol (E_2), estriol (E_3), progesterone and the metabolites of E_1, 2-hydroxyestrone (2-OHE_1), 4-hydroxyestrone (4-OHE_1), 16α-hydroxyestrone (16α-OHE_1), and 2-methoxyestrone (2-$MeOE_1$).

Estrogen

E_1, E_2 and E_3 are usually referred to as estrogens. Estrogens have athero-protective effects, reducing the production of vascular reactive oxygen species [84]. However, Schmidt's group found that estrogens were pro-inflammatory in cells from patients with rheumatoid arthritis [85]. Progesterone may protect women against asthma [86]. Tavares-de-Lima group found that E_2 had a protective role in allergic lung inflammation [87].

Estrone Metabolites

16α-OHE_1 is pro-inflammatory [85]. An increased level of 2-OHE_1 is associated with a decreased risk of breast cancer [88]. Chen's group found that progesterone inhibited NF-κB activation and pro-inflammatory cytokine expression in traumatic brain injury (TBI) [89]. 2-OHE_1 and 2-$MeOE_1$ have strong anti-inflammatory effects, whereas 4-OHE_1 has a pro-inflammatory effect [90]. Each of these female hormones and their metabolites plays a distinct role in the disease progression mechanism, and it is important to measure them in patient samples at different stages of disease.

1.4.2 Link between prostanoids and sex hormones

Studies have found that estrogens play an atheroprotective role by activating cyclooxygenase-2 (COX-2) to produce PGI_2 [91]. Ford, *et al*. found that E_2 increased $PGF_{2\alpha}$ secretion from sheep uterine tissue [92]. Fortier *et al*. found that the production of PGE_2 and $PGF_{2\alpha}$ were reduced by E_2 where increased by progesterone in epithelial cells [93]. The contradictory results tell us that it is very necessary to study the combination of prostanoids and sex hormones from the same biological sample at the same time, so that

we can discover the relationship between sex hormones and prostanoids and the balance between pro- and anti-inflammatory markers, and understand the balance between inflammation and self-protection in cancer patients.

1.5 Inflammation and hypertension

Hypertension is a common cardiovascular disease. It is a chronic medical condition in which the arterial blood pressure is at or above the normal level of 120/80 mmHg. The progression of hypertension is divided into three stages: pre-hypertension (120–139 and/or 80–89 mmHg), stage 1 (140–150 and/or 90–99 mmHg), and stage 2 (≥160 and/or ≥100 mmHg) [104]. Elevated blood pressure is a primary contributor to severe cardiovascular disease, and can increase mortality risk. Based on data reported in 2013 from the National Health and Nutrition Examination Survey between 2007 and 2010, the American Heart Association has estimated that 33.0% of U.S. adults suffer from hypertension [94].

Studies have found that vascular wall inflammation is involved in the pathogenesis and progression of hypertension [95]. Hypertension is also an age-related disease. In the presence of hypertension, arterial stiffness increases, causing organ damage and increasing the risk for cardiovascular morbidity and mortality [96, 97]. While the link between inflammation and hypertension is not clear [98, 99], evidence indicates that essential hypertension patients have higher plasma levels of inflammatory markers including C-reactive protein (CRP), tumor necrosis factor-α (TNF-α) and IL-6 than control subjects [100, 101].

1.6 Analysis of prostanoids and female sex hormones

To date, several analytical techniques have been applied to separate and quantify either prostanoids or sex hormones in biological matrix samples. Noble et al developed a method to determine 8-iso-$PGF_{2\alpha}$ in urine with ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS), and applied the method to evaluate the change of urinary 8-iso-$PGF_{2\alpha}$ from stage 2 hypertension under drug treatment [105]. Fajt et al measured the levels of PGD_2 in bronchoalveolar lavage (BAL) fluid by using enzyme-linked immunosorbent assay (ELISA), and found that the levels of PGD_2 increased in patients with severe and poorly controlled asthma [106]. Zeng et al derivatized PGE_2 with 4-diazomethylpyridine to improve ionization efficiency and measured the levels of PGE_2 with LC–MS/MS in rat brain tissue [107]. Studzinska et al developed a simple and fast method to separate and quantify seven sex hormones in urine with an ultra-high performance liquid chromatography with a diode-array detector [108]. In the method, estrone, estriol, 17-α-estradiol, 17-β-estradiol, progesterone, pregnenolone, and testosterone were determined on an octadecyl columns within 6 min with a gradient elution. In addition, the stereoisomers of estradiol were separated on octadecyl stationary phase due to high column efficiency. Xu et al developed a method to derivatize sex hormones with heptafluorobutyric acid anhydride by under microwave irradiation for three minutes [109]. Then 13 derivatized sex hormones in feeds were determined by gas chromatography-mass spectrometry (GC-MS). Piwowarska et al developed a method to measure the level of estrogens and their metabolites in serum with HPLC and an electrochemical detector [110]. The method was applied to the serum samples from pregnant women.

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CHAPTER 2

ANALYTICAL TECHNIQUES AND INSTRUMENTATION

2.1 Introduction

This chapter focuses on the techniques and instrumentation involved in the development and validation of a method for separating combinations of prostanoids and female sex hormones. In the studies, high pressure liquid chromatography-ultraviolet detection (HPLC-UV) and liquid chromatography- mass spectrometry (LC-MS) were used to separate the mixtures and quantify the level of analytes. Solid phase extraction was used to purify samples before injection to HPLC.

2.2 Principles of HPLC

Liquid chromatography was first invented by the Russian botanist Mikhail Tswett in 1903 [1]. In his studies, a column filled with calcium carbonate as the stationary phase was used to separate the pigments in green leaves. However, LC was not well developed until the sixties and seventies. By the end of the seventies, HPLC was introduced to separation science due to improvements in column materials and instrumentation. Since then, HPLC has become a major analytical instrument in research laboratories and in industry. HPLC is a popular analytical technique to separate the components of a mixture in solution and to quantify each component. The components are separated as the mobile phase flows over the stationary phase. Based on the interaction between these phases, different components are separated.

Based on the separation mechanism, HPLC can be classified into five types: adsorption, partition, size exclusion, affinity, and ion exchange chromatography. Adsorption chromatography is used to separate neutral analytes, and the separation occurs based on the partition of analytes between the liquid mobile phase and a neutral, solid stationary phase. Partition chromatography is similar to adsorption chromatography and is also used to separate neutral analytes, but the stationary phase is a long alkyl chain bonded solid phase. Ion-exchange chromatography (IEC) is used to separate ionic analytes based on ionic interaction. The separation occurs between a charged solute and oppositely charged sites bound to the stationary phase. Size exclusion chromatography (SEC) is a gel permeation chromatography or gel filtration chromatography to separate particles based on molecular size; the stationary phase particles are featured with a wide range of pore sizes. In SEC, the larger size molecules elute first. Affinity chromatography is used to separate biomolecules based on biological-like interactions such as those between antigen and antibody, enzyme and substrate, or receptor and ligand. Normal phase HPLC and reversed phase HPLC are classifications of chromatography based on the adsorption and partition mechanism. In normal phase HPLC, a polar stationary phase and a non-polar mobile phase are used to separate analytes with differing polarities. In reversed phase HPLC, a non-polar stationary phase and an aqueous/organic solvent mobile phase is used to separate relatively non-polar analytes. When surfactant is added into the aqueous mobile phase, PRLC is classified as either ion–pair chromatography (IPC) or micellar liquid chromatography (MLC). Below the critical micelle concentration (CMC), the surfactant molecules present as monomers, and PRLC is called IPC; above CMC, surfactant molecules begin to

aggregate to form micelles, and PRLC is called MLC. In this research study, we mainly used PRLC to study the combination of prostanoids and female sex hermones.

2.3 Basics of chromatography

In the development and validation of a new HPLC separation method, a number of criteria are used to evaluate its performance, including capacity, selectivity, resolution and column efficiency [2]. In general, capacity and selectivity are used to determine the quality of a column, and resolution and column efficiency are used to tell if the separation is acceptable. These terms are defined in the following sections.

Capacity

Capacity factor (k_R') is used to determine the ability of a column to retain samples, and can be calculated according to the following equation

$$k'_R = \frac{t_R - t_0}{t_0} = \frac{V_R - V_0}{V_0}$$

where t_R is the retention time for a solute to reach the detector, t_0 is the time for non-retained species to reach the detector, V_R is the retention volume of solvent for eluting a solute to reach the detector, and V_0 is the void volume of solvent for nonretained species to reach the detector. The properties of the packing material determine the size of the capacity factor. A higher capacity factor will result in more strongly retained solutes. Usually, k_R' values between one and ten are acceptable to achieve reasonable analysis time and resolution.

Selectivity

Selectivity (α) is another parameter to decide if a column can be used to separate particular compounds, and to evaluate how effectively two compounds can be separated. The selectivity can be calculated by the following equation:

$$\alpha = \frac{t_2 - t_0}{t_1 - t_0} = \frac{V_2 - V_0}{V_1 - V_0} = \frac{k_2'}{k_1'}$$

where t_1 and t_2 are retention times of solute 1 and 2 respectively, and V_1 and V_2 are the solvent volumes to elute solutes 1 and 2 respectively. If α is equal to one, there is no separation between the two solutes. The value of α can be adjusted to an acceptable range by changing the composition of the mobile phase.

Resolution

Resolution is used to evaluate the degree of separation between two neighboring solute peaks. It depends on the selectivity (α), efficiency (N) and capacity (k') of the column. It can be calculated by the below equation

$$R = \frac{1}{4}\frac{\alpha - 1}{\alpha}(N^{1/2})\frac{k'}{1 + k'}$$

For accurate quantification measurement, R should be greater than 0.8. R can be improved by adjusting the selectivity and capacity of a column.

Column Efficiency

The column efficiency is evaluated by the number of theoretical plates (N). Plate theory and rate theory are used to interpret column efficiency. In plate theory, an instantaneous equilibrium of the solute is assumed to occur between the stationary and mobile phases, and a column is considered to be made up of many thin theoretical plates. A greater number of theoretical plates (N) indicates better efficiency of the column.

$$H = L/N$$

where L is the length of the column (millimeters), and H is the height equivalent to a theoretical plate (HETP, or H).

However, the plate theory ignores the effects of band broadening and the influence of particle size, stationary phase loading, solvent viscosity, and flow rate on column performance.

In addition, N can be calculated by the following equation.

$$N = (t_R/\sigma)^2 = t_R^2/\sigma^2$$

where t_R is retention time of the solute, σ is the standard deviation of the solute population.

The rate theory is another theory for estimating the plate height of a column, proposed by van Deemter, *et al*. This theory considers the effects of band broadening on plate height.

Eddy diffusion, longitudinal diffusion and resistance to mass transfer are introduced to the equation for evaluating the plate height called the van Deemter equation as follows

$$H = A + B/\mu + C\mu$$

where H is the plate height of the column, μ is the average linear velocity of the mobile phase, A is a constant from the band broadening resulting from eddy diffusion, B is a constant from the band broadening resulting from longitudinal diffusion, and C is a constant related to the resistance of mass transfer.

2.4 Retention mechanisms of Reversed-phase chromatography

Reversed-phase chromatography is the most popular mode among chromatographies, and is used to separate neutral molecules dissolved in water or organic solvents based on their polarities. There are several mechanisms proposed to interpret the retention behavior of analytes in RPLC, including solvophobic and partitioning theories. Solvophobic theory discusses chromatographic retention in terms of classical thermodynamics [3]. In the solvophobic theory, the analyte molecule enters the mobile phase by creating a cavity in it, and then the analyte molecule in the cavity interacts with the surrounding solvent molecules. This process results in free energy changes due to solvation and due to the interaction between an analyte and an alkyl chain.

In the partitioning theory, the retention of a solute depends on the interaction between the stationary phase and solutes. The bonded long alkyl chains in the stationary phase are considered to behave like a liquid. The solute will be surrounded by the alkyl chains in the

stationary phase, and partition between the mobile phase and a stationary phase. The two theories are illustrated in figure 2.1. In general, the partition theory mainly works on long alkyl chain bonded stationary phase, and the solvophobic theory mainly works on shorter chain bonded stationary phase.

In the adsorption model, the solute molecules travel from the liquid phase to the interface between the liquid and stationary phases, and displace the adsorbed solvent molecules at the interface [4]. In HPLC, the adsorption model assumes that the analyte molecules cannot penetrate into the bonded hydrocarbon layer due to its high density of nonpolar hydrocarbon chains, and remain at the interface between the mobile and stationary phases.

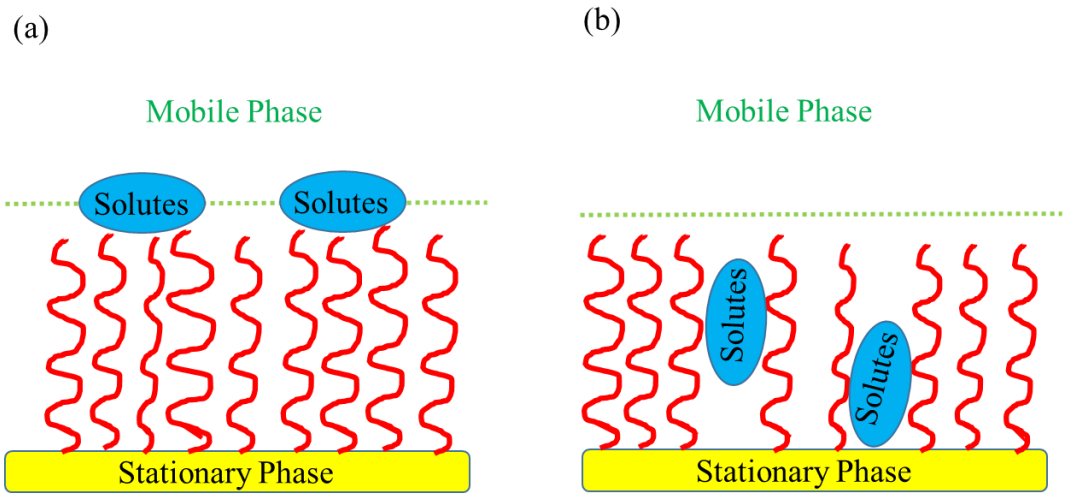

Figure 2.1 Retention models: (a) Solvophobic model; (b) Partitioning model

2.5 Instrumentation of Reversed Phase Liquid chromatography

RPLC mainly consists of a mobile phase, a pump, an injector, a column, a detector, a waste container, and a data collection device. The instrumentation set up of RPLC is illustrated in figure 2.2.

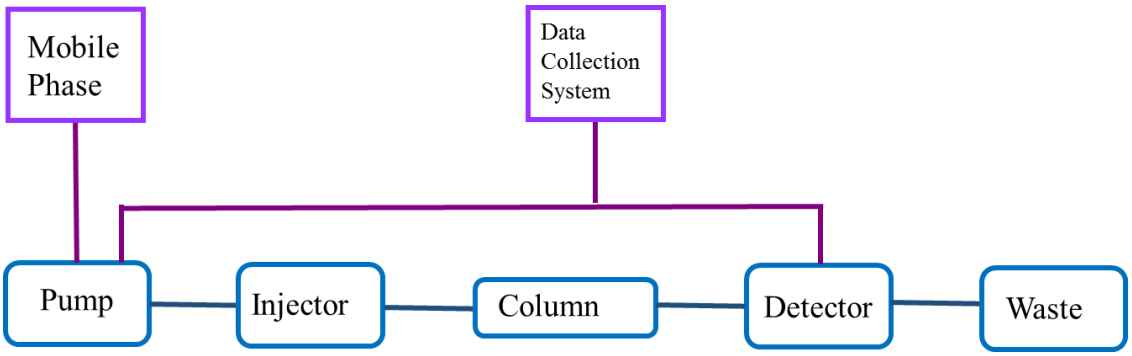

Figure 2.2 Instrumental set up of RPLC

2.5.1 Mobile phase

There are two modes by which HPLC can deliver solvents in the system: isocratic and gradient modes. In isocratic mode, the composition of the mobile phase is kept constant during the analysis. Gradient mode is a very popular mode used in HPLC analysis. In gradient mode, two or three solvents are mixed together to form a gradient elution.

Reverse Phase HPLC (RP-HPLC) consists of an aqueous polar mobile phase and a non-polar organic solvent. It is widely used to separate many non-ionizable compounds. Separation in RPLC is based on the partition of sample molecules between the aqueous mobile phase and the non-polar stationary phase. In RPLC, the order of solvent strength is as follows: water (weakest) < methanol < acetonitrile < ethanol < tetrahydrofuran < propanol < (methylene chloride) (strongest).

2.5.2 Stationary phase

In RPLC, a non-polar alkyl-bonded silica stationary phase such as octadecylated silica (C18) or C8 is used to separate a mixture into components. The selectivity, capacity, and efficiency of the column are all affected by the nature of the packing material and the materials of construction. The retention order of the bonded phase follows the below order: unbonded silica « cyano < Q (TMS) < C3 < C4 < phenyl < Q « C18 (strong).

2.5.3 Detectors

The common detectors connected to HPLC include fluorescence detectors, refractive index (RI) detectors, UV detectors and mass spectrometer (MS) detectors. In this chapter, we will focus on UV/Vis detectors and MS detectors.

UV/Vis detectors

A UV/Vis detector is nondestructive and is widely used in HPLC. It can measure light absorbance in the range of 190-700 nm. Compounds with chromophores can be detected by measuring the absorption of UV radiation. When analytes in the eluent pass through the

detector, they absorb the light, and its measured intensity decreases. By measuring the intensity of transmitted light through the flow cell, the concentration of the analyte can be determined based on the Beer's law. The concentration of analytes is proportional to the absorbance. UV/vis detectors are divided into fixed-wavelength detectors and variable-wavelength and photodiode array detectors. Compared with other detectors, a single-wavelength detector is more sensitive due to less light lost to the environment.

Mass Spectrometric Detectors

A mass spectrometer is a destructive universal detector. It is sensitive, and used to identify the structure of unknown compounds. In my research work, a single quadrupole mass spectrometer with electrospray ionization (ESI) mode is used to detect and measure prostanoids and sex hormones. ESI-MS is a soft ionization technique, and can be used to detect polar or ionic compounds with molecular weights greater than 150,000 amu. The Fenn group at Yale University invented this technique in the 1980s [5]. The instrumental set-up of an ESI ion source is illustrated in Figure 2.3. During ionization, a dilute solution containing analytes is injected through a steel capillary into a high voltage field with a voltage range of 2–6 kV. The high voltage is applied between the tip of the spray needle and the heated capillary. Under the strong electric field, the sample solution is dispersed into small highly charged droplets. A dry gas of N_2 is applied to evaporate the solvent of the droplet. Over time, the size of the droplets keeps decreasing, and the interaction between charged analytes in the droplet becomes stronger and stronger. Finally, the charged analytes are released to enter the mass spectrometric analyzer. ESI-MS is usually used to detect proteins and other macromolecules with multiple charges.

2.5.4 Solid phase extraction

In the current research work, samples were pretreated with solid phase extraction (SPE). A reversed-phase C18 cartridge is used to prepare urine samples. The analytes and impurities in the sample are separated based on their polarities. In the process of SPE, the first step is called condition for wetting the surface of the stationary phase with a polar or slightly polar solvent. Then a sample is loaded on the cartridge. A low polar solvent is used to wash off the interference compounds from the stationary phase, while the analytes are retained on the cartridge. Lastly, a strong polar solvent is used to elute the analytes. In this way, matrix effect from a complicated sample such as urine and blood can be eliminated.

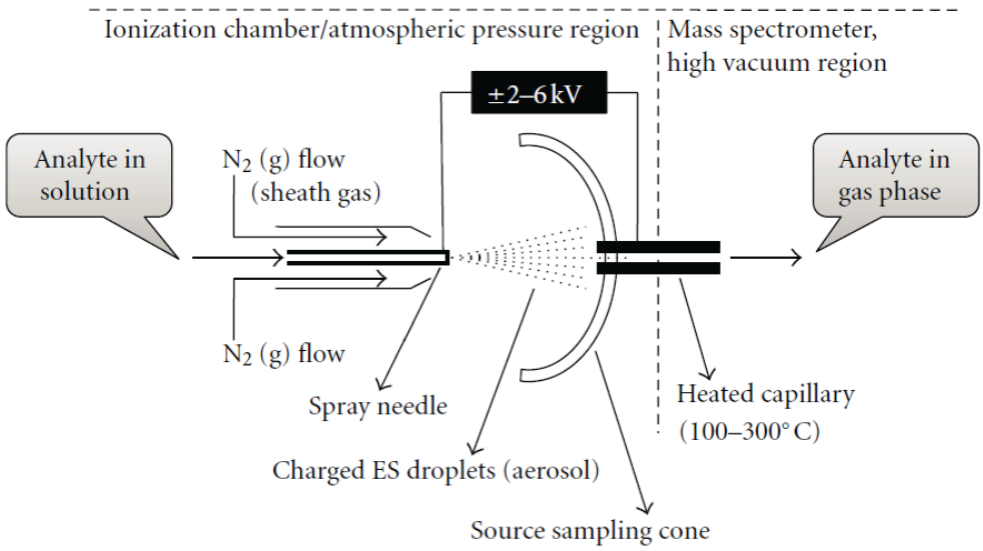

Figure 2.3 Schematic Graph of the ESI-ion source from Ref. [5]

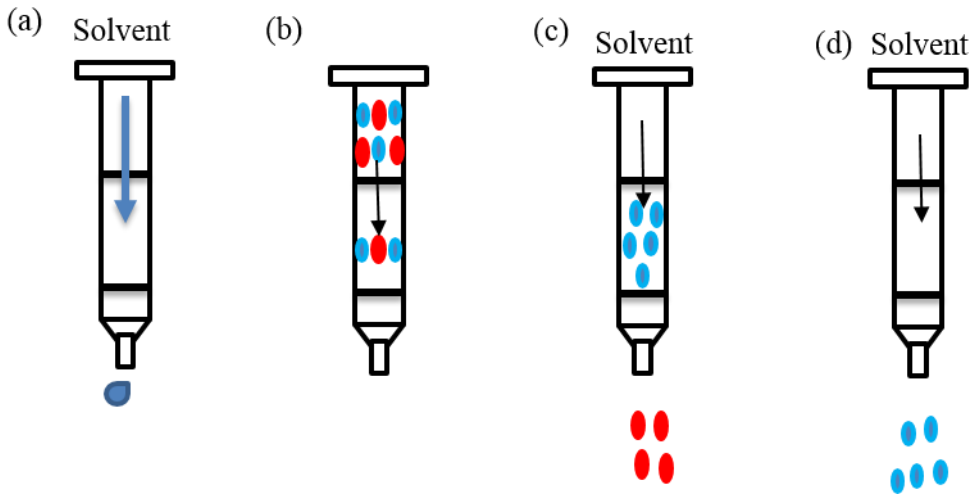

Figure 2.4 Illustration of SPE steps: (a) Conditioning, (b) Loading sample; (c) Washing; (d) Eluting sample

2.6 References

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CHAPTER 3

ASSAY PROFILES OF PROSTANOIDS AND FEMALE SEX HORMONES WITH HPLC-UV

[Reproduced from *J. Hypertens* **3**: 159 (2014).]

3.1 Introduction

Hypertension is a chronic medical condition in which the arterial blood pressure is at or above the normal level of 140/90 mmHg. Elevated blood pressure is a primary contributor to severe cardiovascular disease, and can increase mortality risk. Based on data reported in 2013 from the National Health and Nutrition Examination Survey between 2007 and 2010, the American Heart Association has estimated that 33.0% of U.S. adults suffer from hypertension [1]. The common method to diagnose hypertension is by measuring blood pressure. However, when the symptoms of elevated blood pressure (BP) are present, hypertension has progressed to a late stage. Delayed diagnosis limits the effect of clinical treatment. Therefore, it is critical to find effective biomarkers to diagnose pre-hypertension (BP: 120–139 and/or 80–89 mmHg) or stage 1 hypertension (BP: 140–150 and/or 90–99 mmHg). The first recommendation for treatment of pre-hypertension or Stage 1 hypertension involves lifestyle changes. Changing lifestyle by increasing physical activity and dietary modification changes the overall state of inflammation and expression of estrogenic compounds. Our method that quantifies biomarkers implicated in hypertension disease provides the possibility to predict prehypertension or early stage hypertension before any physical symptom can be detected, and when lifestyle modifications can be most effective. Increases in progesterone are known to lower blood pressure, while some of estrogenic compounds are associated with elevated blood pressure in many women. In

this study, the subjects were considered overweight or obese. The adipose tissue contributes significantly to the quantity of circulating estrogen in pre- and post-menopausal women [2]. In addition, the adipose can significantly raise the level of systemic inflammation. Therefore, monitoring the level of estrogenic compounds and developing a correlation with specific biomarkers known to be implicated in hypertensive disease provides for better understanding not only of the disease but the approaches to intervention as well.

In the past years, hypertension has been the subject of intensive study by the medical community. Men have been found to have a higher risk of hypertension than age-matched women, while women have an increased risk of hypertension after menopause, suggesting that female sex hormones including estrogens and progesterone may play a protective role in the development and progression of hypertension [3, 4]. Studies have found that estradiol has an effect to lower blood pressure, and natural progesterone may enhance the effect of estradiol. However, the role of progesterone during hypertension is not clear due to some contradictory results. Prostanoids as vasodilator have been used to treat hypertension over decades [5]. They are also lipid inflammatory mediators, and can interact with hypertension development and signal endothelial dysfunction involved in hypertension [6]. Studying the combined roles of prostanoids and female sex hormones in hypertension development is very timely, but has not been reported.

To date, several analytical techniques have been applied to separate and quantify either prostanoids or sex hormones in standards or biological samples, including immunoassay methods [7, 8], gas chromatography–mass spectrometry (GC-MS) and GC-MS/MS [9, 10],

and high-performance liquid chromatography with fluorescence (HPLC-FL) detection [11]. However, immunoassay methods lack in specificity on analytes due to cross-reactivity; while GC-MS, GC-MS/MS and HPLC-FL techniques require multiple complicated and time-consuming derivatization steps to be applied to the compounds before analysis. HPLC has therefore become the most popular technique to investigate prostanoids and sex hormones. However, LC-MS and LC-MS/MS have high maintenance and instrument costs. In our studies, HPLC-UV is chosen to do analysis because of easy operation, low maintenance cost, and easy access to most laboratories.

Despite the need to analyze sex hormones and prostanoid lipids together, few previous studies have focused on investigating the *combination* of the two different groups simultaneously in a single run due to their closely-related structures; most methods are limited to analyzing either one or the other [12-14]. Studying either the prostanoid or sex hormone group in isolation neglects the interaction between PGs and sex hormones in the progression of disease, and thus brings only partial evidence to the interpretation of its molecular mechanism. We have developed a method to quickly separate and quantify seventeen bioactive compounds belonging to both the prostanoid and sex hormone classes simultaneously with a short run time. The method is simple, does not require complicated sample preparation, and consumes less of the sample. Our method can be applied to actual urine samples from hypertension patients at different stages to study the level change of prostanoids and female sex hormones and identify potential biomarkers for hypertension development.

Our studies will benefit patients and clinical trial specialists by allowing a correlation of prostanoids and female sex hormones. Diagnosis of early stage of hypertension including pre-hypertension and Stage 1 hypertension is critical so that early medical intervention can be applied to prevent disease progression. In addition, studying the level changes of prostanoids and female sex hormones at different stages of hypertension will provide direct evidence to interpret molecular mechanism of disease progression to understand the pathogenesis of hypertension. Female hormones are known to affect blood pressure and are known to be potent anti-oxidant/anti-inflammatory agents, which can reduce overall inflammation.

3.2 Experimental

3.2.1 Chemicals and materials

Prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were purchased from Biomol (Plymouth Meeting, PA, USA). 11-dehydro-thromboxane B_2 (11-dehydro-TXB_2), 8-iso-$PGF_{2\alpha}$, 13,14-dihydro-15-keto-PGA_2, 13,14-dihydro-15-keto-PGE_2 and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-deoxy-PGJ_2) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Estrone (E_1), 17β-estradiol (E_2), estriol (E_3) and progesterone were purchased from Sigma Aldrich (St. Louis, MO, USA). 2-hydroxyestrone (2-OHE_1), 4-hydroxyestrone (4-OHE_1), 16α-hydroxyestrone (16α-OHE_1), and 2-methoxyestrone (2-$MeOE_1$) were purchased from Steraloids, Inc. (Newport, RI, USA). All prostanoid and sex hormone standards were used without further purification. HPLC-grade solvent water, acetonitrile and methanol and

reagent-grade 85% phosphoric acid were purchased from Fisher Scientific (Waltham, MA, USA). Figure 1 shows the chemical structures of the prostanoids and female sex hormones targeted by our method.

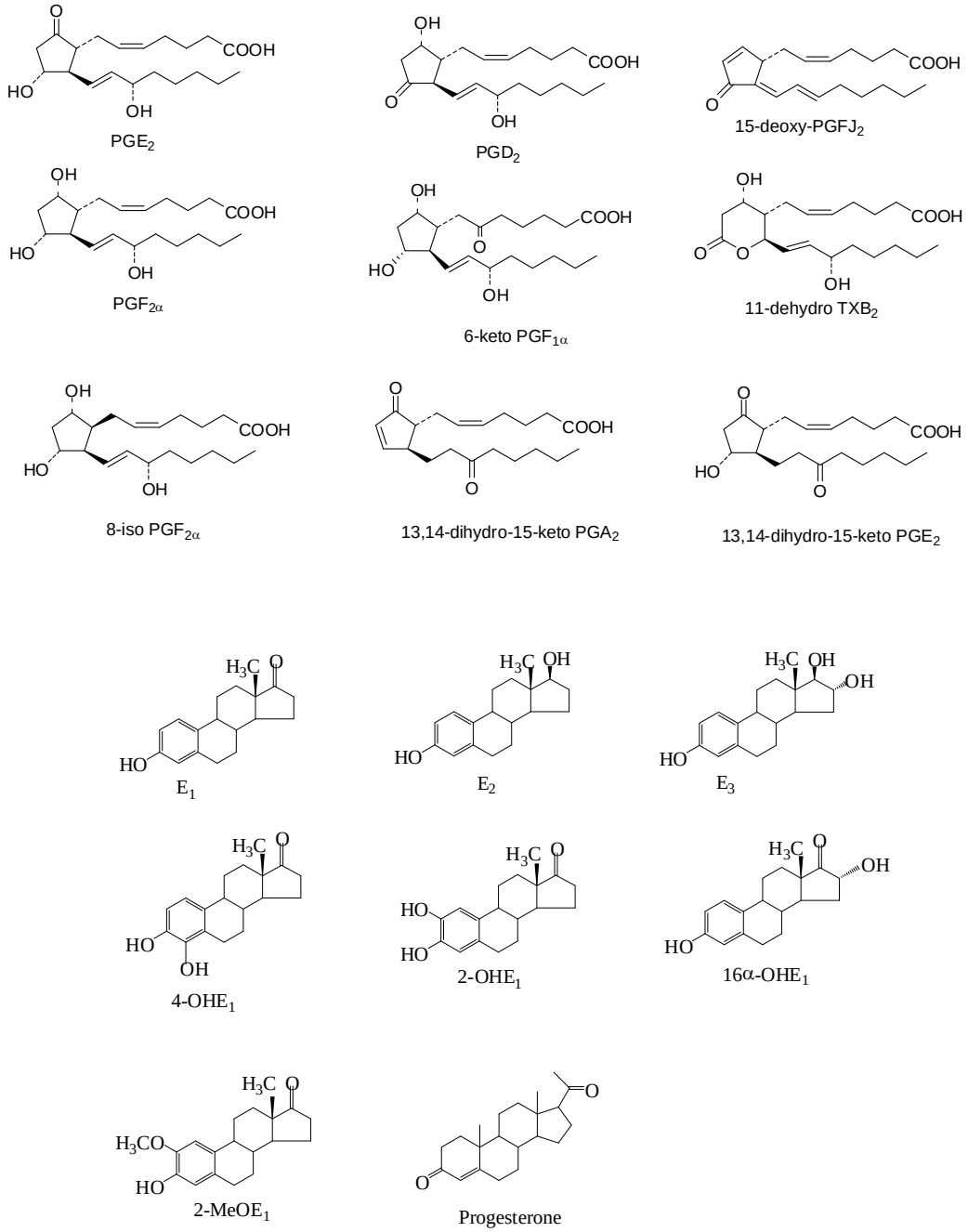


Figure 3.1 Structures of prostanoids and female sex hormones

3.2.2 Instrumental Setup

The high performance liquid chromatograph (HPLC) system was obtained from Jasco Inc. (Easton, MD, USA) with a manual injector (Rheodyne LLC, Rohnert Park, CA, USA). The HPLC system uses two Jasco pumps (PU-980) and a Jasco ultraviolet detector (UV-975). Data were collected with ChromNAV software. Analytes were separated on a Symmetry C18 4.6 × 250 mm column with 5-µm particle size (Waters Corp., Milford, MA, USA). The mobile phase used in this study was 17 mM phosphoric acid (solvent A) and acetonitrile (solvent B). The analytes were separated with a gradient elution at a flow rate of 1.3 mL/min. The gradient elution used is as follows: 34-35% B from 0 to 4 min, 35-45% B from 4 to 5.5 min, hold 45% B from 5.5 to 7 min, 45-32% B from 7 to 7.1 min, hold 32% from 7.1 to 9 min, 32-40% B from 9 to 10 min, hold 40% from 10 to 11 min, 40-85% B from 11 to 15 min, 85-70% B from 15 to 18 min, 70-34% B from 18 to 20 min. The detection wavelength was 196 nm. The total run time for the method was 20.0 min. Following run, five more minutes were needed to re-equilibrate the column. The separation was performed at room temperature with an injection volume of 100 µL.

3.2.3 Sample preparation

PGD_2, PGE_2, 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, E_1, E_2, E_3 and progesterone stock standard solutions were prepared by dissolving 1 mg solid sample into 1 mL methanol respectively to obtain a mass concentration of 1 mg/mL. All other standards were supplied as individual solutions by suppliers with the following concentrations: 11-dehydro-TXB_2 0.5 mg/mL, 8-iso-$PGF_{2\alpha}$ 1 mg/mL, 13,14-dihydro-15-keto-PGA_2 10 mg/mL, 13,14-dihydro-15-keto-

PGE_2 10 mg/mL, 15-deoxy-$\Delta^{12,14}$-PGJ_2 1mg/mL, 2-OHE_1 2 mg/mL, 4-OHE_1 2 mg/mL, 16α-OHE_1 2 mg/mL, and 2-$MeOE_1$ 5 mg/mL. Working standard solutions of each compound at 10 ng/μL were prepared by diluting the stock solutions with acetonitrile. Calibration standard solutions of the individual compounds were prepared by adding 17 mM phosphoric acid and acetonitrile (1:1 v/v) to various volumes of the working standard solutions to achieve a total volume of 500 μL. The concentrations of calibration standard solutions of prostanoids and progesterone are as follows: LOQ, 0.3, 0.8, 0.5, 1, 2, 4, 6, 8 and 10 ng/μL. The concentrations of calibration standard solutions of estrogens include LOQ, 0.05, 0.1, 0.2, 0.5, 1, 2, 3 and 4 ng/μL. Quality control (QC) samples with low, medium and high concentrations in the calibration range were prepared by dilution of working solutions with the mixture of 17 mM phosphoric acid and acetonitrile (1:1 v/v). Triplicate solutions were made at each concentration. All the solutions and quality control samples were stored in a freezer at −80 ◦C until use.

3.2.4 Urine sample preparation

The urine samples were prepared with solid state extraction according to the published procedures in Blewett's paper [15]. A urine sample was completely thawed at room temperature before solid state extraction. Approximately 100 μL of the thawed urine was pushed through a syringe filter with a pore size of 4.5 μm to remove large particles. The filtered urine was then transferred to a 2-mL polypropylene test tube, to which 200 μL of methanol with 0.01 M butylated hydroxytoluene (BHT) and 5 μL of formic acid were added. The mixture was mixed at room temperature with a touch mixer for 30 seconds. HPLC grade water was then added into the urine sample to reach a final volume of 2 mL.

An Oasis solid phase extraction cartridge was used for solid phase extraction. 2 mL 0.1 % formic acid (v/v), 2 mL methanol and 2 mL ethyl acetate were sequentially added to the Oasis cartridge for preconditioning. 2 mL diluted urine sample was then loaded on the cartridge column. 2 mL of 0.1% formic acid (v/v) and 2 ml of 10% methanol with 0.1% formic acid (v/v) were used to clean the column of unwanted compounds. The prostanoids and sex hormones were 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, and collected in a 2-mL polypropylene test tube. The eluent was dried under nitrogen at 0°C. 500 µL of methanol was added into the dry sample to re-dissolve.

3.2.5 Sample preparation for method validation

According to the FDA's guidance on validation of bioanalytical methods [16], method validation usually includes assessment of selectivity, sensitivity, calibration curve, accuracy, precision, recovery and stability of samples. The selectivity of the method was determined by analyzing the urine samples and the solvent blank. A comparison between calibration standards, urine samples and the solvent blank was used to quantify the method's selectivity. The sensitivity of the method is determined by the limit of detection (LOD) and limit of quantitation (LOQ). Based on serial dilution of the working solutions, the LOD was experimentally defined as the concentration of sample load which gave a signal-to-noise ratio of 3, while the LOQ gave signal-to-noise ratio 10. Accuracy was determined by comparing the calculated concentration of each analyte with the nominal concentration. Three sample preparations in 17 mM phosphoric acid and acetonitrile (1:1 v/v) at high, middle and low levels of the linear range were used to measure accuracy. Each

sample was injected three or four times. Recovery was estimated by spiking urine with standard samples at low, middle and high concentration levels. The relative standard deviation (R.S.D.) was used to measure precision. The intra-day precision was determined by analyzing 10 injections of the prostanoid and sex hormone mixture from three independent sample preparations at low, middle and high levels during the same day. The inter-day precision was determined by injecting the standard mixtures with low, middle and high levels on five consecutive days. Triplicate injections were done for each sample.

3.3 Results and discussion

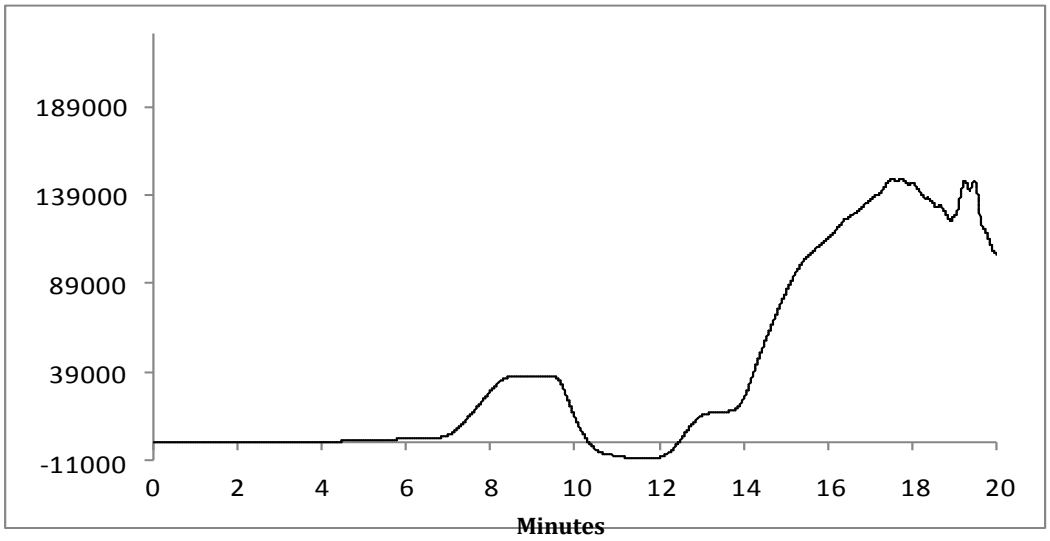
3.3.1 Development of HPLC-UV method

Nine prostanoids and eight female sex hormones were separated by a Symmetry C18 column (4.6mm×250 mm, 5 μm). A flow rate of 1.3 mL/min was chosen to achieve a reasonable separation time. Phosphoric acid was added into HPLC grade water to adjust the mobile phase pH to around 2.3 and improve the shape of the chromatography peak. A gradient elution was optimized to achieve a rapid separation within 20 minutes. Because of its simple operation, easy access in the laboratory and low maintenance cost, a UV detector was chosen to determine prostanoids and female sex hormones. Due to weak chromophore groups on prostanoids, a low wavelength of 196 nm was selected to detect all target compounds. The initial elution condition was set up at 34% acetonitrile to elute E_3 and 6-keto $PGF_{1\alpha}$ after 3.5 minutes to avoid solvent peak interference.

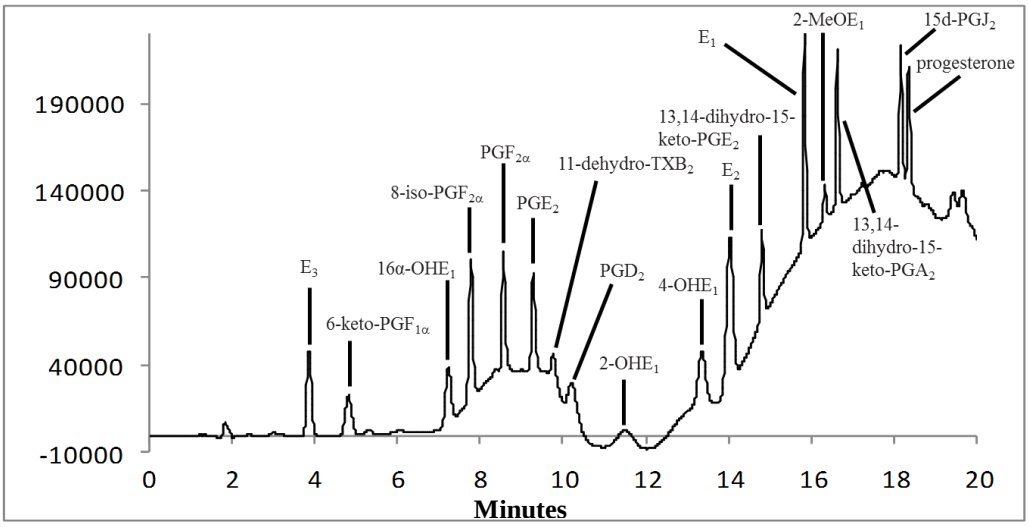
A representative chromatogram obtained from a standard mixture of nine prostanoids and eight female sex hormones is shown in Figure 3.2. The elution order was identified by injecting each individual standard. E_3 was eluted first, followed by 6-keto

$PGF_{1\alpha}$, 16α-OHE_1, 8-iso-$PGF_{2\alpha}$, $PGF_{2\alpha}$, PGE_2, 11-dehydro-TXB_2, PGD_2, 2-OHE_1, 4-OHE_1, E_2, 13,14-dihydro-15-keto-PGE_2, E_1, 2-$MeOE_1$, 13,14-dihydro-15-keto-PGA_2, 15-deoxy-PGJ_2 and finally progesterone. Under current gradient condition, all compounds were separated from each other with a reasonable resolution, where stereoisomers 8-iso-$PGF_{2\alpha}$ and $PGF_{2\alpha}$ were eluted at 7.74 min and 8.52 min, respectively; regioisomers PGE_2 and PGD_2 were eluted at 9.27 min and 10.17 min, respectively; 13,14-dihydro-15-keto-PGE_2, one of functional isomers of PGE_2 and PGD_2, was eluted at 14.81 min; and regioisomers 16α-OHE_1, 2-OHE_1, and 4-OHE_1 were eluted at 7.42 min, 12.23 min and 13.69 min, respectively.

(a)



(b)



(c)

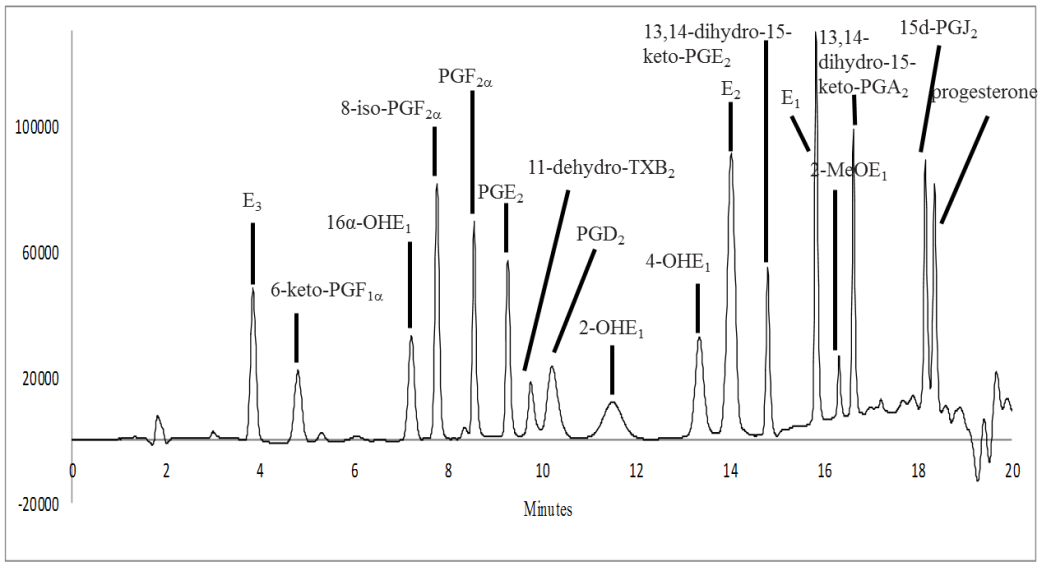


Figure 3.2 Representative chromatograms: (a) solvent blank; (b) standard mixture (c) standard mixture after subtracting solvent blank

3.3.2 Method validation

Selectivity

The selectivity of the method was tested by injecting methanol, water, acetonitrile and a mobile phase mixture of 17mM H_3PO_4 and acetonitrile (1:1 v/v), respectively. The results were compared with the chromatography from the standard mixture. There were no interference peaks showing up near the retention times of the prostanoids or female sex hormones. All peaks from the standard mixture were sharp and well-resolved with good symmetry. The peak parameters are listed in Table 3.1. The peaks of all compounds have resolutions equal to or higher than 1.5, which meets the separation requirement. The plate numbers of all compounds range between 3,556 and 361,009. The symmetry factor for each peak is within the range of 0.97 to 1.29.

Sensitivity

The sensitivity of the method was evaluated by the limit of quantification (LOQ) and limit of detection (LOD) for each individual compound, as defined above. The lowest LOQ has a RSD value lower than 5%. The solutions used to determine LOQ and LOD were prepared by serial dilution of working solutions. The LOD and LOQ of each compound is listed in Table 3.2. The LOQs of sex hormones are less than or equal to 5 ng except for 2-OHE_1 and progesterone at 20 ng; while the LOQs of the prostanoids are 20 ng except for $PGF_{2\alpha}$ and PGE_2 at 1 ng, 10 ng for 6-keto $PGF_{1\alpha}$ and 13,14-dihydro-15-keto-PGA_2, and 30 ng for 11-dehydro TXB_2. All LOQs and LODs are at the ng level. The LODs and LOQs indicate that our method is sensitive enough to analyze bioactive prostanoids and female sex hormones after enriching biological samples by solid phase extraction (SPE).

Compound	RT (min)	Plate No.	Resolution	Symmetry
E_3	3.85	5014	3.7	1.11
6-keto $PGF_{1\alpha}$	4.81	4251	8.9	1.01
16α-OHE_1	7.23	13657	2.6	1.00
8-iso $PGF_{2\alpha}$	7.77	32460	5.1	1.10
$PGF_{2\alpha}$	8.56	59935	4.8	1.23
PGE_2	9.28	52736	2.7	1.17
11-dehydro-TXB_2	9.77	35165	1.5	1.15
PGD_2	10.22	9961	2.2	1.29
2-OHE_1	11.50	3556	3.3	1.03
4-OHE_1	13.35	25139	2.1	1.07
E_2	14.03	33536	3.4	0.97
13,14-dihydro-15-keto-PGE_2	14.81	163010	7.8	1.17
E_1	15.83	287892	4.2	1.26
2-$MeOE_1$	16.33	311235	2.6	1.10
13,14-dihydro-15-keto-PGA_2	16.63	361009	13.0	1.24
15-deoxy-PGJ_2	18.16	333957	1.5	1.13
progesterone	18.36	290509	N/A	1.10

Table 3.1 Peak parameters of prostanoids and female sex hormones

Compound	LOD (ng)	LOQ (ng)	Linear range (ng)	Line equation	R^2
E_3	1	3	3-300	y = 497184x + 4805.3	1.000
6-keto $PGF_{1\alpha}$	3	10	10-1000	y = 138079x - 295.06	0.998
16α-OHE_1	3	5	5-250	y = 670764x - 5429.9	0.997
8-iso $PGF_{2\alpha}$	5	20	20-1000	y = 170680x - 21249	0.997
$PGF_{2\alpha}$	0.3	1	1-1000	y = 179481x + 29395	0.998
PGE_2	0.3	1	1-1000	y = 195950x + 10929	0.999
11-dehydro-TXB_2	5	30	30-1000	y = 155361x - 4146.2	0.999
PGD_2	10	20	20-1000	y = 186770x + 12455	0.997
2-OHE_1	5	20	20-400	y = 516084x - 76783	0.997
4-OHE_1	1	2	2-150	y = 857097x - 12447	0.997
E_2	2	5	5-400	y = 455003x - 9213.2	0.999
13,14-dihydro-15-keto-PGE_2	5	20	20-1400	y = 93711x + 20758	0.998
E_1	0.5	2	2-400	y = 439220x - 10107	1.000
2-$MeOE_1$	0.5	2	2-400	y = 507593x + 1439.7	1.000
13,14-dihydro-15-keto-PGA_2	3	10	10-1000	y = 106262x - 3655	0.999
15-deoxy-PGJ_2	5	20	20-1000	y = 186787x - 15676	1.000
progesterone	5	20	20-1300	y = 68288x + 6046.9	0.997

Table 3.2 Validation results for prostanoids and sex hormones: sensitivity and linearity

Linearity and range

Calibration curves for the prostanoids and sex hormones were generated by plotting peak area against concentration. The linear range was determined by serially diluting the working solution. The calibration standards were injected in triplicate. Calibration curves for all 17 compounds were linear over the tested concentration range. The linear equation, range and correlation coefficients R^2 of each analytical curve are shown in Table 3.2. All prostanoids ranged from LOQ to 1000 ng except 13,14-dihydro-15-keto-PGE_2 with a range from 20 ng to 1400 ng. E_1, E_2, 2-OHE_1 and 2-$MeOE_1$ ranged from LOQ to 400 ng, whereas E_3 ranged from LOQ to 300 ng, 16α-OHE_1 from LOQ to 250 ng, 4-OHE_1 from LOQ to 150 ng, and progesterone from LOQ to 1300 ng. The least-squares regression was chosen to construct the best fit to the linear calibration curve for each compound. The peak area for each compound was plotted against its nominal concentration to obtain a line equation given by Excel. The curves for all compounds were linear with R^2 greater than 0.997 in the tested concentration range.

Accuracy and Precision

The precision of the method was evaluated by determining the repeatability (intra-day assays) and the intermediate precision (inter-day assays). The working solutions were prepared by spiking mobile phase with 17 standards at three different concentration level (high, media and low level) in calibration curve range. Each solution was injected in triplicate or four times. Three solutions prepared at each concentration level were used to determine the intra-day precision. A total of 10 injections (n=10) were made in a single day. The inter-day precision was measured by injecting five solutions at each concentration

level (*n*=15) on five consecutive days. The precision results for all compounds are listed in Table 3.3. The R.S.D. values for intra-day and inter-day precisions at the three concentration levels fall within 0.95-12.58%, which indicates that the separation is reproducible.

Compound	Level 1				Level 2		
	Conc.	Intra-assay precision	Inter-assay precision		Conc.	Intra-assay precision	Inter-assay precision
	(ng/µL)	%R.S.D. (n=10)	%R.S.D. (n=15)		(ng/µL)	%R.S.D. (n=10)	%R.S.D. (n=15)
E_3	0.3	1.07	1.42		0.65	1.49	7.27
6-keto $PGF_{1\alpha}$	1	2.73	2.36		2.1	1.5	3.31
16a-OHE_1	0.3	6.17	3.38		0.75	3.02	6.54
8-iso $PGF_{2\alpha}$	1.2	6.24	2.59		3	2.54	2.81
$PGF_{2\alpha}$	1	2.23	1.7		2.5	1.29	3.49
PGE_2	1	1.31	1.52		2.5	1.15	3.19
11-dehydro TXB_2	1.5	1.56	1.9		3.5	2.01	3.38
PGD_2	1	2.64	9.89		2.5	2.08	8.88
2-OHE_1	0.4	3.77	12.58		1	5.77	11.99
4-OHE_1	0.25	4.53	5.39		0.5	3.93	8.8
E_2	0.3	6.16	1.74		0.75	3.99	5.9
13,14-dihydro-15-keto-PGE_2	1.2	1.55	2.46		3	1.38	3.37
E1	0.5	2.19	1.2		1.4	1.38	6.25
2-$MeOE_1$	0.4	1.09	2.16		1	1.4	7.2
13,14-dihydro-15-keto-PGA_2	1.2	2.25	2.36		2.8	1.76	5.24
15-deoxy-PGJ_2	1.2	1.03	1.91		2.6	1.45	3.36
Progesterone	0.8	2.02	4.33		2.2	3.04	5.9

Compound	Level 3		
	Conc.	Intra-assay precision	Inter-assay precision
	(ng/µL)	%R.S.D. (n=10)	%R.S.D. (n=15)
E_3	1.3	1.2	4.34
6-keto $PGF_{1\alpha}$	4.2	2.06	5.48
16a-OHE_1	1.5	1.56	4.55
8-iso $PGF_{2\alpha}$	6	1.22	4.29
$PGF_{2\alpha}$	5	1.27	4.78
PGE_2	5	1.25	3.81
11-dehydro TXB_2	7	1.94	4.2
PGD_2	5	1.73	10.79
2-OHE_1	2	1.66	6.21
4-OHE_1	1	2.17	6.65
E_2	1.5	2.19	4.84
13,14-dihydro-15-keto-PGE_2	6	1.24	3.85
E1	2.8	2.4	4.87
2-$MeOE_1$	2	1.26	5.17
13,14-dihydro-15-keto-PGA_2	5.6	1.34	2.97
15-deoxy-PGJ_2	5.2	0.95	3.67
Progesterone	4.4	1.92	5.05

Table 3.3 Validation results: inter-day and intra-day precision

Compound	Level 1			Level 2	
	Concentration (ng/µL)	Accuracy % (n=10)		Concentration (ng/µL)	Accuracy % (n=10)
E_3	0.3	86.94		0.65	89.12
6-keto $PGF_{1\alpha}$	1	86.90		2.1	109.94
16a-OHE_1	0.3	88.83		0.75	86.29
8-iso $PGF_{2\alpha}$	1.2	109.04		3	111.50
$PGF_{2\alpha}$	1	101.29		2.5	113.98
PGE_2	1	99.74		2.5	105.43
11-dehydro TXB_2	1.5	108.93		3.5	106.24
PGD_2	1	88.60		2.5	106.34
2-OHE_1	0.4	112.69		1	84.96
4-OHE_1	0.25	101.03		0.5	105.32
E_2	0.3	87.50		0.75	93.99
13,14-dihydro-15-keto-PGE_2	1.2	97.43		3	108.26
E1	0.8	93.89		1.4	90.22
2-$MeOE_1$	0.4	103.83		1	91.47
13,14-dihydro-15-keto-PGA_2	1.2	102.16		2.8	110.88
15-deoxy-PGJ_2	1.2	104.53		2.6	114.13
Progesterone	0.8	97.24		2.2	92.00

Compound	Level 3	
	Concentration (ng/µL)	Accuracy % (n=10)
E_3	1.3	103.88
6-keto $PGF_{1\alpha}$	4.2	98.40
16a-OHE_1	1.5	103.45
8-iso $PGF_{2\alpha}$	6	100.95
$PGF_{2\alpha}$	5	102.19
PGE_2	5	94.81
11-dehydro TXB_2	7	97.55
PGD_2	5	95.83
2-OHE_1	2	109.08
4-OHE_1	1	109.63
E_2	1.5	103.25
13,14-dihydro-15-keto-PGE_2	6	97.38
E1	2.8	97.94
2-$MeOE_1$	2	101.31
13,14-dihydro-15-keto-PGA_2	5.6	97.48
15-deoxy-PGJ_2	5.2	97.78
Progesterone	4.4	108.99

Table 3.4 Validation results: accuracy

The accuracy of the method was evaluated by measuring 10 injections from three solutions prepared at each concentration level. The calculated concentrations for each compound were then compared to the theoretical concentration to determine the accuracy for each compound. Accuracy results are listed in Table 3.4. All compounds have accuracy within 84.86-114.13%, which indicates that the method is sufficiently accurate to quantify concentrations of prostanoids and sex hormones.

Solution stability

During the process of method development, all working and stock solutions were stored in a freezer at -80°C prior to use. Before injection, the tested solutions were taken out to thaw at room temperature and then stored in ice. Therefore, it is important to evaluate whether the tested solutions are stable over the duration of the analysis. Solution stability was tested by quality control solutions through three freeze and thaw cycles, based on FDA bioanalytical guidance [16]. Three solutions at each of the low and high concentrations were prepared and stored in a freezer at -80°C for 24 hours, and naturally thawed at room temperature. After being completely thawed, the solutions were refrozen for 24 hours at -80°C. The freeze–thaw cycle was repeated two more times. On the third cycle, each solution was injected in triplicate. The results of the 9 injections were combined to calculate the R.S.D. at each concentration level, listed in Table 3.5. The R.S.D. values for all compounds were less than 5% at both low and high concentration levels, with the exception of 5.36% for progesterone at the low concentration level and 8.65% for E_1 at the high concentration level. The results indicate that our solutions were stable during the analysis period.

Compound	Level 1			Level 2	
	Concentration (ng/µL)	precision %R.S.D. (n=9)		Concentration (ng/µL)	precision %R.S.D. (n=9)
E_3	0.30	4.12		1.3	2.75
6-keto $PGF_{1\alpha}$	1.00	4.03		4.2	2.18
16a-OHE_1	0.30	3.44		1.5	2.47
8-iso $PGF_{2\alpha}$	1.20	3.49		6.0	2.43
$PGF_{2\alpha}$	1.00	1.98		5.0	2.15
PGE_2	1.00	1.58		5.0	1.99
11-dehydro TXB_2	1.50	3.34		7.0	2.41
PGD_2	1.00	3.09		5.0	2.52
2-OHE_1	0.40	2.43		2.0	3.12
4-OHE_1	0.25	4.08		1.0	3.60
E_2	0.30	4.92		1.5	3.26
13,14-dihydro-15-keto-PGE_2	1.20	2.21		6.0	2.12
E_1	0.80	2.29		2.8	8.65
2-$MeOE_1$	0.40	1.26		2.0	2.66
13,14-dihydro-15-keto-PGA_2	1.20	1.50		5.6	2.55
15-deoxy-PGJ_2	1.20	1.91		5.2	2.11
Progesterone	0.80	5.36		4.4	2.47

Table 3.5 Validation results: solution stability

Recovery

Recovery was estimated by spiking urine with standard samples at low, middle and high concentration levels. The results were listed in Table 3.6. All the compounds fall within 85.4%-114.6%, which indicated that the method is suitable to measure the level of prostanoids and sex hormones in urine.

Compound	Level 1			Level 2	
	Concentration (ng/µL)	Accuracy % (n=3)		Concentration (ng/µL)	Accuracy % (n=3)
E_3	0.3	90.27		0.75	110.90
6-keto $PGF_{1\alpha}$	1.5	109.80		2.00	99.53
16a-OHE_1	0.3	88.85		0.75	100.80
8-iso $PGF_{2\alpha}$	0.9	89.45		2.50	105.20
$PGF_{2\alpha}$	0.6	106.60		2.50	106.10
PGE_2	0.6	105.10		2.30	94.48
11-dehydro TXB_2	1.0	110.10		3.20	103.50
PGD_2	0.4	95.16		1.60	85.43
2-OHE_1	0.3	108.10		0.90	107.10
4-OHE_1	0.1	94.39		0.45	93.51
E_2	0.4	103.10		1.00	106.90
13,14-dihydro-15-keto-PGE_2	1.2	109.40		3.25	114.20
E1	0.5	99.52		1.15	112.50
2-$MeOE_1$	0.4	100.10		1.00	104.60
13,14-dihydro-15-keto-PGA_2	1.0	107.70		2.50	114.60
15-deoxy-PGJ_2	1.2	87.54		2.50	97.02
Progesterone	1.2	98.67		2.50	99.62

Compound	Level 3	
	Concentration (ng/µL)	Accuracy % (n=3)
E_3	1.5	90.72
6-keto $PGF_{1\alpha}$	4.0	94.32
16a-OHE_1	1.5	85.79
8-iso $PGF_{2\alpha}$	5.0	102.00
$PGF_{2\alpha}$	5.0	107.60
PGE_2	4.6	91.43
11-dehydro TXB_2	5.2	106.36
PGD_2	3.2	107.39
2-OHE_1	1.8	94.72
4-OHE_1	0.9	91.80
E_2	2.0	86.55
13,14-dihydro-15-keto-PGE_2	6.5	103.10
E_1	2.3	90.59
2-$MeOE_1$	1.8	93.71
13,14-dihydro-15-keto-PGA_2	5.0	103.39
15-deoxy-PGJ_2	5.0	94.97
Progesterone	5.0	93.14

Table 3.6 Validation results: recovery by spiking urine at low, middle and high concentration levels

3.3.3 Application of method to urine samples

The method was applied to seven urine samples collected from both male and female African Americans with body mass index of 30.5 ± 1.1 kg/m^2 at age 58 ± 2.3 years, having prehypertension or stage 1 hypertension (143 ± 7 / 87 ± 7 mmHg) before and after special 50-minute exercise training. None of patients was under antihypertensive medication. In our study, the urine samples from patients with a lower stage hypertension were investigated in order to see if the biomarkers were present even at low stages. By comparing the peak retention times between the urine samples and the standard mixture, several compounds including E_3, 6-keto $PGF_{1\alpha}$, 16α-OHE_1, 8-iso $PGF_{2\alpha}$, $PGF_{2\alpha}$, PGD_2, 2-OHE_1, E_1, 2-$MeOE_1$ and 15-deoxy-PGJ_2 were identified in most of the urine samples. Figure 3 shows a chromatograph obtained from a urine sample. In our results, five compounds including 6-keto $PGF_{1\alpha}$, 8-iso $PGF_{2\alpha}$, PGD_2, E_1 and PGE_2 were found in both male and female urine. The preliminary results show that after exercise, the level of 6-keto $PGF_{1\alpha}$ decreased for both men and women. However, the results suggest that response is gender dependent. The level change of 8-iso $PGF_{2\alpha}$, PGD_2 and E_1 in man decreased after exercise, which is opposite to the changes observed in woman. The PGE_2 affect was somewhat ambiguous. This study shows the need to extend the analysis to a greater population of patients to better understand the response of PGE_2 to exercise. It is interesting that exercise appears to induce woman to produce more E_1, but decrease the level of E_1 in man.

The above sample analysis shows that the method is capable of determining trace levels of bioactive prostanoids and sex hormones in urine samples after enrichment with the SPE method.

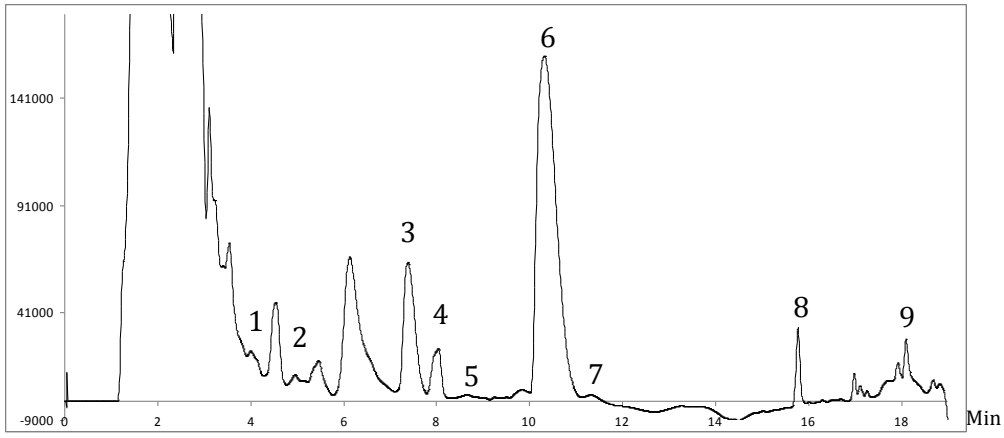


Figure 3.3 Chromatogram of a urine sample: 1. E_3; 2. 6-keto-$PGF_{1\alpha}$; 3. 16α-OHE_1; 4. 8-iso-$PGF_{2\alpha}$; 5. $PGF_{2\alpha}$; 6. PGD_2; 7. 2-OHE_1; 8. E_1; 9. 15d-PGJ_2;

3.4 Discussion

The compounds in our method are bioactive with specific physiological effects on the human body. Some of them are stereoisomers, regioisomers or functional isomers. Their closely related structures present a challenge to achieving simultaneous separation in a single run. Moreover, prostanoids have weak UV chromophores due to a lack of conjugated π-π bonds, which limit the choice of detection wavelengths for the combination of prostanoids and sex hormones. In our method, wavelengths in the low UV, such as 196 nm, had to be chosen to achieve sufficient absorbance from prostanoids at a relative low concentration. We are able to use this wavelength because the detector used in this method has extremely good response at low wavelength. Fortunately, 196 nm also works for sex hormones. Phosphoric acid was added into water with a concentration 17 mM (pH = 2.3) to improve peak shape and modify background absorbance. By adjusting the percentage of acetonitrile in the mobile phase and developing a gradient elution, we finally succeeded in

separating 17 compounds with closely-related structures within 20 minutes. UV detectors are generally less sensitive than mass spectrometers, but are easier to find in most laboratories. The LOQ and LOD values in our method indicate that HPLC with a UV detector can be applied to measure bioactive prostanoids and sex hormones in urine or other biological samples after SPE purification and enrichment. In our method, all compounds were well-resolved by chromatography. The method can be easily adapted to other instruments, such as LC-MS or UPLC by replacing phosphoric acid with formic acid [17].

Examining the structures of the compounds in Figure 1, it is interesting to note that the number and positions of the hydroxyl and ketone substitute groups affect the retention times of the compounds. Among the prostanoids, the retention time of 6-keto $PGF_{1\alpha}$ is lowest because the OH group on the carbon chain is very close to the ketone group on another side carbon chain. 8-iso $PGF_{2\alpha}$ and $PGF_{2\alpha}$ have almost the same chemical structures, but 8-iso $PGF_{2\alpha}$ with its cis bond connection is eluted earlier than $PGF_{2\alpha}$. Compared with $PGF_{2\alpha}$, replacing the OH group with a ketone group on the five-carbon ring increases the retention time of PGE_2. When a ketone group on the five-carbon ring is on the same side as the OH group on the side carbon chain, as in PGD_2, the retention time of PGD_2 increases. 11-dehydro TXB_2 introduces a carboxylic group to form a six-member ring, and is eluted later than PGE_2. Compared with 13,14-dihydro-15-keto PGA_2, 13,14-dihydro-15-keto PGE_2 has one OH group on the five carbon ring; therefore the latter is eluted earlier. The last eluted compound, 15d-PGJ_2, is the least polar due to the loss of one ketone group on the side carbon chain compared with 13,14-dihydro-15-keto PGA_2.

Careful observation of the structures the of sex hormones reveals that the same rule regarding OH and ketone substitute groups can be applied to these compounds. E_3 has the largest number of OH groups (three) on the backbone among the sex hormones in this study, and was therefore eluted first. As the number of OH groups decreases, the retention time of the compounds increases. Therefore, the single-OH-group compounds E_1 and 2-$MeOE_1$ are eluted later than the double-OH-group compounds including 16α-OHE_1, 2-OHE_1, 4-OHE_1 and E_2. Progesterone, without any OH group, is eluted last. With the same number of OH groups, 16α-OHE_1, 2-OHE_1 and 4-OHE_1 with a ketone group substituted into backbone are eluted earlier than E_2. The substitution position of the OH group affects the elution order of 16α-OHE_1, 2-OHE_1 and 4-OHE_1.

A literature search was performed to assess the progress of separation and analysis on the combination of prostanoids and female sex hormones. There were few papers on this topic. The challenge of separating the combination was considered a serious problem. Our method establishes a simple validated method for simultaneously analyzing both groups of inflammatory biomarkers within a short time. We can directly measure changes in the levels of members of both groups in biological samples such as urine to study the interaction between prostanoids and sex hormones as disease progresses. Using our method, we can determine the balance between the inflammatory mediators among the prostanoids and the self-protective effects of sex hormones, which will provide insight into the molecular mechanisms of disease development.

3.5 Conclusions

In this study, we have developed a simple, sensitive, accurate and rapid HPLC-UV method for the separation of the bioactive compound combination of nine prostanoids and eight female sex hormones. The method has been applied to simultaneously quantify endogenous prostanoids and female sex hormones in urine samples from hypertension patients. The levels of prostanoids and female sex hormones were affected by exercise training and recovery time period after the training period.

In a summary, there are several advantages in our method including quantifying seventeen important bioactive compounds with closely related structures, eliminating complex derivatization steps, completely separating the mixture in only twenty-five minutes in a single run, and being easily adaptable to different analytical instruments such as LC-MS or UPLC. A future research task will be to apply this method to various clinical biological fluids and tissues to study the proportions of prostanoids and female sex hormones present in different stages of disease, which will provide useful data to elucidate their roles in disease development.

3.6 References

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CHAPTER 4

DEVELOPMENT AND VALIDATION OF A METHOD FOR SIMULTANEOUS DETERMINATION OF THE COMBINATION OF PROSTANOIDS AND FEMALE SEX HORMONES WITH LC-MS

4.1. Introduction

In our study, we work on separating and quantifying a combination of inflammatory mediators, prostanoids, and female sex hormones by using high performance liquid chromatography (HPLC) with electrospray ionization mass spectrometry (MS). LC-MS with its high sensitivity and wide availability to most laboratories has become a favored instrument to measure bioactive molecules in pharmaceutical industry and clinical research [1-3].

In this chapter, we present a method for simultaneously quantifying the combination of prostanoids and female sex hormones by using LC-ESI-MS in both negative and positive modes. Figure 4.1 shows the chemical structures of seventeen studied bioactive molecules and two deuterated internal standards in our method.

4.2 Experimental

4.2.1 Chemicals and materials

Prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), 6-keto-prostaglandin F1α (6-keto-$PGF_{1\alpha}$), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were purchased from Biomol (Plymouth Meeting, PA, USA). 11-dehydro-thromboxane B_2 (11-dehydro-TXB_2), 8-iso-$PGF_{2\alpha}$, 13,14-dihydro-15-keto-PGA_2, 13,14-dihydro-15-keto-PGE_2 and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-deoxy-PGJ_2) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Estrone (E_1), 17β-estradiol (E_2), estriol (E_3) and progesterone were purchased from Sigma Aldrich

(St. Louis, MO, USA). 2-hydroxyestrone (2-OHE_1), 4-hydroxyestrone (4-OHE_1), 16α-hydroxyestrone (16α-OHE_1), and 2-methoxyestrone (2-$MeOE_1$) were purchased from Steraloids, Inc. (Newport, RI, USA). The deuterated PGE_2 (3,3,4,4-$^2H_4$-PGE_2) used as internal standards for prostanoids was purchased from Cayman Chemical (Ann Arbor, MI, USA). The deuterated β-Estradiol (1,3,5 (10)-Estratriene-2,4-d_2-3,17β-diol) (98 atom % D) used as internal standards for female sex hormones was purchased from Sigma–Aldrich (St. Louis, MO, USA). All prostanoid and female sex hormone standards were used without further purification. HPLC-grade solvent water, acetonitrile, methanol, ethyl acetate and formic acid were purchased from Fisher Scientific (Waltham, MA, USA). Butylated hydroxytoluene (BHT) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Figure 1 shows the chemical structures and molecular weight of the prostanoids, female sex hormones and deuterated internal standards targeted by our method.

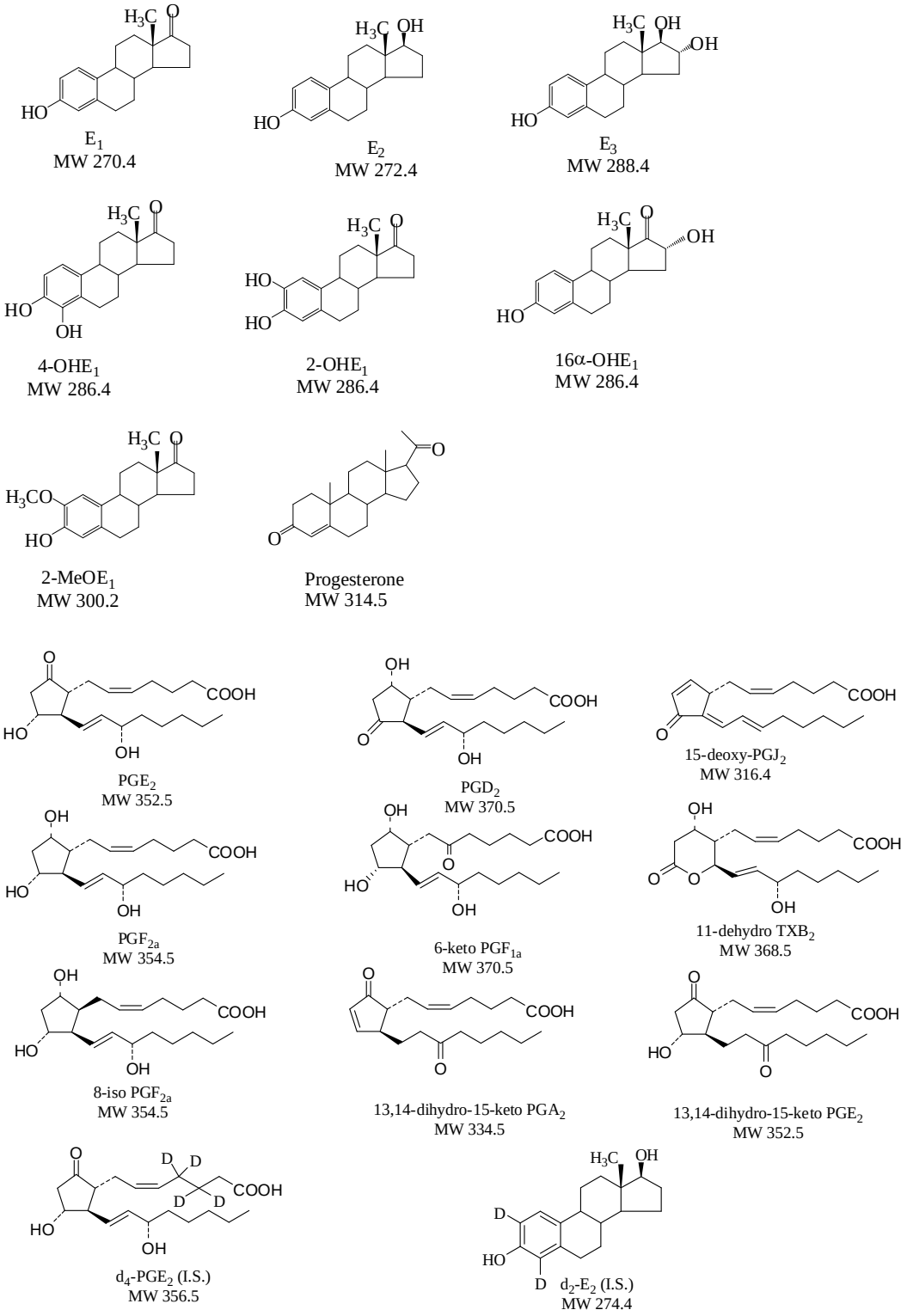


Figure 4.1 Structures of prostanoids, female sex hormones and deuterated internal standards

4.2.2 Instrumentation and Method Parameters

The high performance liquid chromatograph (HPLC) system in the study was an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA) with a binary pump, in-line degasser, and a manual injector (Rheodyne LLC, Rohnert Park, CA, USA). The HPLC was coupled to an Agilent G1946 single quadrapole mass selective detector (MSD). Data were collected with ChemStation software. Analytes were separated on a Symmetry C18 4.6 × 250 mm column with 5-μm particle size (Waters Corp., Milford, MA, USA). The mobile phase used in this study was a mixture of acetonitrile with 0.1% formic acid (v/v) (solvent B) and water with 0.1% formic acid (v/v) (solvent A). The analytes were separated with a gradient elution at a flow rate of 1.3 mL/min. The gradient elution used is as follows: 34-35% B from 0 to 4 min, 35-45% B from 4 to 5.5 min, hold 45% B from 5.5 to 7 min, 45-32% B from 7 to 7.1 min, hold 32% from 7.1 to 9 min, 32-40% B from 9 to 10 min, hold 40% from 10 to 11 min, 40-85% B from 11 to 15 min, 85-70% B from 15 to 18 min, 70-34% B from 18 to 20 min. The total run time for the method was 20.0 min. Following the run, five more minutes were needed to re-equilibrate the column. The separation was performed at room temperature with an injection volume of 20 μL. Electrospray ionization (ESI) in the positive ion mode was used as the ionization source for the measurement of prostanoids, and in the negative ion mode for the measurement of female sex hormones. Nitrogen was used as the nebulizer gas and was maintained at a flow rate of 12.0 L/min with a nebulizer pressure of 45 psi. The gas temperature was set at 350 ^{o}C and the capillary voltage for positive mode was 5500V and for negative mode 4000 V. The fragmentor voltage was optimized for each compound, and listed in Table 4.1. The gain was set up at 2.0. In order to improve sensitivity, a time gradient with the parameters

listed in Table 4.2 was used to collect chromatograms in selective ion mode.

Positive ESI mode		Negative ESI mode	
Compound	Fragmentor voltage (V)	Compound	Fragmentor voltage (V)
E_3	170	6-keto $PGF_{1\alpha}$	110
16α-OHE_1	150	8-iso $PGF_{2\alpha}$	110
2-OHE_1	150	$PGF_{2\alpha}$	110
4-OHE_1	150	PGE_2	90
E_2	130	11-dehydro TXB_2	110
E_1	150	PGD_2	90
2-methoxy E_1	150	13,14-PGE_2	110
progesterone	150	13,14-PGA_2	110
		15-deoxy-PGJ_2	90

Table 4.1 Fragmentor voltages for prostanoids and female sex hormones

Positive ESI mode			Negative ESI mode		
Time (min)	m/z	Ions monitored	Time (min)	m/z	Ions monitored
0.00	289	E_3	0.00	369	6-keto $PGF_{1\alpha}$
5.50	287	16α-OHE_1	6.00	351	PGE_2, PGD_2
10.00	273	E_2		353	8-iso $PGF_{2\alpha}$, $PGF_{2\alpha}$
	275	d_2-E_2		355	d_4-PGE_2
	287	2-OHE_1, 4-OHE_1		367	11-dehydro-TXB_2
14.50	271	E_1	12.50	351	13,14-PGE_2
	301	2-methoxy E_1	15.50	315	15-deoxy-PGJ_2
17.00	315	progesterone		333	13,14-PGA_2

Table 4.2 SIM time gradient for LC-MS analysis

4.2.3 Sample preparation

PGD_2, PGE_2, 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, E_1, E_2, E_3 and progesterone stock standard solutions were prepared by dissolving 1 mg solid sample into 1 mL methanol respectively to obtain a stock concentration of 1 mg/mL. All other standards were supplied as individual solutions by suppliers with the following concentrations: 11-dehydro-TXB_2 0.5 mg/mL, 8-iso-$PGF_{2\alpha}$ 1 mg/mL, 13,14-dihydro-15-keto-PGA_2 10 mg/mL, 13,14-dihydro-15-keto-PGE_2 10 mg/mL, 15-deoxy-$\Delta^{12,14}$-PGJ_2 1mg/mL, 2-OHE_1 2 mg/mL, 4-OHE_1 2 mg/mL, 16α-OHE_1 2 mg/mL, and 2-$MeOE_1$ 5 mg/mL. The stock solution I consisting of PGD_2, PGE_2, 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$, 13,14-dihydro-15-keto-PGA_2, 13,14-dihydro-15-keto-PGE_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2 and 11-dehydro-TXB_2 (10 ng/uL except 11-dehydro-TXB_2 20 ng/uL) was made in methanol. The stock solution II consisting of E1 and its metabolites including 2-OHE_1, 4-OHE_1, 16α-OHE_1 and 2-methoxyE_1 at 10 ng/uL, E_2 at 50 ng/uL, E_3 at 50 ng/uL, and progesterone at 2 ng/uL was made in methanol. The internal standard stock solution III consisting of d_4-PGE_2 at 2.5 ng/uL was made in methanol. The stock solution IV consisting of d_2-E_2 at 10 ng/uL was made in methanol. The working solutions of prostanoids was made by adding different amount of Stock solution I to create the necessary concentrations in methanol. The working solutions of female sex hormones was made by serial dilution of Stock solution II in methanol. The internal standard working solution of d_4-PGE_2 0.5 ng/uL was made by diluting stock solution III in methanol. The working solution of d_2-E_2 at 2 ng/uL was prepared by diluting stock solution IV in methanol. The calibration curve was made based on the results obtained from injecting 20 uL working solutions of prostanoids with 1 ng d_2-PGE_2 and 20 uL working solutions of female sex hormones with 4 ng d_2-E_2, respectively. Quality control (QC) samples with low

and high concentrations in the calibration range were prepared by dilution of Stock Solutions I and II with methanol, respectively. Triplicate solutions were made at each concentration. All the solutions and quality control samples were stored in a freezer at −80 ^{o}C until use.

4.2.4 Recovery samples for method validation

The urine sample was filtered by a syringe filter with 0.45 μm pore size. The filtered urine was spiked with standard samples at low, middle and high concentration levels. Each sample was injected in triplicate. Recovery was calculated by diving the calculated concentration with the nominal concentration.

4.2.5 Urine sample preparation

The urine samples were pretreated with solid state extraction according to the published method in Blewett's paper [4]. A urine sample was completely thawed at room temperature before solid state extraction. Approximately 500 μL of the thawed urine was pushed through a syringe filter with a pore size of 4.5 μm to remove large particles. The filtered urine was then transferred to a 2-mL polypropylene test tube, to which 200 μL of methanol with 0.01 M butylated hydroxytoluene (BHT) and 5 μL of formic acid were added. The mixture was mixed at room temperature with a touch mixer for 30 seconds. HPLC grade water was then added into the urine sample to reach a final volume of 2 mL. An Oasis solid phase extraction cartridge was used for solid phase extraction. 2 mL 0.1 % formic acid (v/v), 2 mL methanol and 2 mL ethyl acetate were sequentially added to the Oasis cartridge for preconditioning. 2 mL diluted urine sample was then loaded on the

cartridge column. 2 mL of 0.1% formic acid (v/v) and 2 ml of 10% methanol with 0.1% formic acid (v/v) were used to clean the column of unwanted compounds. The prostanoids and sex hormones were 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, and collected in a 2-mL polypropylene test tube. 10 uL of an internal standard solution containing d_4-PGE_2 at 0.5 ng/uL and d_2-E_2 at 2 ng/uL was added into the eluent, and the resultant solution was evaporated to dryness under nitrogen at 0°C. 100 μL of methanol was used to re-dissolve the dry sample.

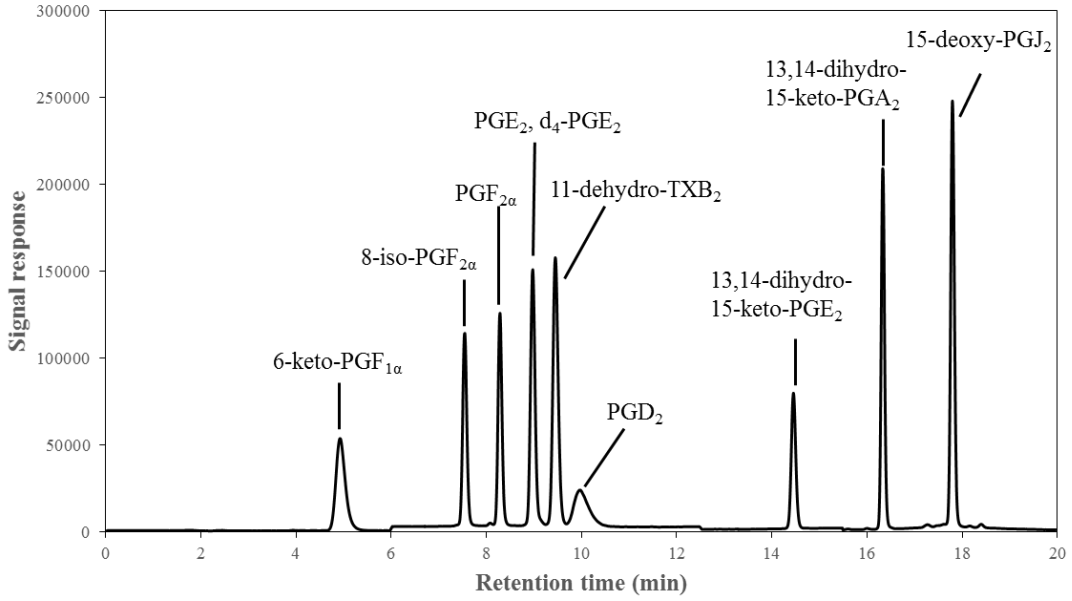
4.3 Results and discussion

4.3.1 Development of the LC-MS method

The separation method for quantifying the combination of prostanoids and female sex hormones by using LC-MS was adapted from the method with HPLC-UV discussed in the previous chapter. However, the mobile phase modifier phosphoric acid in the HPLC-UV method was a nonvolatile acid, which would result in salt deposition in the LC-MS instrument. Therefore, formic acid (FA) was chosen for providing protons and adjusting the pH of the mobile phase in the LC-MS method. The HPLC-grade water and acetonitrile containing 0.1% formic acid was used as mobile phase. Compared with the HPLC method, the replacement of the acid modifier in the mobile phase did not change the elution order of the analytes. The analytical Symmetry C18 column (4.6 mm × 250 mm, 5 μm) was used to separate the combination of prostanoids and female sex hormones. The separation with a satisfactory resolution was done within 20 minutes. The mass spectrometric detection was performed on both positive and negative ion modes with atmospheric pressure electrospray ionization. In positive ion mode, good signal response was obtained from

female sex hormones, their metabolites and 15d-PGJ_2. However, there was no detector response for prostanoids. The pseudo molecular ion of analytes was detected as $[M+H]^+$. In negative ion mode, prostanoids, 2-OHE_1 and 4-OHE_1 showed excellent detector response, and pseudo molecular ion of the analytes was detected as $[M–H]^-$. By compromising the availability of deuterated internal standards and the cost of the method, all female hormones and their metabolites were measured in the positive mode by using d_2-E_2 as internal standard, and all prostanoids in the negative mode by using d_4-PGE_2 as internal standard. In order to improve the sensitivity, the spray chamber parameters including fragmentor voltage, capillary voltage, drying gas flow, nebulizer gas pressure and gain were optimized. The mixture of prostanoids at 1 ng/uL was detected in the negative mode to optimize, and the mixture of female sex hormones at 1 ng/uL in the positive mode. The fragmenor voltages of analytes with best detector response were chosen as a final optimization condition as shown in Table 4.1. The representative LC/MS chromatogram for separation of 9 prostanoids with d_2-PGE_2 in the negative mode and 8 female sex hormones with d_2-E_2 in the positive mode is shown in Fig. 4.2 (a) ad (b), respectively.

(a)



(b)

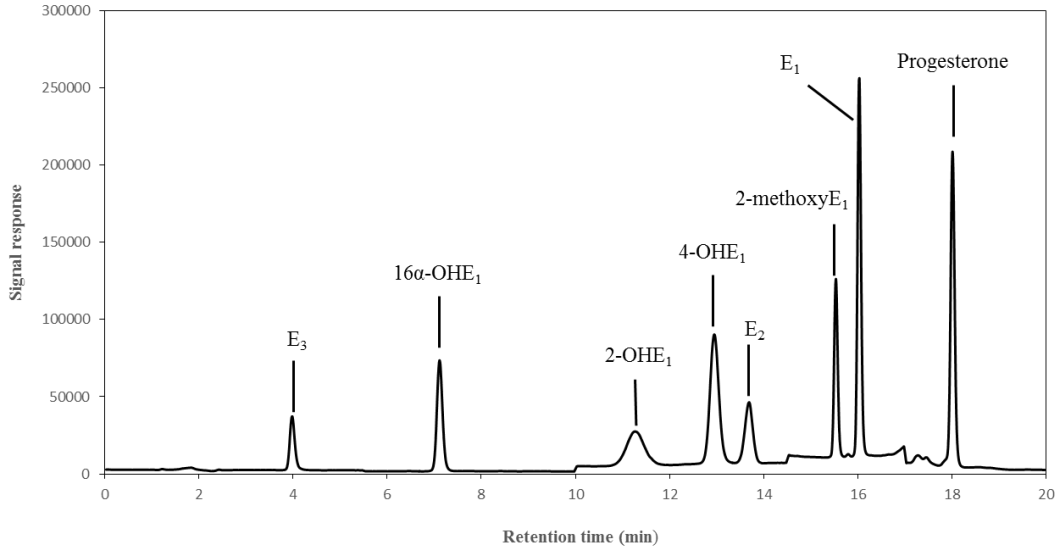


Fig. 4.2 Representative LC–MS chromatogram (a) postanoids in the negative ionization mode; (b) female sex hormones in the positive ionization mode

4.3.2 Method validation

4.3.2.1 Selectivity

The selectivity of the method was tested by injecting methanol, water, acetonitrile, a mobile phase mixture of 0.1% formic acid solution and acetonitrile (1:1 v/v), and elution solvent, respectively. The results were compared with the chromatography from the standard mixture. There were no interference peaks showing up near the retention times of the prostanoids or female sex hormones. All peaks from the standard mixture were sharp and well-resolved with good symmetry.

4.3.2.2 Sensitivity

The sensitivity of the method was evaluated by the limit of quantification (LOQ) and limit of detection (LOD) for each individual compound. The LOD is defined as three times the signal noise ratio, where LOD ten times the signal noise ratio. The lowest LOQ has an RSD value lower than 5%. The solutions used to determine LOQ and LOD were prepared by serial dilution of working solutions. The LOD and LOQ of each compound is listed in Table 4.3. Progesterone has the lowest LOQ equal to 3.6 pg, 16α-OHE_1, 4-OHE_1, E_1 and 2-methoxy E_1 with a LOQ equal to 20 pg, and LOQ of E_3 is equal to 100 pg, 2-OHE_1 240 pg and E2 500 pg. The LOQs of prostanoids are in the range between 15 to 40 pg except PGD_2 100 pg, and 13,14-dihydro-15-keto-PGA_2 0.6 pg. All LOQs and LODs are at the pg level. The LODs and LOQs indicate that our method is sensitive enough to analyze bioactive prostanoids and female sex hormones after enriching biological samples by solid phase extraction (SPE).

Compound	LOD	LOQ	Linear rang	Linear Equation	Weighting factor	R
	pg	pg	pg			
E_3	32	100	100.0-50,000	y = 3.8449x +0.0310	$1/x^2$	0.970
16α-OHE_1	6	20	20.0-10,000	y = 35.9148x + 0.01489	$1/y^2$	0.994
2-OHE_1	80	240	240.0-20,000	y = 30.5790x + 0.1983	$1/x^{0.5}$	1.000
4-OHE_1	6	20	20.0-10,000	y = 65.0100x + 0.26989	$1/y^2$	0.986
E_2	200	500	500.0-200,000	y = 4.3755x + 0.04701	$1/x^2$	0.994
E_1	6	20	20.0-10,000	y = 35.3538x + 0.03840	$1/x^2$	0.997
2-methoxyE_1	6	20	20.0-10,000	y = 77.3171x + 0.0156	$1/y^2$	0.991
progesterone	1.2	3.6	3.6-2,000	y = 319.8373x + 0.0343	$1/x^{0.5}$	0.999
6-keto $PGF_{1\alpha}$	6	20	20.0-20,000	y = 33.5742x + 0.05340	$1/x^2$	0.992
8-iso $PGF_{2\alpha}$	6	20	20.0-20,000	y = 31.1044x + 0.02691	$1/x^2$	0.997
$PGF_{2\alpha}$	6	20	20.0-20,000	y = 32.3939x + 0.02830	$1/x^2$	0.997
PGE_2	6	20	20.0-20,000	y = 42.7386x + 0.02930	$1/x^2$	0.997
11-dehydro-TXB_2	14	40	40.0-40,000	y = 28.6523x + 0.01812	$1/x^2$	0.996
PGD_2	40	100	100.0-20,000	y = 19.2161x + 0.00877	$1/x^2$	0.991
13,14-dihydro-15-keto-PGE_2	9	24	24.0-20,000	y = 24.0484x + 0.00990	$1/x^2$	0.997
13,14-dihydro-15-keto-PGA_2	0.2	0.6	0.6-20,000	y = 50.0518x + 0.07129	$1/x^2$	0.994
15-deoxy-PGJ_2	4	15	15.0-20,000	y = 66.3746x + 0.06252	$1/x^2$	0.990

Table 4.3 Validation results for prostanoids and sex hormones: sensitivity and linearity

4.3.2.3 Linearity and range

The linear ranges of all prostanoids and female sex hormones are listed in table 4.3 and span up to the ng level. Calibration curves for the prostanoids and sex hormones were generated by plotting peak area ratio between analyte and internal standard against concentration. The linear range was determined by serially diluting the working solution. The calibration standards were injected in triplicate. Calibration curves for all 17 compounds were linear over the tested concentration range. The linear equation, range and correlation coefficients (R) of each analytical curve are shown in Table 4.3. All prostanoids ranged from LOQ to 20 ng except 11-dehydro-TXB_2 with a range from LOQ to 40 ng. E_1, 16α-OHE_1 and 4-OHE_1 ranged from LOQ to 10 ng, whereas E_3 ranged from LOQ to 50 ng, E2 from LOQ to 200 ng, 2-OHE_1 from LOQ to 20 ng, and progesterone from LOQ to 2 ng.

A well-designed calibration curve plays an essential role in finding accurate analyte concentrations in unknown samples. However, a concentration range in calibration curve can be up to several orders of magnitude. Szabo et al found that deviations at larger concentrations affected the regression line more than deviations at smaller concentrations, and thus a weighted regression line was required to improve the accuracy of lower concentration in bioanalytical methods [5]. Almeida et al proposed a model to calculate the linear regression by introducing a weighting factor to limit the error at the lower concentration, which is called a weighted least squares linear regression (WLSLR) [6]. In their model, the slope (b) and the intercept (a) of the linear regression equation can be calculated by the following equation.

$$b = \frac{\sum w_i \cdot \sum w_i x_i y_i - \sum w_i x_i \cdot \sum w_i y_i}{\sum w_i \cdot \sum w_i x_i^2 - \left(\sum w_i x_i\right)^2}$$

$$a = \frac{\sum w_i x_i^2 \cdot \sum w_i y_i - \sum w_i x_i \cdot \sum w_i x_i y_i}{\sum w_i \cdot \sum w_i x_i^2 - \left(\sum w_i x_i\right)^2}$$

Where y_i is the data point corresponding to x_i, i means the ith data pair, and w_i is the weighting factor.

In our LC-MS method, empirical weighting factors including 1/x, $1/x^{1/2}$, $1/x^2$, 1/y, $1/y^{1/2}$ and $1/y^2$ were applied to our analytical concentration range to obtain a weighted linear regression equation, respectively. The slope (b) and intercept (a) were calculated based on the above equations, where y is peak area ratio between analyte and internal standard for each compound, and x is the nominal concentration. The effectiveness of a weighting factor is evaluated by the sum of absolute relative error (%RE) values. The best weighting factor gives the smallest sum of % RE values indicating a good fit. Then the best weighing factor is used from the weighted linear regression equation. The value of %RE is calculated based on the following equation in the paper published by Almeida [6]:

$$\%\mathrm{RE} = \frac{C_{\mathrm{found}} - C_{\mathrm{nom}}}{C_{\mathrm{nom}}} \times 100$$

Where C_{found} is the concentration calculated from the linear regression equation, and C_{nom} is the nominal standard concentration.

In our method, most of the analytes have a weighing factor of $1/x^2$ except 16α-OHE_1,

4-OHE_1, and 2-methoxyE_1 with $1/y^2$, and 2-OHE_1 and progesterone with $1/x^{1/2}$. The curves for all compounds were linear with R greater than 0.98 except E_3 with 0.970 in the tested concentration range. Figure 4.3 shows the plots of the %RE versus concentration obtained for all weighting factors from E_2. The best weighting factor for E_2 gave the smallest sum of % RE values is $1/x^2$.

4.3.2.4 Accuracy and Precision

The precision of the method was evaluated by determining the repeatability (intra-day assays) and the intermediate precision (inter-day assays). The working solutions were prepared by spiking 17 standards at two different concentration levels (high and low level) in the calibration curve range. Each solution was injected in triplicate or four times. Three solutions prepared at each concentration level were used to determine the intra-day precision. A total of 10 injections (n=10) were made in a single day. The inter-day precision was measured by injecting five solutions at each concentration level (n=15) on five consecutive days. The precision results for all compounds are listed in Table 4.4. The R.S.D. values for intra-day and inter-day precisions at the high and low concentration levels fall within 0.80-8.56%, which indicates that the separation is reproducible.

The accuracy of the method was evaluated by measuring 10 injections from three solutions prepared at each concentration level. The calculated concentrations for each compound were then compared to the theoretical concentration to determine the accuracy for each compound. Accuracy results are listed in Table 4.5. All compounds have accuracy within 84.66-114.42%, which indicates that the method is sufficiently accurate to quantify the concentrations of prostanoids and sex hormones.

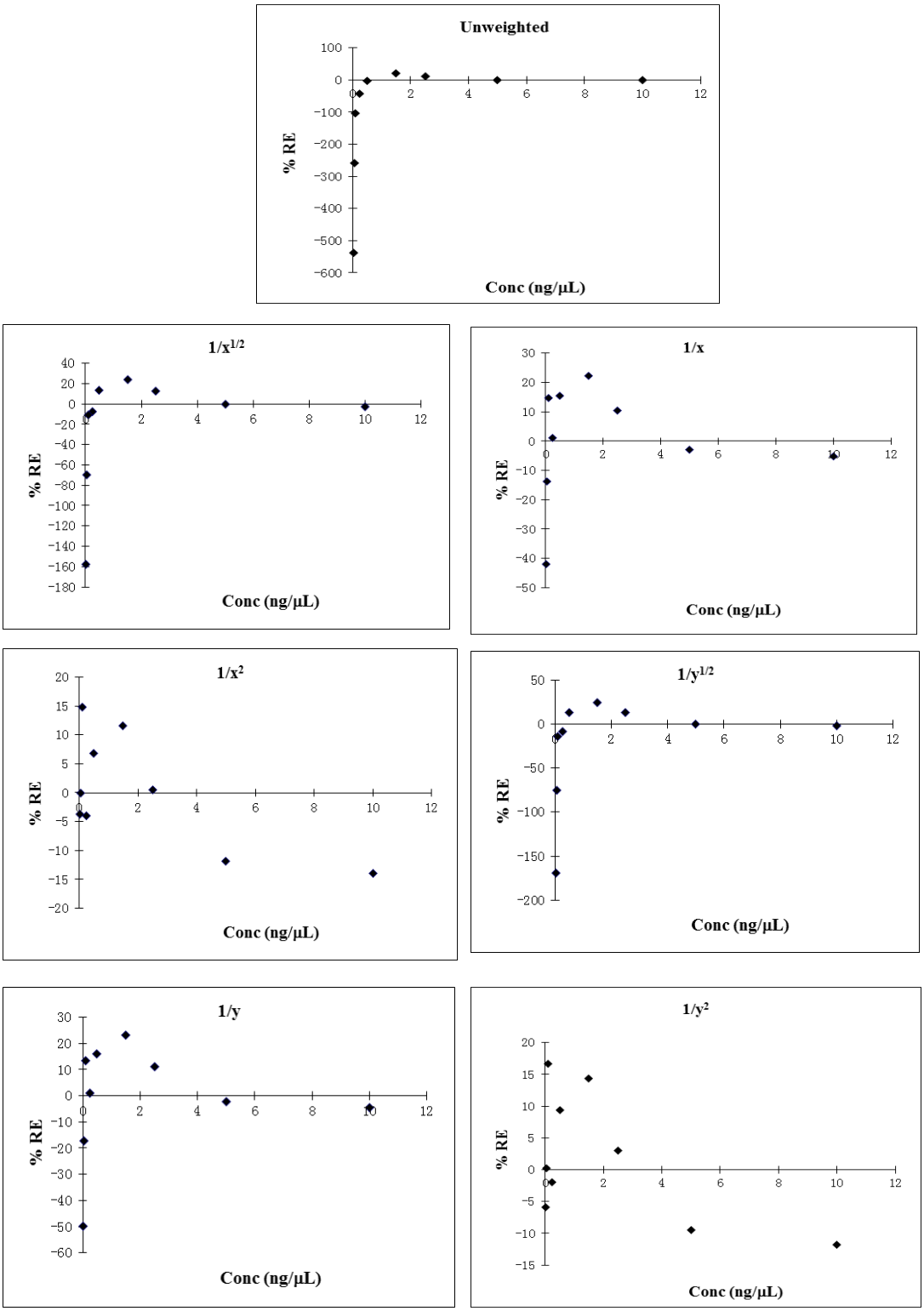


Figure 4.3 Plots of %RE versus concentration obtained for all weighting factors from E_2

Compound	Level 1			Level 2		
	Conc. (ng/µL)	Intra-assay precision %R.S.D. (n=10)	Inter-assay precision %R.S.D. (n=15)	Conc. (ng/µL)	Intra-assay precision %R.S.D. (n=10)	Inter-assay precision %R.S.D. (n=15)
E_3	2.5	4.33	5.64	0.5	2.05	4.94
16α-OHE_1	0.5	1.77	6.22	0.1	0.80	5.13
2-OHE_1	0.5	2.75	7.43	0.1	2.94	8.20
4-OHE_1	0.5	2.52	6.20	0.1	2.36	8.56
E_2	2.5	2.93	3.09	0.5	2.47	5.10
E_1	0.5	5.39	6.68	0.1	3.27	3.09
2-methoxyE_1	0.5	7.01	6.48	0.1	2.28	3.39
progesterone	0.1	2.32	6.78	0.02	1.04	7.08
6-keto $PGF_{1\alpha}$	0.5	1.85	3.16	0.05	6.16	4.14
8-iso $PGF_{2\alpha}$	0.5	1.98	2.51	0.05	5.36	3.63
$PGF_{2\alpha}$	0.5	2.29	2.98	0.05	6.99	3.80
PGE_2	0.5	1.90	2.78	0.05	6.46	4.11
11-dehydro-TXB_2	0.1	2.31	2.61	0.1	3.96	3.85
PGD_2	0.5	2.32	3.63	0.05	5.38	4.48
13,14-dihydro-15-keto-PGE_2	0.5	1.53	6.13	0.05	6.04	3.52
13,14-dihydro-15-keto-PGA_2	0.5	6.06	6.73	0.05	5.74	6.27
15-deoxy-PGJ_2	0.5	7.56	7.41	0.05	4.70	7.52

Table 4.4 Validation results: inter-day and intra-day precision

Compound	Level 1		Level 2	
	Concentration (ng/µL)	Accuracy % (n=10)	Concentration (ng/µL)	Accuracy % (n=10)
E_3	1.75	84.66	0.35	109.2
16α-OHE_1	0.4	95.1	0.08	102.82
2-OHE_1	0.5	99.72	0.1	86.99
4-OHE_1	0.4	98.22	0.08	93.6
E_2	2.5	99.51	0.5	101.59
E_1	0.4	89.54	0.08	97.5
2-methoxyE_1	0.4	84.19	0.08	101.28
progesterone	0.08	103.34	0.016	113.42
6-keto $PGF_{1\alpha}$	0.5	110.98	0.05	95.08
8-iso $PGF_{2\alpha}$	0.5	106.81	0.05	95.28
$PGF_{2\alpha}$	0.5	104.21	0.05	95.38
PGE_2	0.5	105.18	0.05	100.23
11-dehydro-TXB_2	1	102.47	0.1	95.03
PGD_2	0.5	114.42	0.05	100.68
13,14-dihydro-15-keto-PGE_2	0.5	106.62	0.05	96.41
13,14-dihydro-15-keto-PGA_2	0.5	96.61	0.05	105.43
15-deoxy-PGJ_2	0.5	84.82	0.05	99.17

Table 4.5 Validation results: accuracy

4.3.2.5 Solution stability

During the process of method development, all working and stock solutions were stored in a freezer at -80°C until use. Before injection, the tested solutions were taken out to thaw at room temperature and then stored in ice. This is an important procedure to evaluate whether the tested solutions are stable during the analysis. Solution stability was tested by quality control solutions through three freeze and thaw cycles, based on FDA bioanalytical guidance [7]. Three solutions at each of low and high concentrations were prepared and stored in a freezer at -80°C for 24 hours, and naturally thawed at room temperature. After being completely thawed, the solutions were refrozen for 24 hours at -80°C. Then the freeze–thaw cycle was repeated two more times. On the third cycle, each solution was thawed and injected with an injection volume of 20 μL. The total of six injections were made at each concentration level. The R.S.D. at each concentration was calculated based on the results of the six injections as listed in Table 4.6. The R.S.D. values for all compounds were less than 5% at both low and high concentration levels. The results indicate that our solutions were stable during the analysis period.

Compound	Level 1		Level 2	
	Concentration (ng/μL)	precision %R.S.D. (n=6)	Concentration (ng/μL)	precision %R.S.D. (n=6)
E_3	2.5	2.94	0.5	3.47
16α-OHE_1	0.5	3.14	0.1	2.64
2-OHE_1	0.5	2.36	0.1	3.39
4-OHE_1	0.5	2.25	0.1	3.13
E_2	2.5	2.56	0.5	2.59
E_1	0.5	2.35	0.1	3.7
2-methoxyE_1	0.5	2.47	0.1	4
progesterone	0.1	4.26	0.02	3.56
6-keto $PGF_{1\alpha}$	0.5	1.49	0.05	1.44
8-iso $PGF_{2\alpha}$	0.5	1.46	0.05	1.72
$PGF_{2\alpha}$	0.5	1.94	0.05	1.6
PGE_2	0.5	1.41	0.05	0.96
11-dehydro-TXB_2	1	1.1	0.1	1.19
PGD_2	0.5	2.04	0.05	0.67
13,14-dihydro-15-keto-PGE_2	0.5	1.37	0.05	2.82
13,14-dihydro-15-keto-PGA_2	0.5	2.94	0.05	1.81
15-deoxy-PGJ_2	0.5	3.39	0.05	1.48

Table 4.6 Validation results: solution stability

4.3.2.6 Recovery

Recovery was estimated by spiking urine samples with standard samples at low, middle and high concentration levels. The results were listed in Table 4.7. All the compounds fall within 85.4%-114.6%, which indicated that the method is suitable to measure the level of prostanoids and sex hormones in urine.

Compound	Level 1			Level 2	
	Concentration (ng/µL)	Accuracy % (n=3)		Concentration (ng/µL)	Accuracy % (n=3)
E_3	0.07	83.38		0.30	94.56
16α-OHE_1	0.07	102.2		0.20	111.3
2-OHE_1	0.08	102.8		0.25	106.2
4-OHE_1	0.10	105.7		0.30	108.8
E_2	0.05	113.1		0.15	110.8
E_1	0.10	97.94		0.30	99.25
2-methoxyE_1	0.17	93.45		0.50	94.88
progesterone	0.08	100.7		0.25	106.3
6-keto $PGF_{1\alpha}$	0.025	97.67		0.15	107.8
8-iso $PGF_{2\alpha}$	0.025	85.85		0.15	85.29
$PGF_{2\alpha}$	0.03	99.37		0.20	89.4
PGE_2	0.04	100.2		0.25	93.62
11-dehydro-TXB_2	0.08	100.7		0.50	92.08
PGD_2	0.05	90.91		0.25	97.13
13,14-dihydro-15-keto-PGE_2	0.05	93.57		0.30	91.01
13,14-dihydro-15-keto-PGA_2	0.04	115.1		0.25	102.6
15-deoxy-PGJ_2	0.04	111.9		0.25	93.37

Compound	Level 3	
	Concentration (ng/µL)	Accuracy % (n=3)
E_3	0.6	98.78
16α-OHE_1	0.4	96.29
2-OHE_1	0.5	91.66
4-OHE_1	0.6	92.90
E_2	0.3	97.13
E_1	0.6	88.56
2-methoxyE_1	1.0	83.30
progesterone	0.5	90.94
6-keto $PGF_{1\alpha}$	0.3	117.20
8-iso $PGF_{2\alpha}$	0.3	96.08
$PGF_{2\alpha}$	0.4	100.80
PGE_2	0.5	102.60
11-dehydro-TXB_2	1.0	101.10
PGD_2	0.5	112.90
13,14-dihydro-15-keto-PGE_2	0.6	101.00
13,14-dihydro-15-keto-PGA_2	0.5	112.10
15-deoxy-PGJ_2	0.5	97.27

Table 4.7 Validation results: recovery by spiking urine at low, middle and high concentration levels

4.3.3 Application of method to urine samples

The method was applied to seven urine samples collected from both male and female. The medical condition of patients was listed in the paper published by Williamson [8]. African Americans with body mass index of 30.5 ± 1.1 kg/m^2 at age 58 ± 2.3 years, having prehypertension or stage 1 hypertension (143 ± 7 / 87 ± 7 mmHg) before and after special 50-minute exercise training. None of the patients was under antihypertensive medication.

In the LC-MS results, several compounds including eight female sex hormones and 6-keto $PGF_{1\alpha}$, 8-iso $PGF_{2\alpha}$, PGE_2, 13,14-dihydro-15-keto-PGA_2 and 15-deoxy-PGJ_2 were identified in most of the urine samples. The selected ion chromatograms (SIC) obtained from one urine sample are shown in Figure 4.4. The above sample analysis shows that the method is capable of determining trace levels of bioactive prostanoids and sex hormones in urine samples after enrichment with the SPE method.

4.3.4 Comparison between HPLC-UV and LC-MS

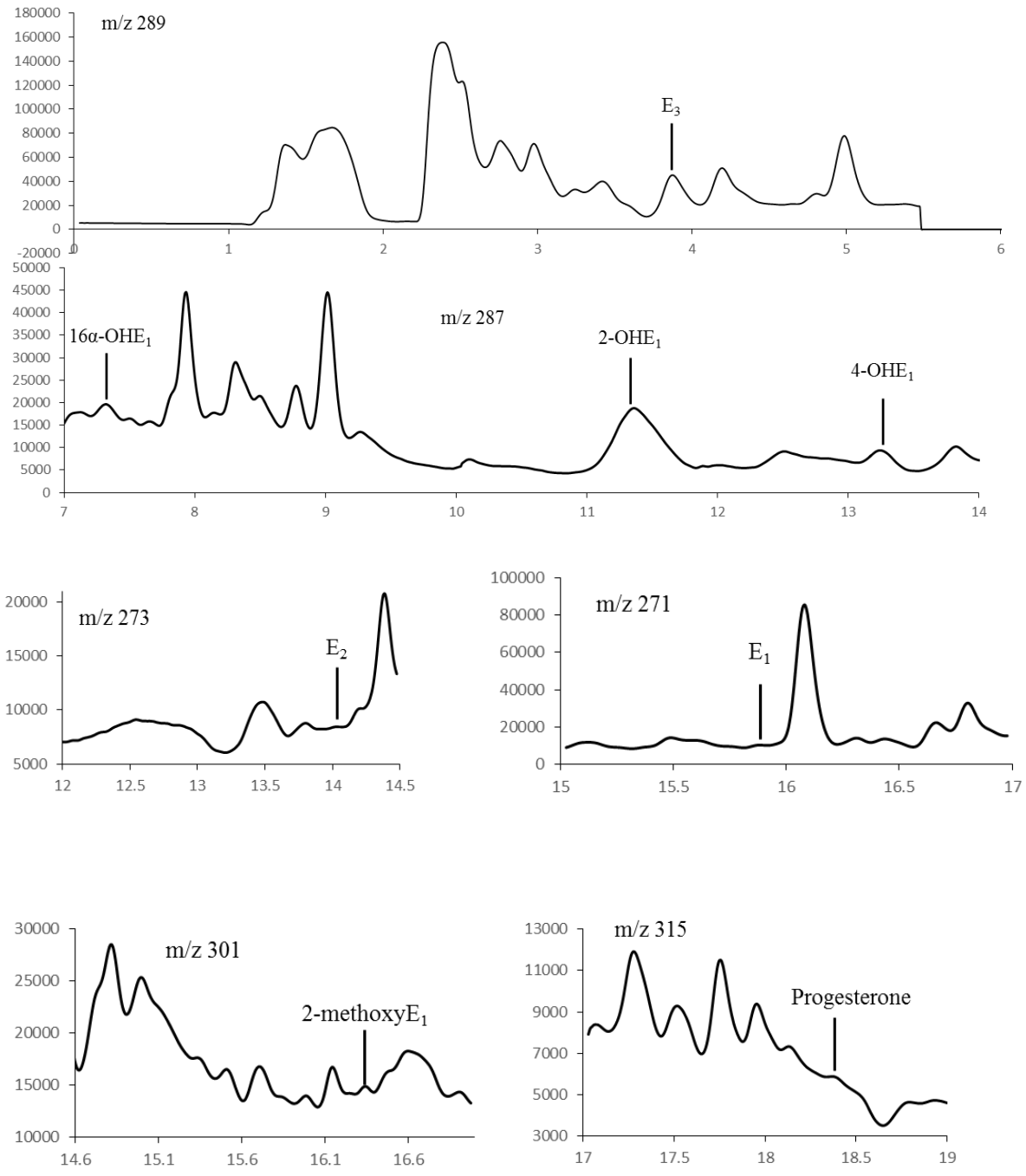
By replacing phosphoric acid with formic acid, the HPLC-UV method was adapted to LC-ESI-MS. Formic acid served as a proton provider. All of the prostanoids were measured in the negative ionization mode, while all of the sex hormones were measured in the positive ionization mode. Compared with HPLC-UV, LC-MS cannot measure the combination of prostanoids and sex hormones in the same run, and have to perform the positive ionization mode and the negative ionization mode, respectively. Otherwise, the sensitivity of LC-MS decreases a lot. In general, mass spectrometers show a higher sensitivity than UV detectors. By comparing the HPLC-UV and LC-MS method, we found

that the values of LOQ of analytes measured by LC-MS are hundreds or thousands lower than that of LOQ measured by HPLC-UV. Table 4.8 listed the LOQ of prostanoids and sex hormones measured by HPLC-UV and LC-MS. The results indicate that the MS detector is much more sensitive than the UV detector. However, LC-MS takes two times longer to measure the combination of prostanoids and sex hormones than HPLC-UV.

Compound	LC-MS LOQ (pg)	HPLC-UV LOQ (ng)
E_3	100	3
16α-OHE_1	20	5
2-OHE_1	240	20
4-OHE_1	20	2
E_2	500	5
E_1	20	2
2-methoxyE_1	20	2
progesterone	3.6	20
6-keto $PGF_{1\alpha}$	20	10
8-iso $PGF_{2\alpha}$	20	20
$PGF_{2\alpha}$	20	1
PGE_2	20	1
11-dehydro-TXB_2	40	30
PGD_2	100	20
13,14-dihydro-15-keto-PGE_2	24	20
13,14-dihydro-15-keto-PGA_2	0.6	10
15-deoxy-PGJ_2	15	20

Table 4.8 LOQ comparison between LC-MS and HPLC-UV

(a)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E3
m/z 287
16α-OHE1
2-OHE1
4-OHE1
m/z 273
E2
m/z 271
E1
m/z 301
2-methoxyE1
m/z 315
Progesterone

(b)

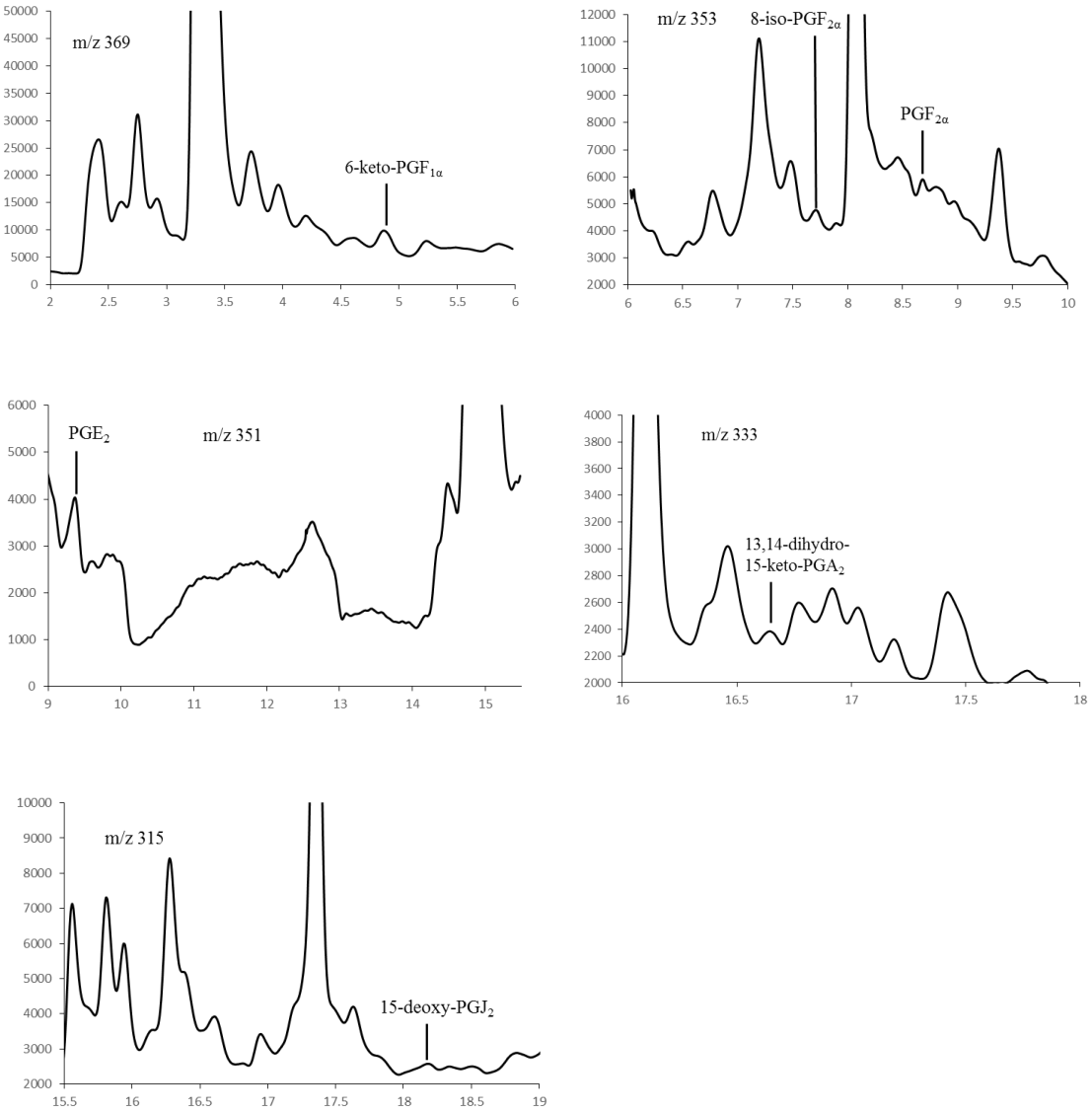

Figure 4.4 Selected ion chromatograms (SIC) of urine (a) sex hormones found in positive ion mode; (b) prostanoids found in negative ion mode

4.4 Conclusion

In this study, we have developed a simple, sensitive, accurate and rapid LC-MS method for the separation of the bioactive compound combination of nine prostanoids and eight female sex hormones. The method has been applied to simultaneously quantify endogenous prostanoids and female sex hormones in urine samples from hypertension patients. The levels of prostanoids and female sex hormones were affected by exercise training and recovery time period after the training period. Our study shows that LC-MS is more suitable to measure the trace amount of bioactive compounds in biological samples due to high sensitivity than HPLC-UV.

4.5 References

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CHAPTER 5

COMBINING QUANTITATIVE STRUCTURE–RETENTION RELATIONSHIPS WITH MULTIPLE LINEAR REGRESSION FOR PREDICTING RETENTION TIME ANALYSIS AND PREDICTION FOR MOLECULES WITH DIVERSE CHEMICAL CHARACTERISTICS

5.1 Introduction

High performance liquid chromatography is a powerful separation tool to separate and quantify analytes in pharmaceutical and industrial analysis. Although the separation method in HPLC is rapid, sensitive and accurate, it requires the injection of each individual standard compound to match the spectra and retention time of analytes during the development of the method, which is time-consuming. Based on the separation mechanism in HPLC, it is known that the chemical properties of analytes and their interaction with the stationary and mobile phases play an important role in determining the elution order and elution time. Therefore, it is possible to develop a theoretical model to predict the retention behavior of analytes in HPLC.

Quantitative structure–retention relationships (QSRRs) were reviewed by Kaliszan and Ukic [1, 2]. The technique focuses on quantitively evaluating the relationships between chromatographic retention and molecular descriptors of analytes derived from chemical properties based on molecular structures [1]. Molecular descriptors are values that characterize chemical and physical properties of molecules. In a general approach, molecular descriptors are calculated by chemical computational software, and then statistical analysis is performed to find effective molecular descriptors correlating with retention behavior and develop a mathematical model for retention prediction in specific chromatographic systems. QSRR can be used for the following purposes [2]: (1) To

identify useful structural descriptors of analytes; (2) To predict retention time for new analytes; (3) To identify unknown analytes; (4) To gain insight into molecular separation mechanism in a given chromatographic system; (5) To quantitatively evaluate properties of different HPLC columns. The studies using QSRR began in 1977 [3]. Since then, there has been significant growth in the area. This method has also been used for gas chromatograph. For example, Fatemi et al proposed a QSRR model to predict the retention indices of amino acids (AAs) and carboxylic acids in gas chromatograph [4].

Several reviews about QSRR studies have been published from early time to recent years [5-7]. In which different statistical methodologies are proposed to build QSRR models, these includes multi-linear regression (MLR), artificial neural network (ANN) and support vector machine (SVM).

A common and simple approach is MLR. In 1941, Martin and Synge correlated the retention time to the solute partition coefficients with MLR analysis [8]. Partition-coefficient is the concentration ratio of a solute distributed in two immiscible phases at equilibrium. Then later, Kaliszan et al introduced the total dipole moment, the electron excess charge of the most negatively charged atom, and the water-accessible molecular surface area into a MLR equation to predict retention time [9]. Gorynski et al applied a MLR analysis to gradient retention time data of 146 drugs and metabolites obtained from reversed-phase (RP-LC–HRMS) and hydrophilic interaction chromatography (HILIC-LC–HRMS) systems [10]. They developed two QSRR models to predict retention times of compounds with different chemical structures. Tosti et al used MLR and partial least square (PLS) regression to develop two retention models based on the experimental retention data from 31 polyoxygenated steroids and selected molecular descriptors [11]. They found that

lipophilicity, steric, and hydrophilic interaction played an important role in the retention process of selected compounds in the reversed phase liquid chromatography. However, the disadvantage of MLR was that the number of analytes should be larger than the number of molecular descriptor in a given MLR equation.

Artificial neural networks (ANN) consists of an input layer, one or more hidden layers and an output layer. Each layer contains some small units. Each small unit contains input, weights, function connecting between layers and output [12]. In ANN models, the combination of linear and non-linear functions improves the accuracy of predicted retention time. The disadvantage of ANN is difficult to interpret because of the complexity of the combination of molecular descriptors. D'Archivio et al performed artificial neural network (ANN) to model the retention times of the combination of selected purines, pyrimidines and nucleosides with the mobile phase of acetonitrile-water in reversed-phase liquid chromatography [13]. In the model, five selected molecular descriptors and the variables related to the gradient profile were combined. In their model, the ANN layers included training, validation and testing sets. The model could be used to predict retention time of analytes in multilinear gradient conditions.

Recently, support vector machine (SVM), a machine learning technique, has been used for classification and regression. Since the SVM technique was applied to QSRS study, it has shown several advantages including global optimization, good generalization ability, and dimensional independence [14]. Luan et al developed a linear model by the heuristic method (HM) and a non-linear model by the SVM technique between the retention time and selected molecular descriptors of 149 volatile organic compounds [15]. One constitutional (number of F atoms), one geometrical (gravitation index of all bonds),

two electrostatic (atomic charge and partial negative surface area) and one quantum chemical (minimum nucleophilic reactive index for a C atom) descriptors were selected by the heuristic method and applied to both the linear and non-linear models. They found that the SVM model had a better performance to predict retention time than the HM model. Du et al developed both HM and SVM models to predict retention time of benzene derivatives, organic acid derivatives, aniline derivatives and other compounds on three columns including Symmetry C18, Chromolith and SG-MIX column, respectively. They found that the SVM model had a better accuracy than the HM method [16].

Zhou et al developed eight statistical modelling methods to predict retention time of peptides by introducing the molecular descriptors related to structural characteristics into the method [20]. The eight methods included MLR, PLS, BPNN, RBFNN, SVM, LSSVM, RF and GP by using 40 molecular descriptors without applying any selection rule. By comparing the eight methods, they found that the MLR modeling showed a low fitting ability, and gave a small value of fitting coefficient of determination, where nonlinear modeling methods showed better results. However, MLR gave much straightforward evidences to interpret the retention mechanism involved in molecular properties and retention behaviors. In this chapter, we optimized the MLR modeling method by introducing more molecular descriptors related to molecular characteristics. Our results showed that a simple MLR method can be used to predict retention time of compounds to achieve satisfactory results.

In our study, the quantitative structure–retention relationship (QSRR) modellings were developed to combine the chemical structures and properties of prostanoids and sex hormones with retention behavior. Prostanoids are unsaturated fatty acids derived from

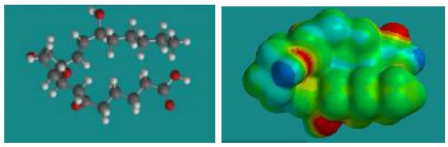
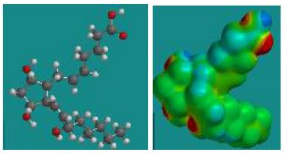
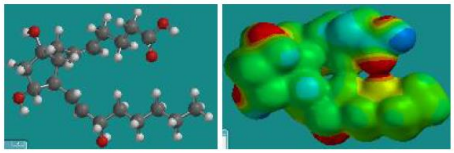
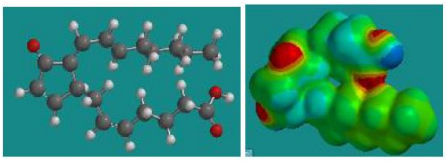
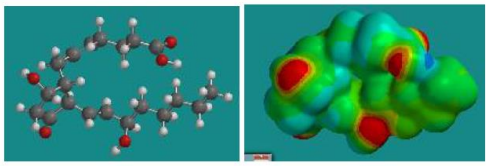
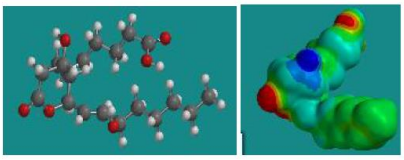
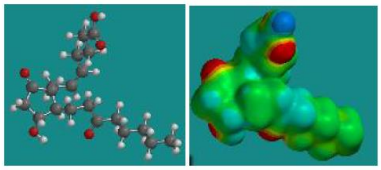
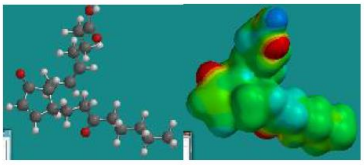
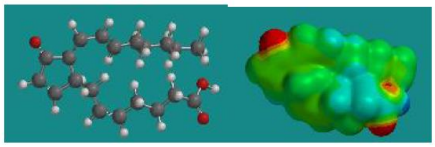
arachidonic acid, a polyunsaturated omega-6 fatty acid. Sex hormones are derivatives of cholesterol. The structures and electrostatic maps of prostanoids and sex hormones are listed in Figure 5.1.

By carefully observing the chemical structures of analytes in Figure 5.1, we can see that all prostanoids are unsaturated fatty acids consisting of a five- or six- membered ring, and two alkyl chains with some substitute groups such as carboxyl group, hydroxyl group and carbonyl group, and all sex hormones have a four-ring structure and some substitute groups such hydroxyl group and carbonyl group. The difference among analytes with close-related structures lies in the number, type and orientation of substitute groups. Comparing with other sex hormones, progesterone is a less polar molecules due to the hydrogenation of the benzyl ring.

In our study, the experimental retention data and the descriptors for characterizing the molecular structures of the analytes were included in QSRR models. The retention data of prostanoids and sex hormones were obtained on a Symmetry C18 column in an isocratic mode by using HPLC-UV. The molecular descriptors including molecular area and volume, polarizability, energy of HOMO (E_{HOMO}), energy of LUMO (E_{LUMO}), log P, dipole moment and solvation energy were calculated with chemical computation software Spartan in semi-empirical AM1 mode. Then statistical analysis was used to create a mathematical model for predicting retention time by combing the selected molecular descriptors and experimental retention data. The resultant QSRR models can be used to perform retention analysis on either existing or hypothetical analytes.

Our goal is to develop a simple common MLR model to predict retention time of compounds with big structural dissimilarity. Our study will be designed to aim

experimental researchers without strong mathematical and simulation background to develop a retention model to predict retention time of analytes and identify unknown compounds. This is particularly challenging as the chemical nature of the studied molecules is quiet diverse.

6-keto $PGF_{1\alpha}$ 	8-iso $PGF_{2\alpha}$ 
$PGF_{2\alpha}$ 	PGE_2 
PGD_2 	11-dehydro TXB_2 
13, 14-dihydro-15-keto-PGE_2 	13, 14-dihydro-15-keto-PGA_2 
15-d PGJ_2 	

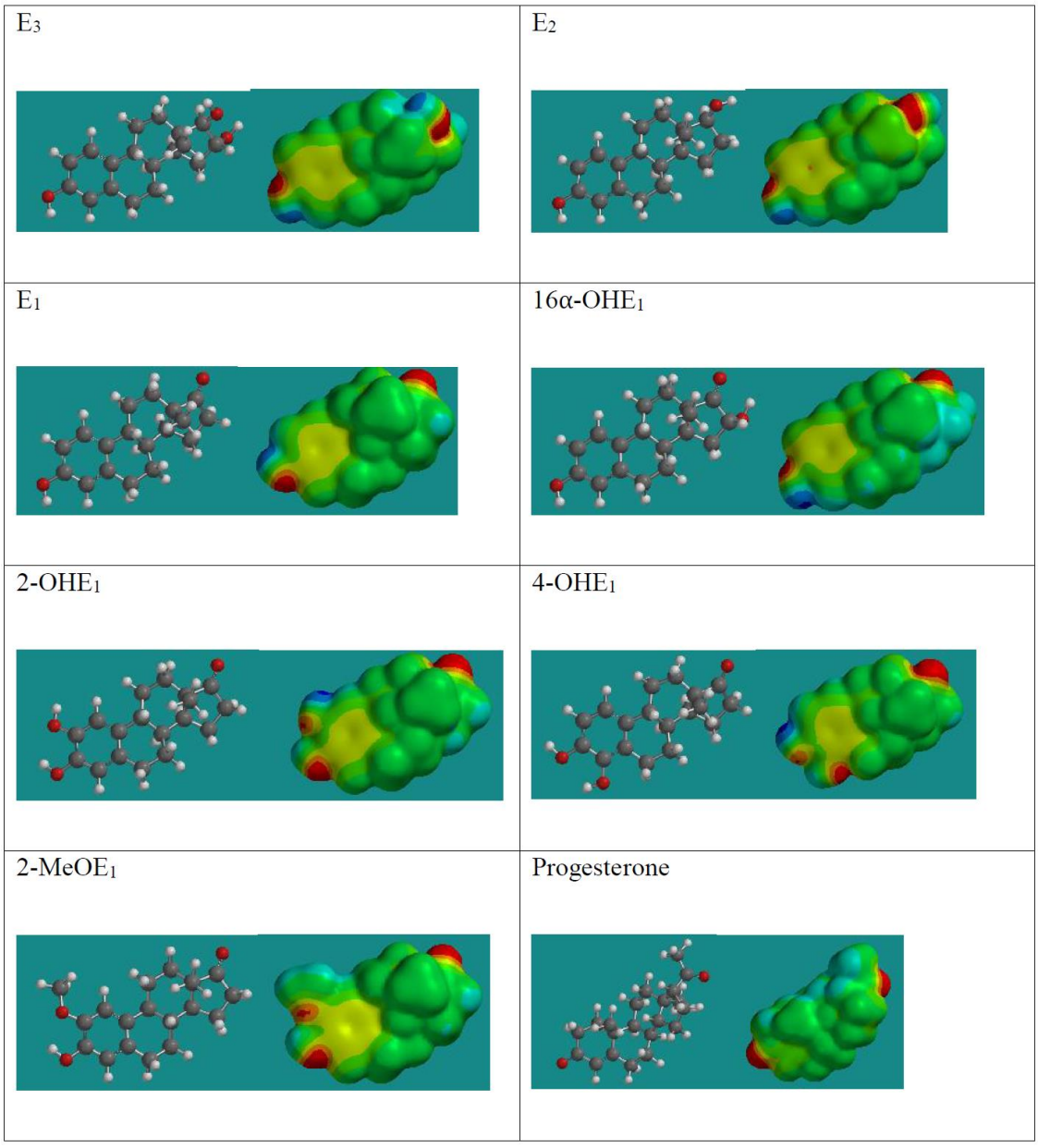


Figure 5.1 Structures and electrostatic maps of prostanoids and female sex hormones

5.2 Experimental

5.2.1 Chemicals and materials

Prostaglandin D_2 (PGD_2), prostaglandin E_2 (PGE_2), 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were purchased from Biomol (Plymouth Meeting, PA, USA). 11-dehydro-thromboxane B_2 (11-dehydro-TXB_2), 8-iso-$PGF_{2\alpha}$, 13,14-dihydro-15-keto-PGA_2, 13,14-dihydro-15-keto-PGE_2 and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15-deoxy-PGJ_2) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Estrone (E_1), 17β-estradiol (E_2), estriol (E_3) and progesterone were purchased from Sigma Aldrich (St. Louis, MO, USA). 2-hydroxyestrone (2-OHE_1), 4-hydroxyestrone (4-OHE_1), 16α-hydroxyestrone (16α-OHE_1), and 2-methoxyestrone (2-$MeOE_1$) were purchased from Steraloids, Inc. (Newport, RI, USA). All prostanoid and sex hormone standards were used without further purification. HPLC-grade solvent water, acetonitrile and methanol and reagent-grade 85% phosphoric acid were purchased from Fisher Scientific (Waltham, MA, USA).

5.2.2 Instrumental setup

The high performance liquid chromatograph (HPLC) system was obtained from Jasco Inc. (Easton, MD, USA) with a manual injector (Rheodyne LLC, Rohnert Park, CA, USA). The HPLC system used two Jasco pumps (PU-980) and a Jasco ultraviolet detector (UV-975). Data were collected with ChromNAV software. Analytes were separated on a Symmetry C18 4.6 × 250 mm column with 5-µm particle size (Waters Corp., Milford, MA, USA). The mobile phase used in this study was 17 mM phosphoric acid (solvent A) and acetonitrile (solvent B). The analytes were separated with an isocratic mode at a flow rate

of 1.3 mL/min. The detection wavelength was 196 nm. The separation was performed at room temperature with an injection volume of 100 µL.

5.2.3 Sample preparation

PGD_2, PGE_2, 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, E_1, E_2, E_3 and progesterone stock standard solutions were prepared by dissolving 1 mg solid sample into 1 mL methanol respectively to obtain a mass concentration of 1 mg/mL. All other standards were supplied as individual solutions by suppliers with the following concentrations: 11-dehydro-TXB_2 0.5 mg/mL, 8-iso-$PGF_{2\alpha}$ 1 mg/mL, 13,14-dihydro-15-keto-PGA_2 10 mg/mL, 13,14-dihydro-15-keto-PGE_2 10 mg/mL, 15-deoxy-$\Delta^{12,14}$-PGJ_2 1mg/mL, 2-OHE_1 2 mg/mL, 4-OHE_1 2 mg/mL, 16α-OHE_1 2 mg/mL, and 2-$MeOE_1$ 5 mg/mL. Working standard solutions of each compound at 10 ng/µL were prepared by diluting the stock solutions with acetonitrile. The standard solutions of the individual compounds at 1 ng/uL were prepared by adding 17 mM phosphoric acid and acetonitrile (1:1 v/v) to achieve a total volume of 500 µL. All the solutions and quality control samples were stored in a freezer at −80 ◦C until use.

5.3 Calculation of molecular descriptors

The computational software Spartan"14 ES V1.1.4 was used to calculate a set of physicochemical parameters for prostanoids and sex hormones. All three-dimensional structures of studied compounds at zero charge were optimized in the semi-empirical Austin Method 1 (AM1) mode at equilibrium state. AM1 reduced the complexity of computation and worked on the valence electrons [21]. Parameterization 3 (PM3), a modification of AM1, is another commonly used semi-empirical method in computational

chemistry. Compared with PM3, AM1 gives more reliable results, because AM1 has been successfully applied to a wide range of chemical reactions and chemical problems [22]. Therefore, AM1 is selected to compute molecular descriptors. There are eight molecular descriptors including dipole moment, solvation energies, log P, polarizability, E_{HOMO}, E_{LUMO}, molecular area and molecular volume obtained from the calculation results. All the parameters of the molecular descriptors are listed in Table 5.1.

Compounds	$RT_{exp.}$	$E_{solvation}$ (aq)	E_{HOMO}	E_{LUMO}	Dipole moment	Area	Volume	LogP	Polarizability
	min	kJ/mol	ev	eV	debye	Å2	Å3		
E_3	3.842	-48.25	-8.84	0.37	1.22	301.07	300.24	1.15	63.56
6-keto $PGF_{1\alpha}$	4.633	-61.19	-10.1	0.73	2.05	435.33	399.35	2.1	71.22
16a-OHE_1	7.392	-47.46	-8.9	0.31	2.94	296.62	295.65	1.8	63.19
8-iso $PGF_{2\alpha}$	8.242	-56.12	-9.83	0.89	2.53	426.28	391.96	2.86	70.65
$PGF_{2\alpha}$	11.208	-55.71	-9.85	0.86	2.14	435.49	393.44	2.86	70.77
PGE_2	14.317	-48.1	-9.93	0.64	1.38	426.85	388.54	3.18	70.4
11-dehydro TXB_2	15.942	-61.72	-10.18	0.53	4.11	425.37	395.42	2.78	70.93
PGD_2	17.283	-50.93	-9.99	0.55	2.03	417.2	387.52	3.18	70.33
2-OHE_1	16.375	-43.95	-8.67	0.25	0.68	295.76	295.28	1.51	63.22
4-OHE_1	18.508	-39.33	-8.81	0.22	2.22	294.85	295.14	1.51	63.19
E_2	20.433	-32.42	-8.77	0.44	2.4	292.04	292.73	2.1	62.95
13,14-dihydro-15-keto-PGE_2	26.255	-49.6	-10.03	0.65	3.21	424.9	387.52	3.51	70.3
E1	34.742	-35.34	-8.87	0.34	3.34	287.69	288.2	2.59	62.58
2-$MeOE_1$	43.342	-31.16	-8.59	0.33	0.39	316.61	315.42	1.62	64.86
13,14-dihydro-15-keto-PGA_2	74.183	-38.31	-10	-0.02	2.42	411.87	376.24	4.69	69.55

Table 5.1 Parameters of molecular descriptors

5.4 Statistical Analysis

The possible correlation between the retention time and each individual molecular descriptor was evaluated by using linear regression with Excel 2010 software. Then the multiple molecular descriptors were chosen for further multiple linear regression (MLR) and correlation analysis. The MLR equation combining multiple molecular descriptors to predict retention time was performed by using the statistical software Medcalc. A correlation coefficient greater than 0.9 was considered as good correlation. Prostanoids are fatty acids derived from arachdonic acid (AA), and their backbone has a similar structure, while sex hormones are derived from cholesterol, and all have a similar backbone structure. Therefore, in our studies, correlation analysis and MLR analysis were performed on the combination of prostanoids and sex hormones, the group of prostanoids and the group of sex hormones, respectively. Table 5.2a, b and c listed the slope, intercept and correlation coefficient when linear regression correlation analysis was applied on prostanoids, sex hormones and the combination of prostanoids and sex hormones, respectively.

(a)

Molecular Discriptor	Slope	Intercept	R
E_{LUMO}	-0.0085	0.631	0.6151
solvation energy	0.3171	-52.542	0.5908
LogP	0.0286	1.9639	0.5531
Volume	-0.0865	348.45	0.0332
Polarizability	-0.005	67.273	0.0245
Area	-0.0664	367.1	0.0173
Dipole moment	0.0002	2.1995	0.0045
E_{HOMO}	-0.00008	-9.4225	0.0024

(b)

Molecular Discriptor	Slope	Intercept	R
LogP	0.0315	2.5464	0.9373
E_{LUMO}	-0.0118	0.8277	0.9233
Volume	-0.2785	395.29	0.8941
Polarizability	-0.0199	70.897	0.8776
solvation energy	0.2892	-58.207	0.8355
Area	-0.2811	430.75	0.7767
E_{HOMO}	-0.0005	-9.9789	0.0975
Dipole moment	0.0032	2.4226	0.0854

(c)

Molecular Discriptor	Slope	Intercept	R
solvation energy	0.4333	-47.57	0.8698
E_{HOMO}	0.0044	-8.8581	0.5519
LogP	0.0166	1.4537	0.4974
Polarizability	0.0192	63.015	0.3751
Volume	0.2257	293.42	0.3670
Area	0.2187	293.83	0.3335
Dipole moment	-0.01	2.0653	0.1237
E_{LUMO}	0.0002	0.3185	0.0458

Table 5.2 Correlation for (a) the combination of PGs and hormones; (b) PGs; (c) hormones

5.5 Retention time prediction

One of the advantages of the QSRR model is its ability to predict the retention time of known analytes. In our study, the molecular descriptors of 15-d PGJ_2 and progesterone were calculated in semi-emperical AM1 mode using the Spartan software. Then the theoretical retention time was calculated by using the MLR equations from the QSRR analysis.

5.6 Results and discussion

5.6.1 Correlation analysis between molecular descriptors and retention time

Excel software was used to perform correlation analysis to find the correlation between individual molecular descriptors and retention time. In Table 5.2a, it can be seen that E_{HOMO}, solvation energy and log P had better correlations than other molecular descriptors, with a correlation coefficient (R) greater than 0.5. However, the correlation was not good when applying the correlation analysis to the combination of prostanoids and sex hormones.

Due to the similar backbone structure of molecules in the prostanoid group, the correlation analysis was simply performed on the prostanoid group. The results were listed in Table 5.2b. Log P and E_{LUMO} showed a good correlation with retention time, with R greater than 0.9. Molecular volume and polarizability had less correlation, with R greater than 0.87. Solvation energy and molecular volume also showed correlations, with values of R of 0.89 and 0.88 respectively. Molecular area had a value of R equal to 0.78. E_{HOMO} and dipole moment showed no correlation with retention time.

All the sex hormones have a similar structure. When correlation analysis is applied to the molecular descriptors of sex hormones as in Table 5.2c, better correlation coefficients are achieved compared with those from the combination of prostanoids and sex hormones. Solvation energy has the best correlation, with a correlation coefficient of 0.87. E homo and log P show less correlation, with correlation coefficients of 0.55 and 0.50 respectively. Polarity, volume and area show similar values of correlation coefficient. Dipole moment and E_{LUMO} have almost no correlation with retention time.

5.6.2 Retention models

In the past decades, the retention mechanism of HPLC has been extensively discussed. The most popular theory about the retention process is solvophobic theory. It was proposed in 1976, and considered the retention related to the mass transfer of solutes between mobile phase and stationary phase from thermodynamic principles [5]. Nowadays, the solvophobic theory alone cannot explain the retention mechanism of HPLC. It is also necessary to consider hydrophobic effects, oil–water partitioning and adsorption from a dilute solution. On the basis of the solvophobic theory, a partition model and adsorption model were proposed to interpret the retention mechanism and predict the retention behavior of analytes [6].

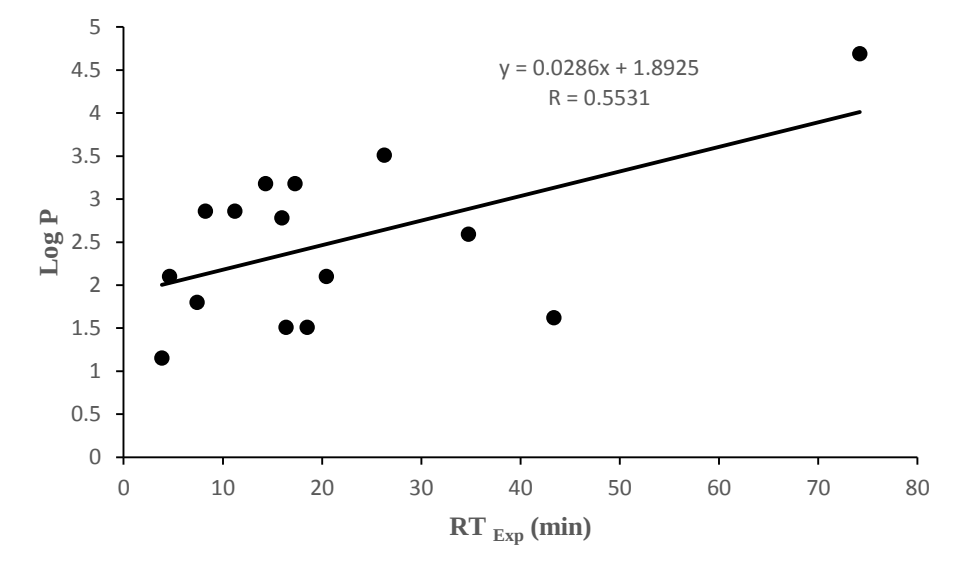
5.6.2.1 Partition model

In the partition model, it was assumed that the organic phase and aqueous phase were mutually insoluble, and the solutes partition between them [6]. In HPLC, the hydrocarbon chains bonded on the stationary phase were assumed to have a bulk liquid-like behavior due to hydrophobic interaction among them, and the solute molecules were partitioned between the hydro-organic mobile phase and the stationary phase. The partition model only worked on studying the retention behavior of small nonpolar molecules on stationary phases bonded with long hydrocarbon chains longer than 12 carbon atoms.

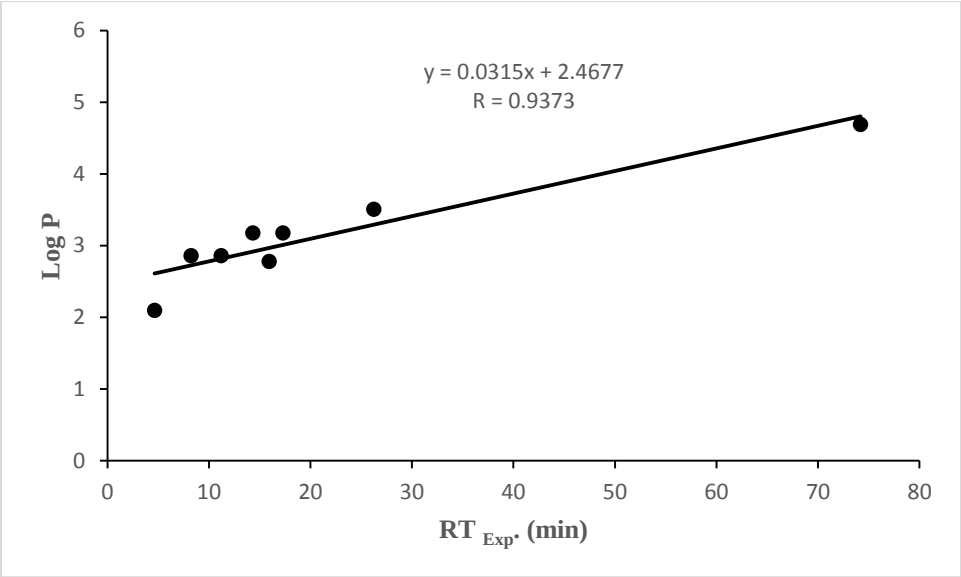
Log P is the logarithm of the partition coefficient, which is a ratio of concentrations of neutral solute distributed between the two immiscible liquid phases. In HPLC, log P is related to the distribution of solutes between mobile phase and stationary phase. Log P is an evaluation of the hydrophobicity of solutes. In the partition model, the linear regression

between the calculated log P and experimental retention was plotted on the combination of prostanoids, the group of prostanoids and the group of sex hormones, respectively. The log P calculated by the Spartan software were listed in Table 5.1. The linear regression results were showed in Figure 5.2 a, b and c. We can see that the retention behavior of prostanoids had a better correlation than that of the combination of prostanoids and sex hormones and that of sex hormones. According to the value of R, the correlation order was as follows: prostanoids (R=0.94) > combination of prostanoids (R=0.55) > sex hormones (R=0.50). The retention of sex hormones had a very poor correlation with log P. This may be due to the molecular structure of sex hormones, which had greater steric hindrance than that of prostanoids. It indicated that the partition model worked better on prostanoids than on sex hormones to predict the retention behavior in the C18 column.

(a)



(b)



(c)

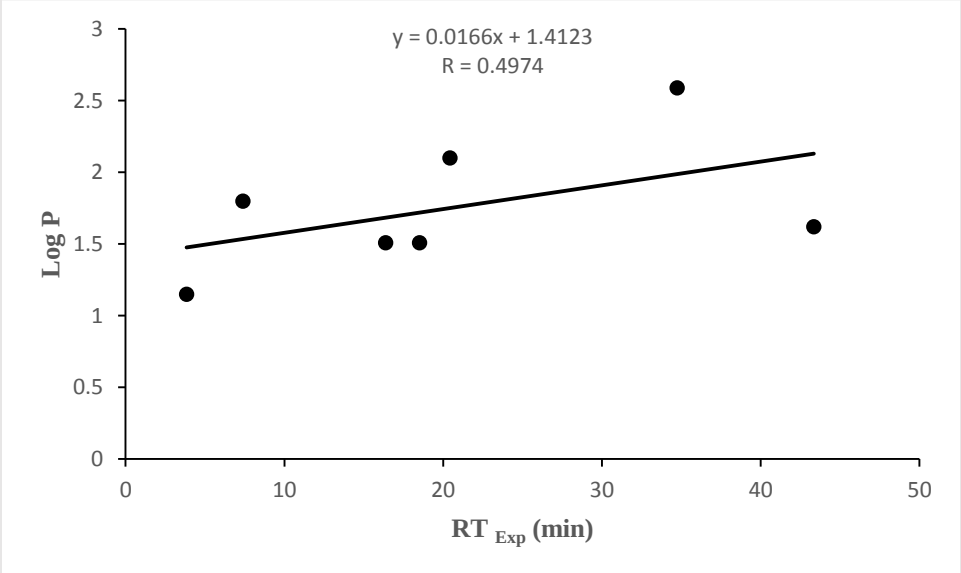


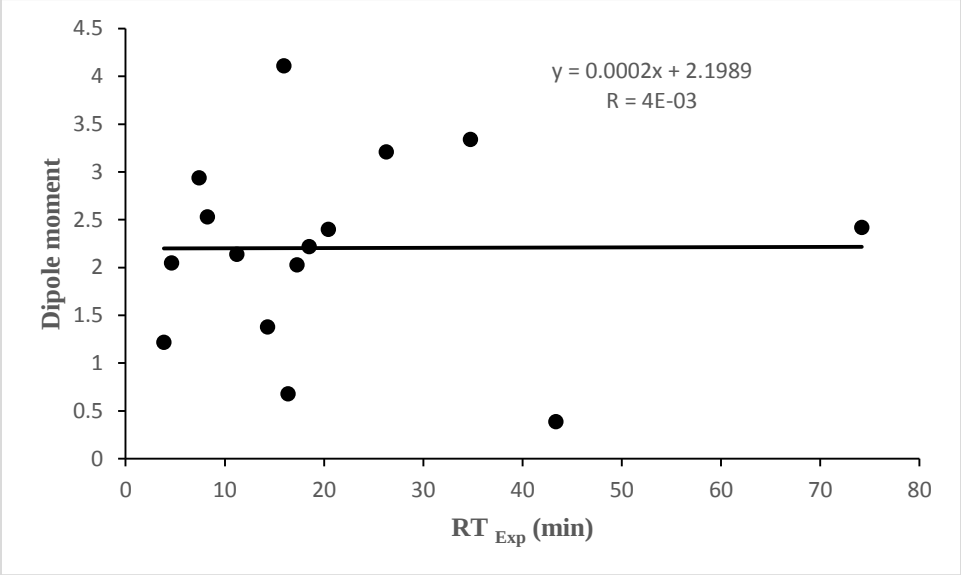
Figure 5.2 Partition model for (a) the combination of PGs and hormones; (b) PGs; (c) hormones: log P vs. $RT_{exp.}$ (min)

5.6.2.2 Adsorption model

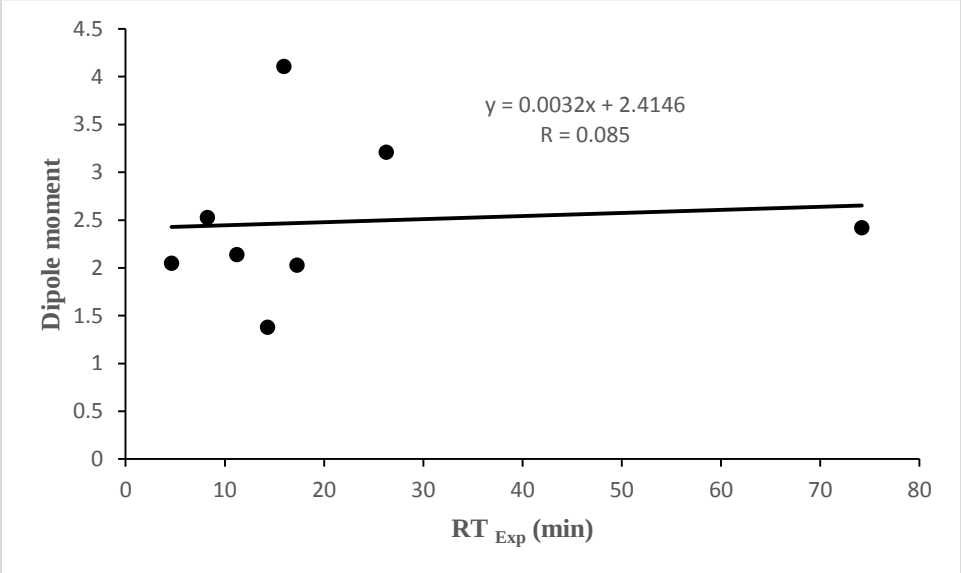
In the adsorption model, the solute molecules travel from the liquid phase to the interface between the liquid phase and the stationary phase, and displace the adsorbed solvent molecules at the interface [6]. In HPLC, the adsorption model assumes that the analyte molecules cannot penetrate into the bonded hydrocarbon layer due to the high density of nonpolar hydrocarbon chains, and they remain at the interface between the mobile and stationary phases.

In HPLC, the adsorption of solutes on the stationary phase depends on the interaction of solutes and the hydrocarbon chains bonded on the stationary phase. The interaction can be estimated by the dipole moment and polarizibility of solutes [7]. Dipole moment is the measure of molecular polarity. Polarizability is the ability for a molecule to respond to the changing electric field by changing electron distribution. By plotting the calculated dipole moment and polarizibility of solutes (Table 5.4), linear regression equations are obtained on the combination of prostanoids and sex hormones, sex hormones and prostanoids as shown in Figure 5.2 and 5.3. The values of R are 4×10^{-3} on the combination, 0.085 on PGs and 0.124 on hormones, respectively. The low R indicates that there is no significant correlation between the dipole moment of the prostanoids and sex hormone molecules (Figure 5.2) and the RT (min). However, there is a correlation between the retention behavior and the polarizability of prostanoids with R = 0.8776. This indicates that the adsorption model is involved in the retention mechanism of prostanoids in the C18 bonded stationary phase.

(a)


y = 0.0002x + 2.1989
R = 4E-03
Dipole moment
RT Exp (min)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
0
10
20
30
40
50
60
70
80

(b)


y = 0.0032x + 2.4146
R = 0.085
Dipole moment
RT Exp (min)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
0
10
20
30
40
50
60
70
80

(c)

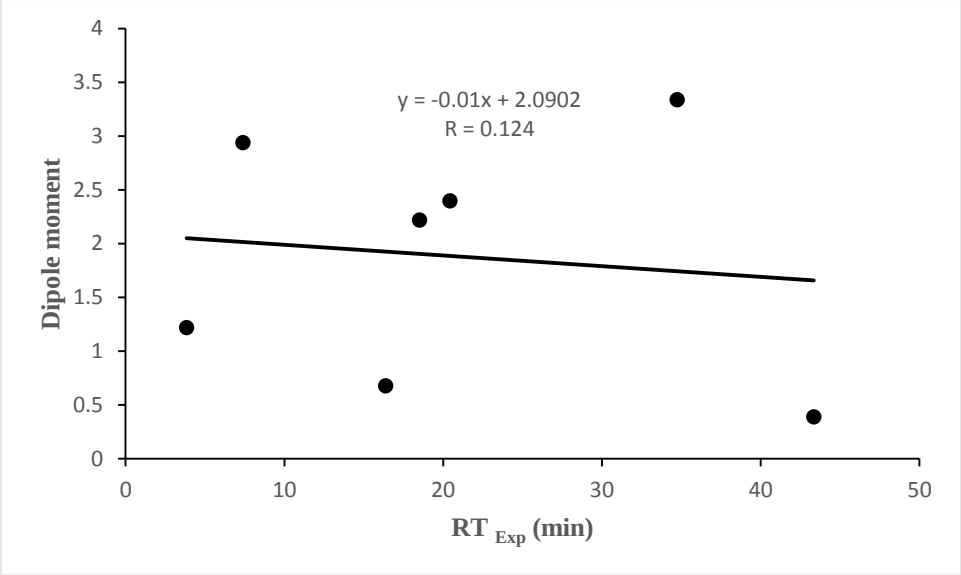
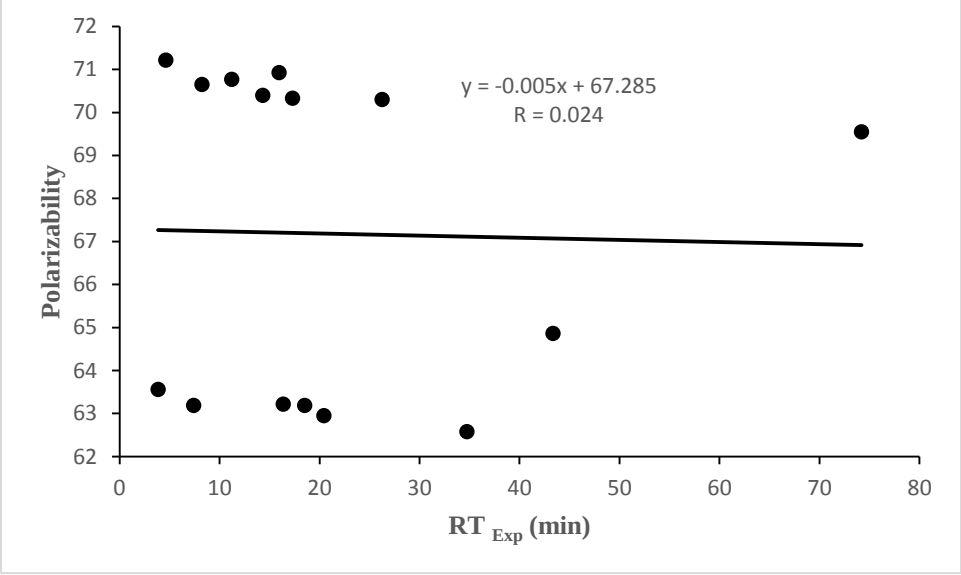
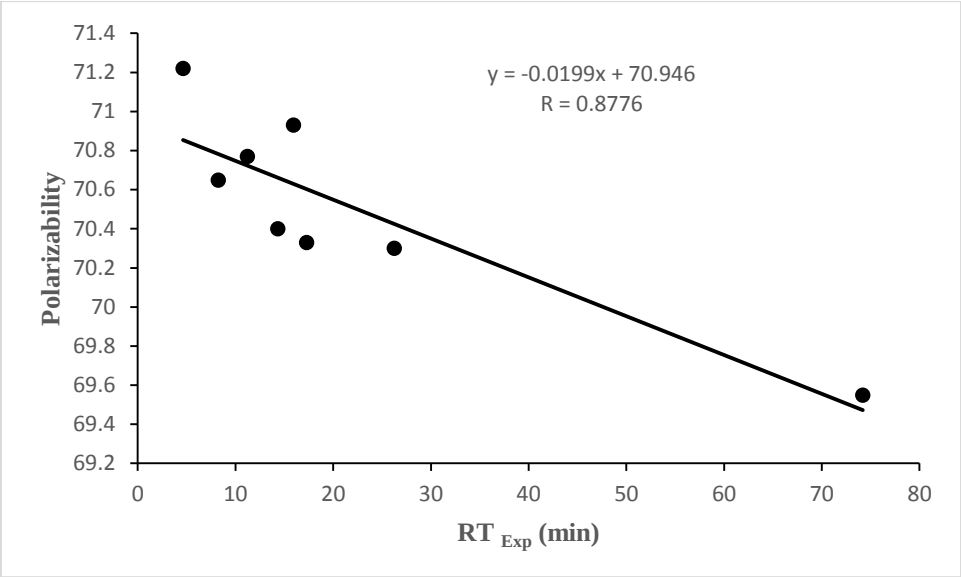


Figure 5.3 Adsorption model for (a) the combination of PGs and hormones; (b) hormones; (c) hormones: Dipole moment vs. $RT_{exp.}$ (min)

(a)



(b)



(c)

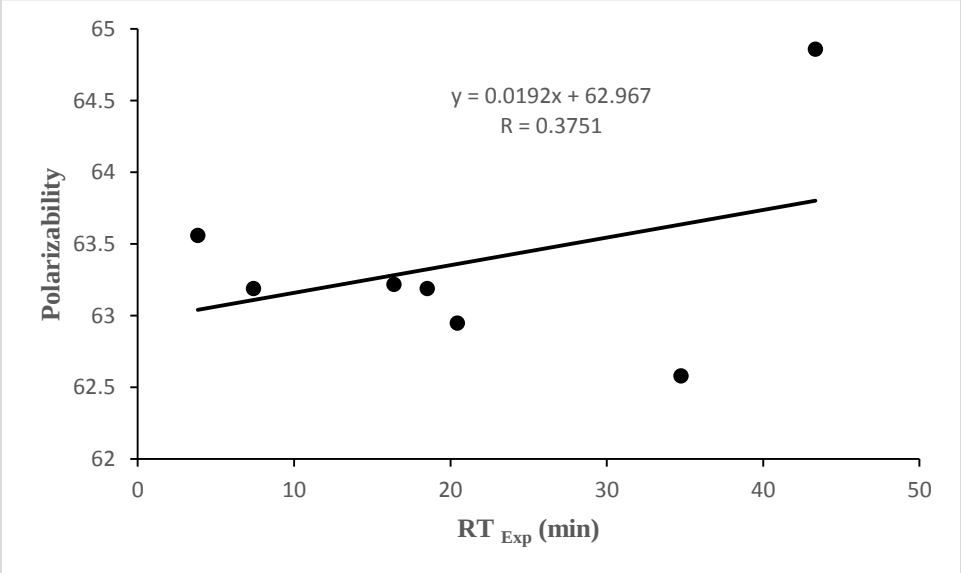
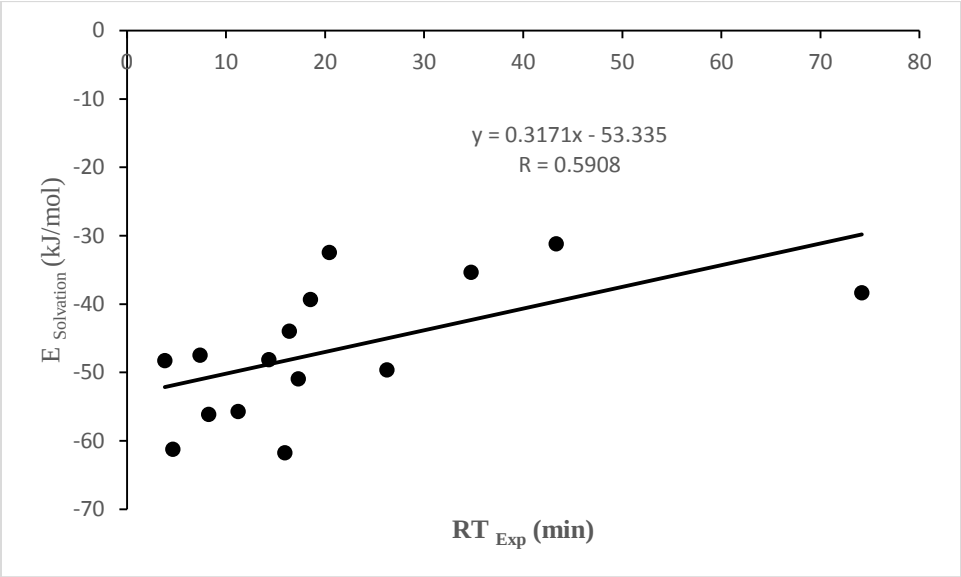


Figure 5.4 Adsorption model for (a) the combination of PGs and hormones; (b) hormones; (c) hormones: Polarizability vs. $RT_{exp.}$ (min)

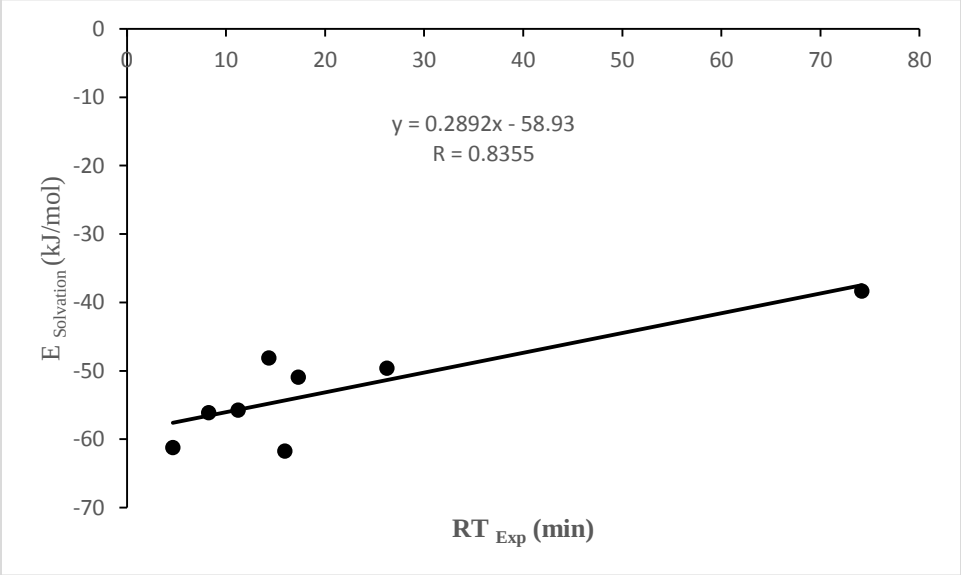
5.6.2.3 Linear solvation energy correlation model

Solvation energy is related to the difficulty in surrounding solutes by solvent molecules. In HPLC, when the analytes migrate from the stationary phase to the mobile phase, the analyte molecules will be surrounded by solvent molecules to travel along the column, and the system will lose equilibrium temporarily as more analyte molecules transfer from the stationary phase to the mobile phase. All analytes are soluble, and stationary phase with long carbon chains is solvated to analytes. The solvation energy of analytes would affect their retention behavior [23, 24]. The plots of solvation energy versus retention on the combination of prostanoids and sex hormones, prostanoids and sex hormones were shown in Figure 5.4 a, b and c. From the graphs, we can see that the retention time increased with increasing solvation energy, that the retention time of prostanoids was correlated with the solvation energy with R = 0.8355, and the retention time of sex hormones was correlated with the solvation energy with R = 0.8698. When correlation analysis was applied to the combination of prostanoids and sex hormones, it showed less correlation to solvation energy, with R= 0.5908. The reduced correlation in the combination may be due to the large difference in molecular structure between prostanoids and sex hormones. Our results showed that the correlation analysis should be performed either on prostanoids or on sex hormones, but not on their combination, and that the solvation energies of analytes played an important role in their retention behavior in HPLC.

(a)


y = 0.3171x - 53.335
R = 0.5908
E Solvation (kJ/mol)
RT Exp (min)

(b)


y = 0.2892x - 58.93
R = 0.8355
E Solvation (kJ/mol)
RT Exp (min)

(c)

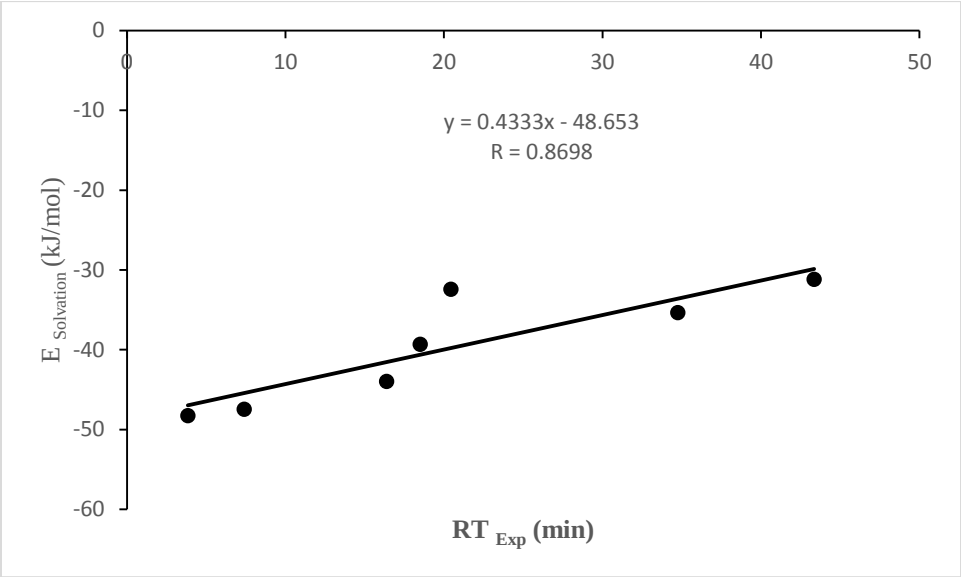


Figure 5.5 Solvation model for (a) the combination of PGs and hormones; (b) hormones; (c) hormones: $E_{Solvation}$ vs. RT_{exp} (min)

5.7 Multiple linear regression (MLR) on selective molecular descriptors

In the study, the statistical software Medcalc was used to apply MLR analysis on the targeted analytes. The MLR regression equation had a general format

$y = b_0 + b_1x_1 + b_2x_2 + \cdots + b_nx_n$,

where y was the dependent variable, x_i was an independent variable, b_0 was the intercept, and b_i was the regression coefficient for x_i.

The method of least squares can be used to solve the multiple linear equation. In the method, a "best fitting" line is drawn, where some of data points lie above and some lie below the line. According to the method of least squares, the fitting line can be expressed as

$$\hat{y} - b_0 = b_1(x_1 - \bar{x}_1) + b_2(x_2 - \bar{x}_2) + \cdots + b_k(x_k - \bar{x}_k)$$

Where a set of data points can be expressed as (x_{11}, …, x_{1k}, y_1), … , (x_{n1}, …, x_{nk}, y_n). In order to find a best fitting line, we have to find the values of the b_j coefficients to obtain the minimum of the sum of the squares as below:

$$\sum_{i=1}^{n}(\hat{y}_i - y_i)^2$$

where $\hat{y}_i$ is the y-value on the best fitting line corresponding to $x_{11}, \ldots, x_{ik}$.

Because of complexity of retention mechanism, the MLR analysis was performed on the multiple molecular descriptors of prostanoids and sex hormones calculated by the computational software Spartan. By selecting different combinations of molecular descriptors, we could obtain different regression equations to predict the retention behavior of analytes, where some equations offered a better correlation coefficient than the others.

In the current study, MLR was applied to the selected molecular descriptors on the combination of prostanoids and sex hormones, the group of prostanoids, and the group of sex hormones, respectively. By comparing regression coefficients, the equation with the best linear regression was selected to predict the retention time of analytes.

5.7.1 Combination of prostanoids and sex hormones

From Spartan software, eight molecular descriptors were obtained including $E_{solvation}$, E_{HOMO}, E_{LUMO}, dipole moment, area, volume, log P and polarizability. Different molecular descriptors were selected to find MLR equations with the best regression coefficient. The MLR analysis was first applied to the combination of prostanoids and sex hormones. Table 5.3 showed the results obtained from different combination of molecular descriptors.

From Table 5.3, we could see that the regression coefficient (R) was 0.878, when solvation energy, dipole moment and log P were selected to find the equation between the structure of analytes and the retention time. When E_{LUMO} was added into the above combination, the regression got improved with the value of R equal to 0.926. Then later, the different combination of molecular descriptors were performed, we obtain the values of equal to 0.973, 0.949 and 0.950, respectively. Comparing with all the values of R, the best regression was R=0.973, where all the molecular descriptors were selected except the molecular volume. It indicated that more parameters related to the structure of analytes would be helpful to develop a theoretical model to predict retention behavior.

Later, the resultant QSRR equation was used to calculate the retention time of prostanoids and sex hormones. By plotting the retention time calculated with this QSSR equation against the experiemental RT, a graph with $R^2 = 0.9458$ was shown in Figure 5.5.

Intercept	$E_{solvation}$ (aq)	E_{HOMO}	E_{LUMO}	Dipole moment	Area	Volume	LogP	Polarizability	R
	kJ/mol	eV	eV	debye	Å2	Å3			
46.7111	1.2522			-1.092			13.105		0.878
36.6768	0.7227		-29.099	-1.748			13.321		0.926
15.267	0.9829	46.949	-56.408	5.7074	0.239		7.085	5.9628	0.973
0.1593	1.3567		-41.303			0.2563	4.9578		0.949
-130.72	1.3464		-40.793				5.0541	3.2568	0.95

Table 5.3 MLR coefficients for the combination of PGs and sex hormones

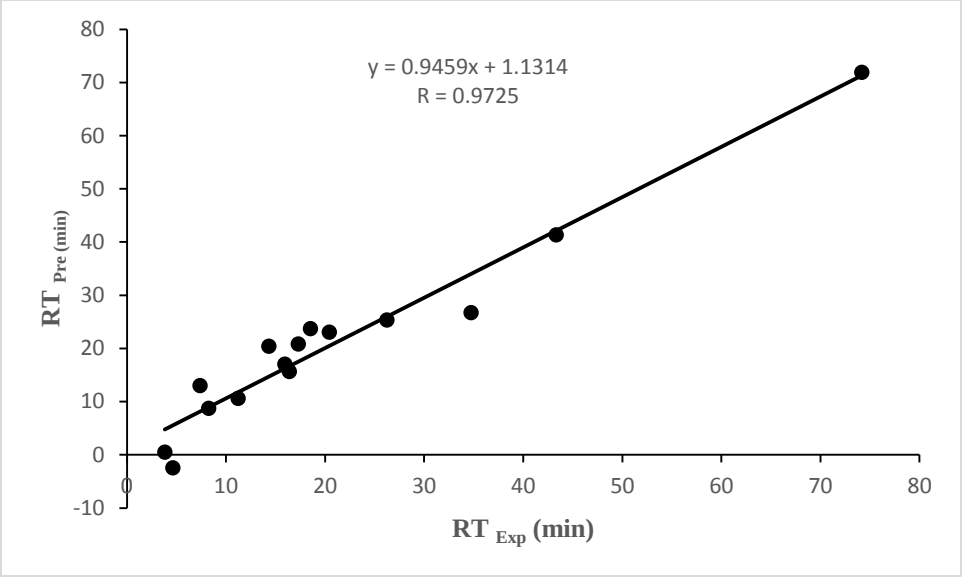


Figure 5.6 Chromatographically determined RT (min) for PGs and hormones vs. theoretically determined RT (min)

According to the reference from Zhou [20], the unbiased ability (U) of modelling was calculated based on the following equation:

$$U = 1 - \frac{|\theta - 45^\circ|}{45^\circ}$$

Where θ was the angle of the line fitting the predicted *vs.* experimental retention times of analytes. In Zhou's reliability standard [20], the systematic error was significant when U was less than 0.889.

The model 1 equation was listed below

Calculated RRT = 15.267+ 7.085 (log P) +5.7074 (dipole moment) + 0.9829 ($E_{solvation}$) +46.9489 (E_{HOMO}) -56.4084(E_{LUMO}) +0.239 (area) +5.9628 (polarizability) (1)

Compounds	RT $_{Exp.}$ min	RT $_{model\ 1}$ min
E_3	3.84	0.50
6-keto $PGF_{1\alpha}$	4.63	-2.45
16α-OHE_1	7.39	13.00
8-iso $PGF_{2\alpha}$	8.24	8.75
$PGF_{2\alpha}$	11.21	10.60
PGE_2	14.32	20.39
11-dehydro TXB_2	15.94	17.02
PGD_2	17.28	20.85
2-OHE_1	16.38	15.65
4-OHE_1	18.51	23.71
E_2	20.43	23.07
13,14-dihydro-15-keto-PGE_2	26.26	25.38
E_1	34.74	26.74
2-$MeOE_1$	43.34	41.35
13,14-dihydro-15-keto-PGA_2	74.18	71.94

Table 5.4 Predicted retention time from Model 1 (combination)

From Table 5.4, we could see that the retention time of E_3, 6-keto $PGF_{1\alpha}$ and 16α-OHE_1 did not match well with the experimental retention time.

5.7.2 Prostanoids

Different molecular descriptors were selected to find MLR equations with the best regression coefficient. The analysis of MLR was applied to the group of prostanoids. Table 5.5 shows the results obtained from different combinations of molecular descriptors.

From Table 5.5, we could see that the regression coefficient R was 0.947 when $E_{solvation}$, dipole moment and log P were selected to find the equation between the structure

of analytes and the retention time. When E_{LUMO} was added into the above combination, the regression improved to R=0.981. For various combinations of molecular descriptors, we obtained values of R equal to 0.989, 0.984, 0.992, 0.990 and 0.991 respectively. Comparing all the values of R, the best regression was R=0.992, where all the molecular descriptors were selected except the polarizability.

Later, the resultant QSRR equation was used to calculate the retention time of the prostanoids. By plotting the retention time calculated with this QSRR equation against the experimental RT, a graph with R = 0.9915 is shown in Figure 5.7.

Intercept	$E_{solvation}$ (aq)	E_{HOMO}	E_{LUMO}	Dipole moment	Area	Volume	LogP	Polarizability	R
	kJ/mol	eV	eV	debye	Å2	Å3			
-259.64	-2.4693			-5.699			51.715		0.947
-145.18	-1.7302		-35.358	-4.666			33.682		0.981
-420.52	-1.4334		-43.271	-3.381	0.682		34.442		0.989
-9874.6	1.7397			-7.556	-1.69		93.803	147.85	0.984
1057.85	3.6277	111.8	-107.83	19.29	0.927		-34.31		0.992
9931.11	-4.9952		-86.732	0.9818	3.212		-28.92	-161.67	0.99
944.923	-2.8464		-54.34		1.852	-6.959	-0.105	12.529	0.991

Table 5.5 MLR coefficients for prostanoids

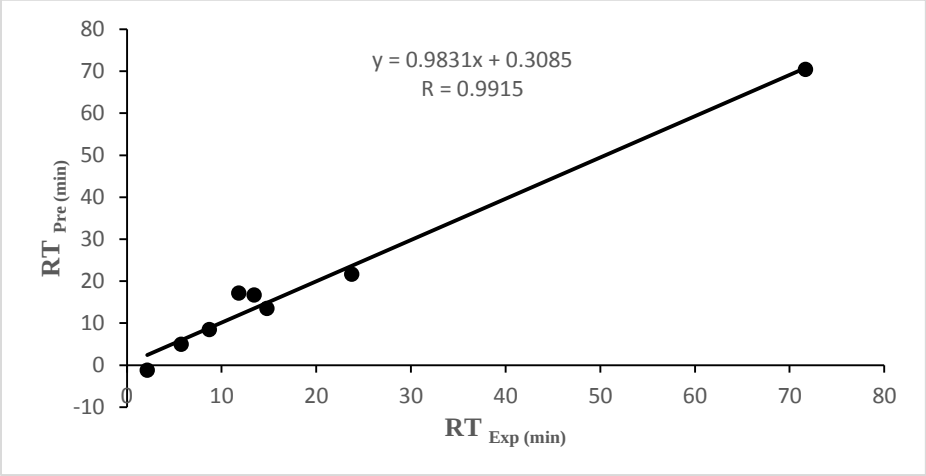


Figure 5.7. Chromatographically determined RT (min) for PGs vs. theoretically determined RT (min)

The predicted Retention time results were listed in Table 5.6. In order to describe easily the equation used in the study, we called it the Model 2a equation. The model 2a equation was listed below

Calculated RT = 1057.8541-34.3068 (log P) + 19.2904 (dipole moment) + 3.6277 ($E_{solvation}$) + 111.8002 (E_{HOMO}) -107.8308 (E_{LUMO}) + 0.9266 (area) (2a)

Compounds	RT min	Cal RT
6-keto $PGF_{1\alpha}$	4.63	-1.15
8-iso $PGF_{2\alpha}$	8.24	4.98
$PGF_{2\alpha}$	11.21	8.48
PGE_2	14.32	17.22
11-dehydro TXB_2	15.94	16.73
PGD_2	17.28	13.55
13,14-dihydro-15-keto-PGE_2	26.26	21.69
13,14-dihydro-15-keto-PGA_2	74.18	70.45

Table 5.6 Predicted Retention time from Model 2a (PGs)

5.7.3 Sex hormones

Different molecular descriptors were selected to find the MLR equations with the best regression coefficient. The analysis of MLR was applied to the group of sex hormones. Table 5.7 showed the results obtained from different combinations of molecular descriptors.

Intercept	$E_{solvation}$	E_{HOMO}	E_{LUMO}	Dipole moment	Area	Volume	Log P	Polarizability	R
47.2692	1.1546			-6.31			16.315		0.913
64.4675	1.257		-49.7626	-6.244			17.917		0.944
-1055.3	2.0229	-133.1	-79.5337	-19.75			27.593		0.963
-189.29	0.8519		-56.9446	-3.675		0.7528	24.285		0.982
-162.49	0.9316		-56.5984	-3.614	0.677		23.477		0.981
-8533.8	0.7617	-21.82				-16.04	20.464	207.19	0.969

Table 5.7 MLR coefficients for sex hormones

From Table 5.7, we could see that the regression coefficient R was 0.913, when $E_{solvation}$, dipole moment and log P were selected to find the equation between the structure of analytes and the retention time. When E_{LUMO} was added into the above combination, the regression improved to R=0.944. As different combinations of molecular descriptors were analyzed, we obtained values of R equal to 0.963, 0.982, 0.981, and 0.969, respectively. Comparing all the values of R, the best regression was R=0.982, where all the molecular descriptors were selected except the polarizability.

Later, the resultant QSRR equation was used to calculate the retention time of sex hormones. By plotting the retention time calculated with this QSRR equation against the experimental RT, a graph with R = 0.9818 was shown in Figure 5.8.

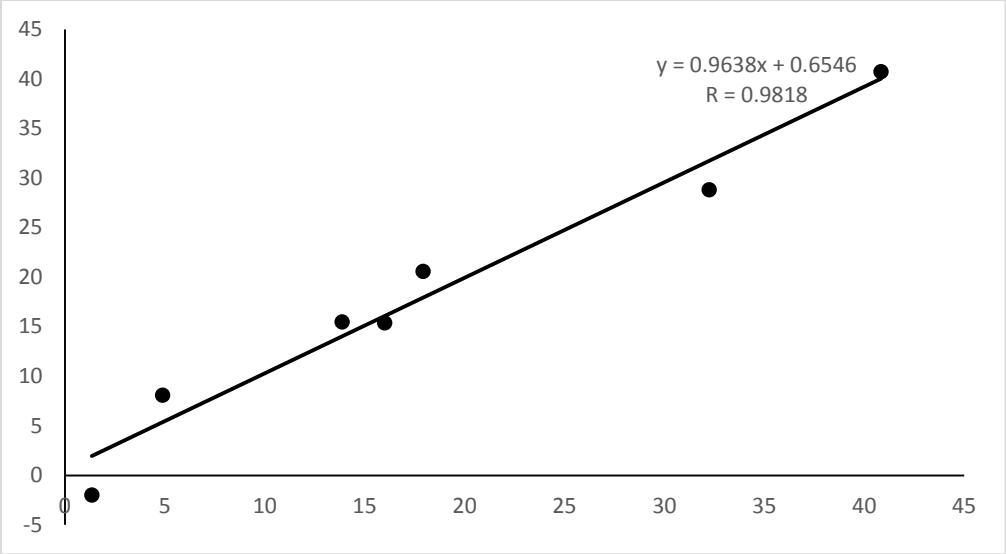


Figure 5.8 Chromatographically determined RT (min) for hormones vs. theoretically determined RT (min)

The Predicted Retention time results are listed in Table 5.8. In order to conveniently refer to the equation used in the study, we called it the Model 2b equation. The model 2b equation was listed below

Calculated RT = -189.2868 +24.2852 (log P)-3.6753 (dipole moment) + 0.8519 ($E_{solvation}$) -56.9446 (E_{LUMO}) +0.7528(volume) (2b)

Compound	RT min	Cal RT
E_3	3.84	-2.00
16α-OHE_1	7.39	8.10
2-OHE_1	16.38	15.49
4-OHE_1	18.51	15.37
E_2	20.43	20.58
E_1	34.74	28.83
2-$MeOE_1$	43.34	40.73

Table 5.8 Predicted retention time from Model 2b (Hormones)

5.8 Modification of MLR model

From the predicted Retention time results in Model 1 and model 2, we could see that both of models did not give good results, where six compounds showed good results in Model 1, and five compounds in Model 2 did not show satisfactory predicted retention time. A modification of the MLR model had to be made to improve the accuracy of the prediction.

By carefully observing the experimental retention time and the chemical structures of prostanoids and sex hormones, we found that the retention times of analytes were affected by the number and the position of OH groups, and the number of C=O groups. The more OH groups in compounds, the less retained in column. The more electron-withdrawing groups, the more polar the analyte was. From the structure of analytes, we could see that E_3, 6-keto $PGF_{1\alpha}$, 8-iso $PGF_{2\alpha}$ and $PGF_{2\alpha}$ had three OH groups, they were the most polar compounds among the analytes, and eluted first. However, 6-keto $PGF_{1\alpha}$ had one more C=O group comparing E_3, 8-iso $PGF_{2\alpha}$ and $PGF_{2\alpha}$, therefore 6-keto $PGF_{1\alpha}$ was more polar than the others, and eluted first; the compound without OH group, 13,14-dihydro-15-keto-PGA_2, was the least polar and eluted last; cis-8-iso $PGF_{2\alpha}$ eluted earlier than trans-PGF_{2a}. 16α-OHE_1, 2-OHE_1 and 4-OHE_1 had two OH groups, where one of OH groups in 16α-OHE_1 was located on a five-membered ring with an electron-withdrawing group nearby, thus making 16α-OHE_1 more polar than 2-OHE_1 and 4-OHE_1 and eluted earlier; 2-OHE_1 eluted earlier than 4-OHE_1 due to OH groups located on the meta position of a benzene ring. The major difference between prostanoids and sex hormones was the number of rings in molecular structure.

After carefully observing the structure of analytes, six more molecular descriptors listed in Table 5.9 were introduced into Model 1 including the number of carbon atoms (No. of C), molecular weight (M.W.), the number of OH groups (No. of OH group), the number of C=O groups (No. of C=O), the number of COO groups (No of COO), and the number of rings (No of ring). Model 3 was obtained by adding No. of C, M.W. and No. of OH group into Model 1, and R was improved from 0.9725 to 0.9913. When No. of C=O was added into Model 3 to form Model 4, the linearity got improved a little with R = 0.9916. However, when No. of COO was added into Model 4 to form Model 5, the value of R decreased to 0.9894 which indicated that No. of COO may be not a proper molecular descriptor for retention model. In Model 6, No. of ring was added into Model 4, and the resulting R was 0.9925. When all six molecular descriptors were added into Model 1 to form Model 7, the greatest R was obtained with the value of 0.9935. The intercept and coefficients of molecular descriptors from Model 3 to Model 7 were listed into Table 5.10. Model 7 did not give a good prediction of E_3 and 6-keto $PGF_{1\alpha}$ possibly due to the formation of hydrogen bonds between molecules and inside molecules. Based on the reliability standard proposed by Zhou [20], Models 3-7 had the values of U greater than 0.889, which indicates that the system errors of our models 3-7 are small, and can be negligible. The statistical analysis of t-test was applied to the linear regression lines obtained from Model 3-7 and the ideal linear regression line when the slope equal to 1 to compare the slope obtained from Models 3-7 and the slope equal to 1, respectively. The resultant values of t and p from Models 3-7 were listed in Table 5.10. From Table 5.10, we can see that all the values of p from Models 3-7 are in the range of 0.6 to 0.7, and are greater than 0.05, which indicates that the difference between two slopes is significantly small.

Both the estimation of the unbiased ability (U) and the statistical t-test are proved that our models are reliable to predict retention times for unknown analytes.

Compound	RT exp min	No. of C	M.W.	No. of OH	No of CO	No. of COO	No. of ring
E_3	3.842	18	288.39	3	0	0	4
6-keto $PGF_{1\alpha}$	4.633	20	370.49	3	1	1	1
16a-OHE_1	7.392	18	286.37	2	1	0	4
8-iso $PGF_{2\alpha}$	8.242	20	354.49	3	0	1	1
$PGF_{2\alpha}$	11.208	20	354.49	3	0	1	1
PGE_2	14.317	20	352.47	2	1	1	1
11-dehydro TXB_2	15.942	20	368.47	2	0	2	1
PGD_2	17.283	20	352.47	2	1	1	1
2-OHE_1	16.375	18	286.37	2	1	0	4
4-OHE_1	18.508	18	286.37	2	1	0	4
E_2	20.433	18	272.39	2	0	0	4
13,14-dihydro-15-keto-PGE_2	26.255	20	352.47	1	2	1	1
E1	34.742	18	270.37	1	1	0	4
2-$MeOE_1$	43.342	19	300.398	1	1	0	4
13,14-dihydro-15-keto-PGA_2	74.183	20	334.46	0	2	1	1

Table 5.9 Parameters of molecular descriptors

Based on Models 3-7, we obtained the corresponding Equations 3-7 to predict retention time.

Calculated RT = 1396.5013 - 0.5371 ($E_{solvation}$) + 30.9837 (E_{HOMO}) -60.0123 (E_{LUMO}) + 5.9174 (dipole moment) + 0.1957 (area) -28.3127 (log P) + 44.255 (Polarizability) -6.7724 (No. of C) -3.4281 (M.W.) -20.9778 (No. of OH group) (3)

Calculated RT = -1543.7267-0.55 ($E_{solvation}$) + 34.6837 (E_{HOMO}) -61.4154 (E_{LUMO}) + 6.581 (dipole moment) + 0.08714 (area) -28.6316 (log P) + 48.7756 (Polarizability) -8.7556 (No. of C) -3.5769 (M.W.) -20.0523 (No. of OH group) +1.5619 (No. of CO) (4)

Calculated RT = -707.212-0.1262 ($E_{solvation}$) + 35.1851 (E_{HOMO}) -57.9652 (E_{LUMO}) + 4.7358 (dipole moment) + 0.01775 (area) -5.1097 (log P) + 41.2093 (Polarizability) -80.3401 (No. of C) -46.5122 (No. of OH group) -30.7952 (No. of CO) -65.7794 (No. of COO) (5)

Calculated RT = 353.2955-1.1756 ($E_{solvation}$) + 27.9758 (E_{HOMO}) -74.5581 (E_{LUMO}) + 9.0594 (dipole moment) + 0.92 (area) -71.2564 (log P) + 72.4952 (No. of C) -4.4531 (M.W.) -29.0198 (No. of OH group) -3.6286 (No. of CO) -67.0292 (No. of ring) (6)

Calculated RT = 1456.478-0.9317 ($E_{solvation}$) + 35.7537 (E_{HOMO}) -74.8817 (E_{LUMO}) + 7.6441 (dipole moment) + 0.6777 (area) -51.3498 (log P) -41.1351 (No. of C) -78.2869 (No. of OH group) -54.707 (No. of CO) -104.754 (No. of COO) -82.5329 (No. of ring) (7)

	Model 3	Model 4	Model 5	Model 6	Model 7
Intercept	-1396.501	-1543.7267	-707.212	353.2955	1456.48
solvation energy	-0.5371	-0.55	-0.1262	-1.1756	-0.9317
E_{HOMO}	30.9837	34.6837	35.1851	27.9758	35.7537
E_{LUMO}	-60.0123	-61.4154	-57.9652	-74.5581	-74.882
Dipole moment	5.9174	6.581	4.7358	9.0594	7.6441
Area	0.1957	0.08714	0.01775	0.92	0.6777
LogP	-28.3127	-28.6316	-5.1097	-71.2564	-51.35
Polarizability	44.255	48.7756	41.2093		
No. of C	-6.7724	-8.7556	-80.3401	72.4952	-41.135
M.W.	-3.4281	-3.5769		-4.4531	
No. of OH group	-20.9778	-20.0523	-46.5122	-29.0198	-78.287
No. of CO		1.5619	-30.7952	-3.6286	-54.707
No of COO			-65.7794		-104.75
No of ring				-67.0292	-82.533
R	0.9913	0.9916	0.9894	0.9925	0.9935
U	0.9889	0.9893	0.9864	0.9904	0.9916
t	-0.4782	-0.4637	-0.5285	-0.4459	-0.4141
p-value	0.6365	0.6467	0.6016	0.6594	0.6822

Table 5.10. MLR coefficients in Model 3-7

Compound	Model 3	Model 4	Model 5	Model 6	Model 7
E_3	6.27	5.7	5.24	6.73	6.55
6-keto $PGF_{1\alpha}$	0.87	0.97	1.54	0.04	1.02
16a-OHE_1	10.02	9.98	11.73	8.15	8.49
8-iso $PGF_{2\alpha}$	6.09	6.2	6.67	6.45	7.18
$PGF_{2\alpha}$	11.85	11.21	10.91	12.59	11.59
PGE_2	14.77	13.64	14.97	14.56	13.6
11-dehydro TXB_2	16.74	15.56	16.07	16.03	15.94
PGD_2	18.69	18.67	18.45	19.93	19.26
2-OHE_1	14.86	14.53	14.85	14.33	14.97
4-OHE_1	17.45	17.57	17.12	20.33	19.07
E_2	22.89	22.43	23.6	19.81	21.47
13,14-dihydro-15-keto-PGE_2	29.53	29.54	29.6	29.44	29.97
E_1	29.73	29.28	28.59	31.89	30.75
2-$MeOE_1$	43.61	43.23	43.61	43.33	43.34
13,14-dihydro-15-keto-PGA_2	73.5	73.29	73.67	72.95	73.52

Table 5.11. Predicted Retention time (min) based on Model 3-7

5.9 Prediction of retention time

Models 1, 2 and 7 were applied to predict the retention times of 15-deoxy PGJ_2 and progesterone. The predicted retention times are listed in Table 5.12. When the isocratic condition with a mobile phase of 35% 17mM H_3PO_4: 65% ACN was used to find the retention time of 15-deoxy-PGJ_2 and progesterone, there was no peak eluted after 120 min. Therefore, the retention time of 15-deoxy-PGJ_2 and progesterone should be more than 120 min. Because these two compounds had a retention time close to each other in the gradient method described in Chapter 3, the retention times of 15-deoxy PGJ_2 (174.8 min) and progesterone (155.2 min) in Model 2 were considered as good predictions. In Model 7 also gave a prediction of 15-deoxy PGJ_2 (137.47 min) and progesterone (170.94 min).

Compound	Calculated RT		
	Model 1	Model 2	Model 7
15-deoxy-PGJ_2	125.7	174.8	137.47
Progesterone	48.2	155.2	170.94

Table 5.12 Predicted Retention time of 15d-PGJ_2 and progesterone

In our study, the chromatographic mobile phase was isocratic. The properties of mobile phase such as dielectric constant kept constant during a single run. Dielectric constant is a value to measure the insulation ability of a substance to charges. In HPLC, the dielectric constant of mobile phase is related to the interactions of polar groups between analytes and mobile phase. The dielectric constant of mobile phase increases with the concentration of organic solvent decreasing. In our QSRR model, the effect of the dielectric constant of mobile phase is not accounted, which remains to be studied in more detail. Without this included explicitly, our model meets the reliability standard established by Zhou [20].

5.10 Conclusion

From our studies, it is possible to use a simple and common MLR method to develop a retention model to predict retention time of compounds with a satisfactory result. Molecular descriptors related to geometry, energy, hydrophobicity, polarity and numbers of featured substituent groups should be included into the model. In Model 7, thirteen molecular descriptors were included to form a multiple linear equation of retention time. In the future, a larger data set should be used to develop a retention model to predict

retention time of compounds with a big structural difference. Our study will help experimental researchers without strong computation background to develop a retention model. It has the advantages of saving solvent, research cost and experimental time.

5.11 References

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CHAPTER 6

STUDYING THE EFFECTS OF EXERCISE ON URINARY LEVELS OF PROSTANOIDS AND SEX HORMONES IN HYPERTENSIVE PATIENTS

6.1 Introduction

Hypertension is a chronic medical condition in which the arterial blood pressure is at or above the normal level of 140/90 mmHg. Elevated blood pressure is a primary contributor to severe cardiovascular disease, and can increase mortality risk. Based on data reported in 2013 from the National Health and Nutrition Examination Survey between 2007 and 2010, the American Heart Association has estimated that 33.0% of U.S. adults suffer from hypertension [1]. Many factors are considered to be involved in the development of hypertension such as life style, family disease history, gender, age and obesity. Compared with Caucasians, African Americans suffer a higher incidence of hypertension, especially in older age.

High blood pressure may promote a low-grade inflammation. Vascular wall inflammation and vascular stiffening with age are involved in the development and progression of hypertension [12]. Vascular wall inflammation will increase vascular stiffness and result in elevated blood pressure. During inflammation, the inflammatory mediators play an important role in inflammatory response. Prostanoids are short-chain fatty acids, and act as mediators to promote or suppress inflammation. PGD_2 induces vasodilatation and bronchoconstriction, and inhibits platelet aggregation. PGE_2 and $PGF_{2\alpha}$ are pro-inflammatory mediators. 6-keto-$PGF_{1\alpha}$ is a marker of PGI_2, which inhibits platelet aggregation and acts as a vasodilator. 8-iso-$PGF_{2\alpha}$ is a biomarker of oxidative stress and an effective vasoconstrictor. 11-dehydro TXB_2 is a stable metabolite of TXA_2, a marker to

assess platelet activation. 15d-PGJ_2 has an anti-inflammatory effect by inhibiting the NF-κB signaling pathway. 13,14-dihydro-15-keto PGE_2 is a marker to evaluate the biosynthesis and disposition of PGE_2 in plasma. 13,14-dihydro-15-keto PGA_2 is a byproduct of PGE_2 metabolism. Evaluating changes in the levels of inflammatory mediators will provide direct evidence to understand the mechanism of hypertension at the molecular level, to help predict hypertension at different stages, and to provide an effective treatment to prevent the progression of hypertension.

Compared with age-matched men, women before menopause have a lower incidence of hypertension. Female sex hormones are expected to play a protective role during disease development and progression. From research studies, it has been found that estrogens including E_1, E_2 and E_3 have an athero-protective effect by reducing the production of vascular reactive oxygen species. 16α-OHE_1 has a pro-inflammatory effect, where 2-OHE_1 and 2-$MeOE_1$ have strong anti-inflammatory effects. Moreover, female sex hormones can regulate the production of prostanoids to affect the inflammation process.

Physical exercise can reduce the risk of cardiovascular disease and diabetes [1, 2]. Ginsburg et al. found that in men estradiol with an antioxidant effect increased 58%, and testosterone reduced 58%, while in women, there was no significant change after exercise [3]. Chan et al. found that physical activity lowered endogenous testosterone and estradiol concentrations in postmenopausal women [4]. High levels of endogenous estradiol and testosterone were a predictor of increased breast cancer risk [5]. Martınez found that physical activity and low obesity reduced the concentration of PGE_2 in the rectal mucosa [6]. Lovatel et al found that exercise for improving learning and memory decreased the level of PGE_2 in hippocampal tissue in rats [7]. McTiernan found that exercise decreased

the concentrations of sex hormones including estrone, estradiol and testosterone in postmenopausal women [8]. Exercise can increase the production of reactive oxygen species (ROS), thus increasing the production of prostanoids. It has been found that the serum levels of prostacyclin (PGI_2) and PGE_2 increases after exercise. ROS and prostanoids together play a role in regulating blood pressure after exercise. Smith found that in premenopausal women, moderate aerobic exercise did not significantly change sex hormone levels [9]. Hawkins found that moderate-intensity aerobic exercise increased DHT and SHBG levels, but it did not change in middle-aged to older men [10]. Wilson found that exercise increased the release of vasodilatory $PGF_{1\alpha}$ and PGE_2 [11].

In this chapter, a method for separating the combination of prostanoids and sex hormones was applied to urine samples from hypertension patients. By studying the changes in the levels of prostanoids and sex hormones before and after exercise, the effective biomarkers of hypertension can be found to predict, prevent and treat hypertension.

6.2 Protocol

The physical exercise protocol was proposed by Dr. Brown's group. The procedure for collecting urine samples was described in their paper [12]. Ten African Americans volunteers from Baltimore, Maryland and Washington DC with pre-hypertension (pre-HTN) or stage I hypertension (systolic blood pressure: 143±7 and diastolic pressure 87±7 mm Hg) participated in the study. These participants had the following characteristics: Age – 57±3 yrs; BMI (Body Mass Index) – 30.46± 3.50.; VO_2 (oxygen consumption or aerobic capacity) (mL/kg) – 22.587 ±3.724. All of participants were not under anti-hypertensive medical treatment. Urine collected for a 24- hour period one week before the exercise

session started was used as baseline samples. After a period of exercise, post exercise (PE) urine samples were collected for a 24-hour period. All urine samples were in a freezer at -80°C.

6.3 Instrumental analysis

The urine samples were prepared by solid phase extraction before injection to LC-MS. The instrumental set-up was as described in Chapter 4. Prostanoids and sex hormones were determined and quantified by LC-MS.

6.4 Results

6.4.1 Prostanoids

By using the LC-ESI-MS method, most of the prostanoids in the urine samples were detected and quantified, including 6-keto $PGF_{1\alpha}$, 11-dehydro TXB_2, 8-iso $PGF_{2\alpha}$, $PGF_{2\alpha}$, PGE_2 and 15-d PGJ_2. However, some prostanoids had very low concentrations, and did not show changes before and after exercise. Here we focus on 6-keto $PGF_{1\alpha}$, 11-dehydro TXB_2, 8-iso $PGF_{2\alpha}$ and $PGF_{2\alpha}$. The concentrations of urinary prostanoids may vary due to a hydration effect. Therefore, the level of 6-keto $PGF_{1\alpha}$, 11-dehydro TXB_2, 8-iso $PGF_{2\alpha}$ and $PGF_{2\alpha}$ were normalized to the urinary creatinine (ng/μg) level, where the data of creatinine level in urine samples were collected by Varma in Dr. Jansen's group.

Table 6.1 listed the normalized concentration of urinary 6-keto $PGF_{1\alpha}$ and 11-dehydro TXB_2 with creatinine before and after acute exercise. It shows that in four participants, the level of 6-keto $PGF_{1\alpha}$ was reduced after exercise, while the other two people had an increased level of 6-keto $PGF_{1\alpha}$. For 11-dehydro TXB_2, there were three

people with a reduced level of 11-dehydro TXB_2 after exercise, two with increased levels, and one under a detectable level. It is interesting that after exercise, both of the levels of 6-keto $PGF_{1\alpha}$ and 11-dehydro TXB_2 either decreased or increased at the same time.

Subject No.	6-keto $PGF_{1\alpha}$/creatinine			11-dehydro TXB_2/creatinine		
	Baseline	Post-Exercise	Change	Baseline	Post-Exercise	Change
1	345.5	20.26	-	125	12.53	-
2	2.75	1.64	-	6.51	5.73	-
3	25.54	7.1	-	13.5	8.03	-
4	23.42	6.42	-	Not detected	61.28	Not detected
5	3.63	38.2	+	18.1	114.2	+
6	6.36	23.57	+	6.08	70.23	+

Table 6.1. Urinary levels of 6-keto PGF1α and 11-dehydro TXB_2 normalized by creatinine × 10^6

Note: B= baseline; PE = Post exercise

Subject No.	8-iso $PGF_{2\alpha}$/creatinine		
	Baseline	Post-Exercise	Change
1	84.4	5.19	-
2	3.01	Not detected	Not detected
3	4.79	7.13	+
4	9.35	2.13	-
5	28.68	10.58	-
6	Not detected	12.93	Not detected

Table 6.2. Urinary levels of 8-iso $PGF_{2\alpha}$ normalized by creatinine × 10^6

Table 6.2 listed the normalized concentration of urinary 8-iso $PGF_{2\alpha}$ with creatinine before and after acute exercise. It shows that three people had a reduced level of 8-iso $PGF_{2\alpha}$ after exercise, with only one person with an increased level, and two people under a detectable level.

Subject No.	$PGF_{2\alpha}$/creatinine		
	Baseline	Post-Exercise	Change
1	656.6	53.2	-
2	3.69	5.46	+
3	8.01	4.75	-
4	9.03	4.09	-
5	23.2	21.5	-
6	1.91	17	+

Table 6.3 Urinary levels of $PGF_{2\alpha}$ normalized by creatinine × 10^6

From Table 6.3, we can see that after exercise, there were four people with a reduced level of $PGF_{2\alpha}$, where the other two people showed an increased level.

6.4.2 Female sex hormones

In LC-ESI-MS positive mode, eight sex hormones including E_1, E_2, E_3 and 16a-OHE_1, 2-OHE_1, 4-OHE_1, and progesterone were determined in a single run. Female sex hormones may have a different effect on men from on women. Therefore, we will discuss the level change of female sex hormones on the basis of gender.

Table 6.4 lists the change in levels of E_2 after exercise. E_2 is a vasodilator and has an effect in lowering blood pressure. From Table 6.3, we can see that among three women, one woman had a 2-fold reduced level of E_2 after exercise, while the other two women had

too low a level of E_2 to be detected. Among the three men, after exercise, one had an increased level of E_2, and the other two had a reduced level.

Subject No.	Gender	E_2/creatinine		
		Baseline	Post-Exercise	Change
1	F	9.05	4.51	-
2	F	Not detected	Not detected	Not detected
3	F	49.91	Not detected	Not detected
4	M	37.65	3.72	-
5	M	182.58	2.55	-
6	M	0.11	11.793	+

Table 6.4. Urinary levels of E_2 normalized by creatinine × 10^5

Subject No.	Gender	2-OHE_1/4-OHE_1		
		Baseline	Post-Exercise	Change
1	F	6.92	5.39	-
2	F	6.29	5.77	-
3	F	7.07	2.45	-
4	M	3.95	14.95	+
5	M	0.02	10.51	+
6	M	2.68	1.39	-

Table 6.5 Ratio of 2-OHE_1 to 4-OHE_1 in urine

E_1 has several metabolites including 2-OHE_1, 4-OHE_1, 2-methoxyE_1 and 16α-OHE_1 after hydrolysis. These metabolites are isomers. The position of the OH group on

the molecular structure decides the clinical effect of the metabolites. For example, 4-OHE_1 and 16α-OHE_1 have a pro-inflammatory effect, while 2-OHE_1 and 2-methoxyE_1 have an anti-inflammatory effect. Table 6.5 listed the ratio change between 2-OHE_1 and 4-OHE_1. We can see that in women after exercise, the ratio between 2-OHE_1 and 4-OHE_1 decreased a little bit, and a shift from 2-OHE_1 to 4-OHE_1 occurred, which indicates that exercise induced a pro-inflammatory signal in women. In men, two had an increased ratio between 2-OHE_1 to 4-OHE_1, and a shift from 4-OHE_1 to 2-OHE_1 occurs, which indicates exercise has a helpful effect in inhibiting inflammation in men. One man has a decreased ratio change, which indicates that exercise may play a pro-inflammatory role for him. The contradictory results suggest that differences between individuals result in different effects of exercise on inflammation.

Subject No.	**Gender**	2-MethoxyE_1/creatinine		
		Baseline	Post-Exercise	Change
1	F	19.94	5.78	-
2	F	0.08	0.04	-
3	F	1.46	0.16	-
4	M	1.16	0.64	-
5	M	18.41	1.16	-
6	M	0.009	0.52	+

Table 6.6 Urinary levels of 2-MethoxyE_1 normalized by creatinine × 10^5

2-methoxyE_1 has an anti-inflammatory effect during inflammation. From Table 6.6, we can see that after exercise, three women and two men had a reduced level of 2-methoxy E_1, and the change is significant, while only one man had an increased level.

6.5 Discussion

6-keto-$PGF_{1\alpha}$ is a biomarker of PGI_2, which inhibits platelet aggregation. Our results show that the level of 6-keto-$PGF_{1\alpha}$ increased in some people following exercise and decreased in others. Exercise may play a beneficial role for some elderly people by decreasing the activity of platelet aggregation. But it may play an opposite role in others. It is interesting that when the level of 6-keto-$PGF_{1\alpha}$ decreased, the level of 11-dehyro TXB_2, a marker to assess platelet activation, is also reduced. This indicates that there is a balance between 6-keto-$PGF_{1\alpha}$ and 11-dehyro TXB_2. Whether exercise inhibits inflammation or not after exercise depends on the balance between 6-keto-$PGF_{1\alpha}$ and 11-dehyro TXB_2. In addition, the beneficial role of exercise may also depend on the body condition of individuals such as diet and obesity.

8-iso-$PGF_{2\alpha}$ is a biomarker of oxidative stress. Our study shows that after exercise, the level of 8-iso-$PGF_{2\alpha}$ decreased, which indicates that exercise reduced oxidative stress, thus deactivating cytokines to inhibit inflammatory signals and lower blood pressure.

After exercise, there were four people with a reduced level of $PGF_{2\alpha}$, which indicates that exercise may reduce the pro-inflammatory effect.

We also study the female sex hormone level change before and after exercise. Exercise did not promote the production of E_2 in woman, however it increases the production of E_2 in one man, but decrease in other men. This suggests that differences in body condition such as age and diet among men may results in the inconsistent results. Exercise promotes the shift from 2-OHE_1 to 4-OHE_1 in women, and induces a pro-inflammatory signal. However, exercise promotes a shift between 2-OHE_1 and 4-OHE_1 in men in both ways.

6.6 Conclusion

In this study, it was found that exercise changed the level of prostanoids such as 6-keto $PGF_{1\alpha}$, 11-dehydro TXB_2 and 8-iso PGF_{2a}. After exercise, a shift from 2-OHE_1 to 4-OHE_1 occurred in women to promote inflammation, however, an opposite shift happened to men to inhibit inflammation. It indicates that gender really plays a role in affecting the effect of exercise. The shortcoming of the study is that the sample size is too small. In the future, a larger population should be involved in the study to obtain a statistically significant result.

6.7 References

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