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Assay Profiles of Prostanoids and Female Sex Hormones for Studying Potential Biomarkers of Hypertension

Guoxiu Wei[1], Susan A Jansen[1]*, Sheara Williamson[2] and Michael Brown[2]

[1]*Department of Chemistry, Temple University, 1901 N. 13th St., Philadelphia, PA 19122, USA*
[2]*Department of Kinesiology, Hypertension, Molecular and Applied Physiology Laboratory, Temple University, USA*

Abstract

Aim: Prostanoids and female sex hormones play an important role in the progression of hypertension. However, their close-related structures make simultaneous separation and analyses difficult. A method was developed to separate and analyze seventeen important bioactive compounds including nine prostanoids and eight sex hormones in a short time. By applying the method to quantify prostanoids and female sex hormones in urine samples from hypertension patients, it becomes possible to find effective biomarkers for clinical diagnosis, prevention and treatment of hypertension.

Materials and methods: A reversed phase high performance liquid chromatography is used to develop the separation method. A combination of up to nine prostanoids (PGD2, PGE2, 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 PGE2 and 15-deoxy $\Delta^{12,14}$ PGJ_2) and eight female sex hormones (E_1, E_2, E_3, progesterone, 2-OHE1, 4-OHE1, 16α-OHE1, and 2-$MeOE_1$) are chosen as analytes. The separation is performed on Symmetry C18 4.6x250 mm column with 5 µm particle size. A UV detector is selected to determine the levels of analytes in a single 25-minute run.

Results: The method is the first we are aware of successfully demonstrating the separation of the two different groups of bioactive compounds prostanoids and female sex hormones. It has been shown to have excellent selectivity, sensitivity, and accuracy over biologically relevant concentration ranges and has been tested on urine samples from hypertension patients.

Keywords: Hypertension; Prostanoid; Female sex hormones; Urine

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).

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 [2,3]. 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 [4]. They are also lipid inflammatory mediators, and can interact with hypertension development and signal endothelial dysfunction involved in hypertension [5]. 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 [6,7], gas chromatography–mass spectrometry (GC-MS) and GC-MS/MS [8,9], and high-performance liquid chromatography with fluorescence (HPLC-FL) detection [10]. 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 [11-13]. 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

***Corresponding author:** Susan A Jansen, Department of Chemistry, Temple University, 1901 N. 13th St., Philadelphia, PA 19122, USA, Tel: +1 215 204 6390; Fax: +1 215 204 1532;E-mail: suebee@temple.edu

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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.

Experimental

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.

Instrumental Setup

The high performance liquid chromatography (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.

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.

Urine sample preparation

The urine samples were prepared with solid state extraction according to the published procedures in Blewett's paper [14]. 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.

Sample preparation for method validation

According to the FDA's guidance on validation of bioanalytical methods [15], 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

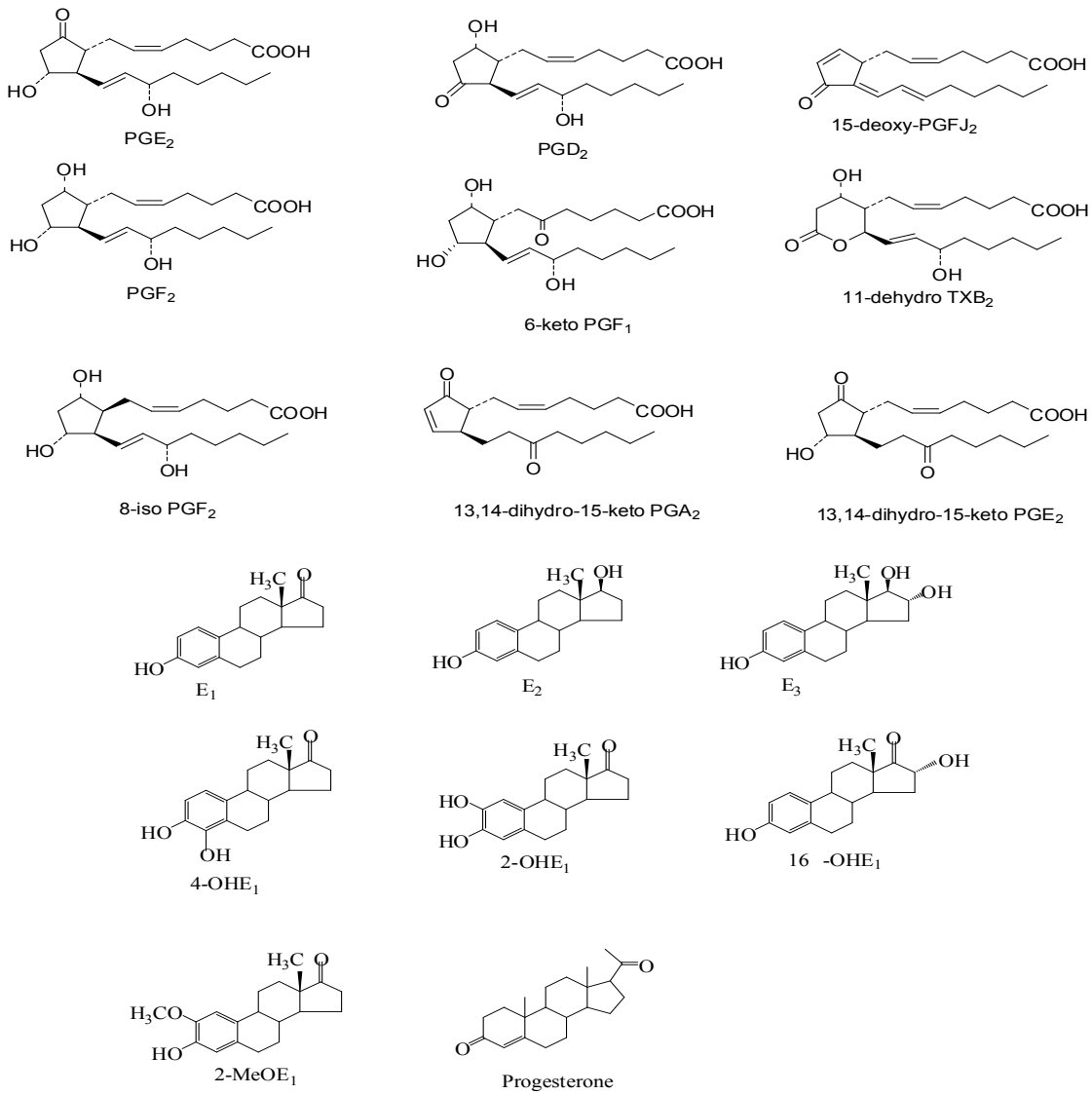

Figure 1: Immunohistochemical staining of removed adrenal gland using the anti-IL-6 antibody (x20). Positive staining was observed at the surrounded cells of the tumor.

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.

Results and Discussion

Development of HPLC-UV method

Nine prostanoids and eight female sex hormones were separated by a Symmetry C18 column (4.6 mm×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 2. The elution order was identified by injecting each individual

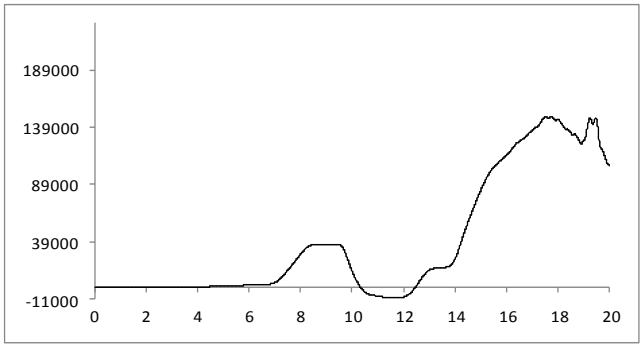

Figure 2A: Chromatogram of solvent blank.

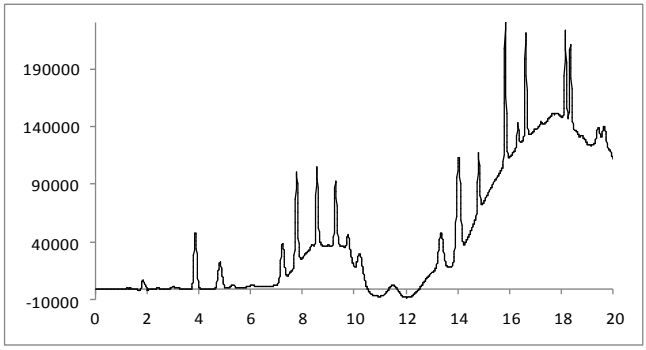

Figure 2B: Chromatogram of a standard mixture containing nine prostanoids and eight female sex hormones: 1. E_3; 2. 6-keto-PGF_1; 3. 16α-OHE_1; 4. 8-iso-$PGF_{2\alpha}$; 5. $PGF_{2\alpha}$; 6. PGE_2; 7. 11-dehydro-TXB_2; 8. PGD_2; 9. 2-OHE_1; 10. 4-OHE_1; 11. E_2; 12. 13,14-dihydro-15-keto-PGE_2; 13. E_1; 14. 2-$MeOE_1$; 15. 13,14-dihydro-15-keto-PGA_2, 16. 15d-PGJ_2; 17. Progesterone.

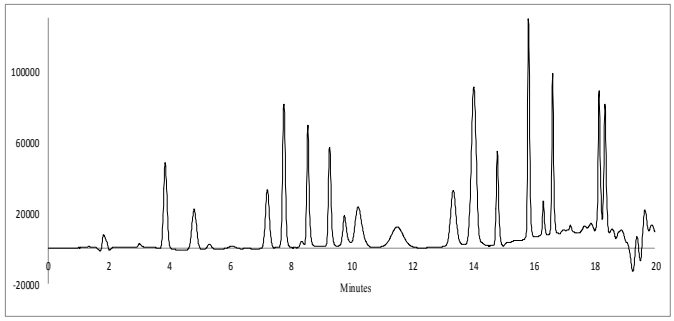

Figure 2C: Chromatogram of a standard mixture containing nine prostanoids and eight female sex hormones after subtracting solvent blank: 1. E_3; 2. 6-keto-$PGF_{1\alpha}$; 3. 16α-OHE_1; 4. 8-iso-$PGF_{2\alpha}$; 5. $PGF_{2\alpha}$; 6. PGE_2; 7. 11-dehydro-TXB_2; 8. PGD_2; 9. 2-OHE_1; 10. 4-OHE_1; 11. E_2; 12. 13,14-dihydro-15-keto-PGE_2; 13. E_1; 14. 2-$MeOE_1$; 15. 13,14-dihydro-15-keto-PGA_2, 16. 15d-PGJ_2; 17.

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, E1, 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.

Method Validation

Selectivity

The selectivity of the method was tested by injecting methanol, water, acetonitrile and a mobile phase mixture of 17 mM 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 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 are listed in Table 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-PGA2, 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).

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 2. All prostanoids ranged from LOQ to 1000

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 1: Peak parameters of prostanoids and female sex hormones.

Compound	LOD (ng)	LOQ (ng)	Linear range (ng)	Line equation	R^2
E_3	1.0	3.0	3-300	y=497184x+4805.3	1.000
6-keto $PGF_{1\alpha}$	3.0	10.0	10-1000	y=138079x-295.06	0.998
16α-OHE_1	3.0	5.0	5-250	y=670764x-5429.9	0.997
8-iso $PGF_{2\alpha}$	5.0	20.0	20-1000	y=170680x-21249	0.997
$PGF_{2\alpha}$	0.3	1.0	1-1000	y=179481x+29395	0.998
PGE_2	0.3	1.0	1-1000	y=195950x+10929	0.999
11-dehydro-TXB_2	5.0	30.0	30-1000	y=155361x-4146.2	0.999
PGD_2	10.0	20.0	20-1000	y=186770x+12455	0.997
2-OHE_1	5.0	20.0	20-400	y=516084x-76783	0.997
4-OHE_1	1.0	2.0	2-150	y=857097x-12447	0.997
E_2	2.0	5.0	5-400	y=455003x-9213.2	0.999
13,14-dihydro-15-keto-PGE_2	5.0	20.0	20-1400	y=93711x+20758	0.998
E_1	0.5	2.0	2-400	y=439220x-10107	1.000
2-$MeOE_1$	0.5	2.0	2-400	y=507593x+1439.7	1.000
13,14-dihydro-15-keto-PGA_2	3.0	10.0	10-1000	y=106262x-3655	0.999
15-deoxy-PGJ_2	5.0	20.0	20-1000	y=186787x-15676	1.000
progesterone	5.0	20.0	20-1300	y=68288x+6046.9	0.997

Table 2: Validation results for prostanoids and sex hormones: sensitivity and linearity.

Compound	Level 1			Level 2			Level 3		
	Conc.	Intra-assay precision	Inter-assay precision	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)	(ng/μL)	% R.S.D. (n=10)	% R.S.D. (n=15)
E_3	0.30	1.07	1.42	0.65	1.49	7.27	1.30	1.20	4.34
6-keto $PGF_{1\alpha}$	1.00	2.73	2.36	2.10	1.50	3.31	4.20	2.06	5.48
16a-OHE_1	0.30	6.17	3.38	0.75	3.02	6.54	1.50	1.56	4.55
8-iso $PGF_{2\alpha}$	1.20	6.24	2.59	3.00	2.54	2.81	6.00	1.22	4.29
$PGF_{2\alpha}$	1.00	2.23	1.70	2.50	1.29	3.49	5.00	1.27	4.78
PGE_2	1.00	1.31	1.52	2.50	1.15	3.19	5.00	1.25	3.81
11-dehydro TXB_2	1.50	1.56	1.90	3.50	2.01	3.38	7.00	1.94	4.20
PGD_2	1.00	2.64	9.89	2.50	2.08	8.88	5.00	1.73	10.79
2-OHE_1	0.40	3.77	12.58	1.00	5.77	11.99	2.00	1.66	6.21
4-OHE_1	0.25	4.53	5.39	0.50	3.93	8.80	1.00	2.17	6.65
E_2	0.30	6.16	1.74	0.75	3.99	5.90	1.50	2.19	4.84
13,14-dihydro-15-keto-PGE_2	1.20	1.55	2.46	3.00	1.38	3.37	6.00	1.24	3.85
E1	0.50	2.19	1.20	1.40	1.38	6.25	2.80	2.40	4.87
2-$MeOE_1$	0.40	1.09	2.16	1.00	1.40	7.20	2.00	1.26	5.17
13,14-dihydro-15-keto-PGA_2	1.20	2.25	2.36	2.80	1.76	5.24	5.60	1.34	2.97
15-deoxy-PGJ_2	1.20	1.03	1.91	2.60	1.45	3.36	5.20	0.95	3.67
Progesterone	0.80	2.02	4.33	2.20	3.04	5.90	4.40	1.92	5.05

Table 3: Validation results: inter-day and intra-day precision.

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. 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.

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. 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

Compound	Level 1		Level 2		Level 3	
	Concentration	Accuracy	Concentration	Accuracy	Concentration	Accuracy
	(ng/μL)	% (n=10)	(ng/μL)	% (n=10)	(ng/μL)	% (n=10)
E_3	0.30	86.94	0.65	89.12	1.30	103.88
6-keto $PGF_{1\alpha}$	1.00	86.90	2.10	109.94	4.20	98.40
16a-OHE_1	0.30	88.83	0.75	86.29	1.50	103.45
8-iso $PGF_{2\alpha}$	1.20	109.04	3.00	111.50	6.00	100.95
$PGF_{2\alpha}$	1.00	101.29	2.50	113.98	5.00	102.19
PGE_2	1.00	99.74	2.50	105.43	5.00	94.81
11-dehydro TXB_2	1.50	108.93	3.50	106.24	7.00	97.55
PGD_2	1.00	88.60	2.50	106.34	5.00	95.83
2-OHE_1	0.40	112.69	1.00	84.96	2.00	109.08
4-OHE_1	0.25	101.03	0.50	105.32	1.00	109.63
E_2	0.30	87.50	0.75	93.99	1.50	103.25
13,14-dihydro-15-keto-PGE_2	1.20	97.43	3.00	108.26	6.00	97.38
E_1	0.80	93.89	1.40	90.22	2.80	97.94
2-$MeOE_1$	0.40	103.83	1.00	91.47	2.00	101.31
13,14-dihydro-15-keto-PGA_2	1.20	102.16	2.80	110.88	5.60	97.48
15-deoxy-PGJ_2	1.20	104.53	2.60	114.13	5.20	97.78
Progesterone	0.80	97.24	2.20	92.00	4.40	108.99

Table 4: Validation results: accuracy.

Compound	Level 1		Level 2	
	Concentration	precision	Concentration	precision
	(ng/μL)	% R.S.D. (n=9)	(ng/μL)	% R.S.D. (n=9)
E_3	0.30	4.12	1.30	2.75
6-keto $PGF_{1\alpha}$	1.00	4.03	4.20	2.18
16a-OHE_1	0.30	3.44	1.50	2.47
8-iso $PGF_{2\alpha}$	1.20	3.49	6.00	2.43
$PGF_{2\alpha}$	1.00	1.98	5.00	2.15
PGE_2	1.00	1.58	5.00	1.99
11-dehydro TXB_2	1.50	3.34	7.00	2.41
PGD_2	1.00	3.09	5.00	2.52
2-OHE_1	0.40	2.43	2.00	3.12
4-OHE_1	0.25	4.08	1.00	3.60
E_2	0.30	4.92	1.50	3.26
13,14-dihydro-15-keto-PGE_2	1.20	2.21	6.00	2.12
E_1	0.80	2.29	2.80	8.65
2-$MeOE_1$	0.40	1.26	2.00	2.66
13,14-dihydro-15-keto-PGA_2	1.20	1.50	5.60	2.55
15-deoxy-PGJ_2	1.20	1.91	5.20	2.11
Progesterone	0.80	5.36	4.40	2.47

Table 5: Validation results: solution stability.

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 [15]. 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 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.

Recovery

Recovery was estimated by spiking urine with standard samples at low, middle and high concentration levels. The results were listed in Table 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.

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

Compound	Level 1		Level 2		Level 3	
	Concentration	Accuracy	Concentration	Accuracy	Concentration	Accuracy
	(ng/μL)	% (n=3)	(ng/μL)	% (n=3)	(ng/μL)	% (n=3)
E_3	0.3	90.27	0.75	110.9	1.5	90.72
6-keto $PGF_{1\alpha}$	1.5	109.8	2.0	99.53	4.0	94.32
16a-OHE_1	0.3	88.85	0.75	100.8	1.5	85.79
8-iso $PGF_{2\alpha}$	0.9	89.45	2.5	105.2	5.0	102.0
$PGF_{2\alpha}$	0.6	106.6	2.5	106.1	5.0	107.6
PGE_2	0.6	105.1	2.3	94.48	4.6	91.43
11-dehydro TXB_2	1.0	110.1	3.2	103.5	5.2	106.36
PGD_2	0.4	95.16	1.6	85.43	3.2	107.39
2-OHE_1	0.3	108.1	0.9	107.1	1.8	94.72
4-OHE_1	0.1	94.39	0.45	93.51	0.9	91.80
E_2	0.4	103.1	1.0	106.9	2.0	86.55
13,14-dihydro-15-keto-PGE_2	1.2	109.4	3.25	114.2	6.5	103.1
E_1	0.5	99.52	1.15	112.5	2.3	90.59
2-$MeOE_1$	0.4	100.1	1.0	104.6	1.8	93.71
13,14-dihydro-15-keto-PGA_2	1.0	107.7	2.5	114.6	5.0	103.39
15-deoxy-PGJ_2	1.2	87.54	2.5	97.02	5.0	94.97
Progesterone	1.2	98.67	2.5	99.62	5.0	93.14

Table 6: Validation results: recovery by spiking urine at low, middle and high concentration level.

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.

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 x-LC by replacing phosphoric acid with formic acid [16].

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

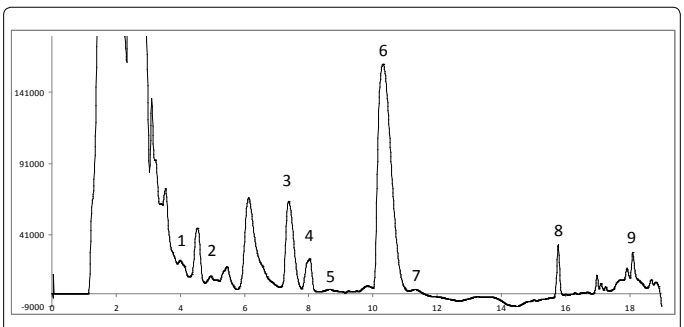

Figure 3: Chromatogram of a urine sample: 1. E_3; 2. 6-keto-$PGF_{1\alpha}$; 3. 16α-OHE_1; 4. 8-iso-$PGF_{2\alpha}$; 5. $PGF_{2\alpha}$; 6. PGD_2; 7. 2-OHE_1; 8. E_1; 9. 15d-PGJ_2.

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 16-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.

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 x-LC. 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. 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 [17]. 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.

References

1. Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, et al. (2013) Heart disease and stroke statistics--2013 update: a report from the American Heart Association. Circulation 127: e6-6e245.
2. Dubey RK, Oparil S, Imthurn B, Jackson EK (2002) Sex hormones and hypertension. Cardiovasc Res 53: 688-708.
3. Boschitsch E, Mayerhofer S, Magometschnigg D (2010) Hypertension in women: the role of progesterone and aldosterone. Climacteric 13: 307-313.
4. Zheng Y, Yang T, Chen G, Hu E, Gu Q, et al. (2014) Prostanoid therapy for pulmonary arterial hypertension: a meta-analysis of survival outcomes. Eur J Clin Pharmacol 70: 13-21.
5. Williamson S, Varma D, Brown M, Jansen S (2011) Eicosanoid Production following One Bout of Exercise in Middle-Aged African American Pre- and Stage 1 Hypertensives. J Aging Res 2011: 302802.
6. Patrignani P, Panara MR, Greco A, Fusco O, Natoli C, et al. (1994) Biochemical and pharmacological characterization of the cyclooxygenase activity of human blood prostaglandin endoperoxide synthases. J Pharmacol Exp Ther 271: 1705-1712.
7. Farré M, Kuster M, Brix R, Rubio F, López de Alda MJ, et al. (2007) Comparative study of an estradiol enzyme-linked immunosorbent assay kit, liquid chromatography–tandem mass spectrometry, and ultra performance liquid chromatography–quadrupole time of flight mass spectrometry for part-per-trillion analysis of estrogens in water samples. J. Chromatogr. A 1160: 166-175.
8. Tsukamoto H, Hishinuma T, Mikkaichi T, Nakamura H, Yamazaki T, et al. (2002) Simultaneous quantification of prostaglandins, isoprostane and thromboxane in cell-cultured medium using gas chromatography–mass spectrometry. J. Chromatogr. B 774: 205-214.
9. Temes TA, Andersen H, Gilberg D, Bonerz M (2002) Determination of estrogens in sludge and sediments by liquid extraction and GC/MS/MS. Anal Chem 74: 3498-3504.
10. Yue H, Strauss KI, Borenstein MR, Barbe MF, Rossi LJ, et al. (2004) Determination of bioactive eicosanoids in brain tissue by a sensitive reversed-phase liquid chromatographic method with fluorescence detection. J. Chromatogr. B 803: 267-277.
11. Xu X, Veenstra TD, Fox SD, Roman JM, Issaq HJ, et al. (2005) Measuring fifteen endogenous estrogens simultaneously in human urine by high-performance liquid chromatography-mass spectrometry. Anal Chem 77: 6646-6654.
12. Xu X, Roman JM, Veenstra TD, Van Anda J, Ziegler RG, et al. (2006) Analysis of fifteen estrogen metabolites using packed column supercritical fluid chromatography-mass spectrometry. Anal Chem 78: 1553-1558.
13. Furugen A , Yamaguchi H, Tanaka N, et al. (2011) Quantification of intracellular and extracellular prostanoids stimulated by A23187 by liquid chromatography/electrospray ionization tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 879: 3378- 3385.
14. Blewett AJ, Varma D, Gilles T, Libonati JR, Jansen SA (2008) Development and validation of a high-performance liquid chromatography-electrospray mass spectrometry method for the simultaneous determination of 23 eicosanoids. J Pharm Biomed Anal 46: 653-662.
15. US FDA Guidance for Industry (2001) Bioanalytical Method Validation. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Center for Veterinary Medicine.
16. Varma D, Ganti S, Gilles T, Diaz M, Thompson T, et al. (2011) Comparison of UHPLC and HPLC methods for the assay of prostanoids: “are the methods equivalent in terms of accuracy and precision?”. Bioanalysis 3: 853-862.
17. Kershaw EE, Flier JS (2004) Adipose tissue as an endocrine organ. J Clin Endocrinol Metab 89: 2548-2556.