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Development and validation of a high-performance liquid chromatography–electrospray mass spectrometry method for the simultaneous determination of 23 eicosanoids

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Abstract

Inflammation is implicated in the pathogenesis of a number of diseases, including cardiovascular disease. Current research is focused on developing assays to search for biomarkers for inflammation. Eicosanoids are the oxidative metabolites of arachidonic acid (eicosatetraenoic acid, AA), a long chain polyunsaturated fatty acid common in Western diets. AA can be oxidized by one of three pathways to form prostaglandins (PGs), leukotrienes (LTs), or a number of hydroxyl and epoxy compounds. These eicosanoids have a variety of physiological functions, including regulating inflammation.

We have developed a method utilizing LC–MS to separate and quantitate 23 different eicosanoids from all the three oxidative pathways. The eicosanoids were separated using a gradient elution of acetonitrile with 0.1% formic acid (v/v) and water with 0.1% formic acid (v/v) at a flow rate of 1 mL/min with a Symmetry C18 column (250 mm × 4.6 mm). Deuterated eicosanoids were used as internal standards for quantitation. Mass spectrometric detection was carried out using an Agilent 1100-series LC–MSD with an electrospray ionization interface. Electrospray ionisation (ESI) mass spectra were acquired using negative ionization and selective ion monitoring. The method was validated and shown to be sensitive (LOQ at pg levels for most compounds), accurate (recovery values 75–120%) and precise (R.S.D. < 20 for all compounds) with a linear range over several orders of magnitude. The method was applied to rat kidney tissue and shown to be indicative of the eicosanoid levels within a specific organ. The analysis of eicosanoids can provide insight into the inflammatory mechanisms associated with cardiovascular disease.

Keywords: Eicosanoids; Inflammation biomarkers; Liquid chromatography–mass spectrometry; Prostaglandins; Leukotrienes; Arachidonic acid; Cardiovascular disease

1. Introduction

1.1. Inflammation

Inflammation is the body's natural physiological response to infection or injury, acting as a defense mechanism to remove and repair damaged tissue. The cardinal features of inflammation (heat, pain, redness, and swelling) are caused in large part by an increase in blood flow and vascular permeability and the ability for larger inflammatory mediators to cross the endothelial surface and travel and adhere to the site of the injury. While inflammation is clearly evident in superficial injuries such as lacerations, scratches and burns, inflammation is also widely implicated, both directly and indirectly, in the pathogenesis of a large number of diseases. Rheumatoid arthritis [1], asthma and chronic obstructive pulmonary disease (COPD) [2], irritable bowel syndrome (IBS) [3], cystic fibrosis [4], Alzheimer's disease [5], and cancer [6] are all caused or mediated by inflammation. Recently, increasing evidence has suggested that cardiovascular disease (CVD) is, in fact, mediated in large part by inflammatory events, especially the onset of atherosclerosis and coronary heart disease (CHD) [7–11]. As a result, current research is focused on developing quantitative analytical techniques to discover biomarkers of inflammation that can be

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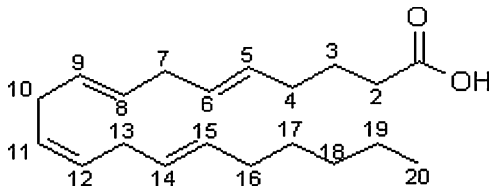

Fig. 1. Structure of arachidonic acid.

utilized to more accurately predict future CVD risk. Promising results have been shown using protein biomarkers including C-reactive protein (CRP), fibrinogen, and cytokines such as tumor necrosis factor-α (TNF-α) and some of the interleukins IL-1 and IL-6 [6,8,12,13].

1.2. Eicosanoids

Another group of localized inflammatory biomarkers that shows promise is the eicosanoids. Eicosanoids are the biological oxidative metabolites of arachidonic acid (eicosatetraenoic acid, AA), responsible for a large variety of physiological functions including regulating inflammation. AA is a 20-carbon ω-6 polyunsaturated fatty acid (PUFA) with four unsaturated double bonds (all *cis*) at positions 5, 8, 11, and 14 (Fig. 1). Esterified AA is released from the membrane phospholipids, usually by cytosolic phospholipase A_2 (c-PLA_2), in response to an inflammatory stimulus. The liberated AA can then become the substrate for one of three oxidative enzymatic pathways: cyclooxygenase (COX), lipoxygenase (LOX) or cytochrome P450 (CYP) (Fig. 2). The COX pathway produces the prostanoids (which include the prostaglandins (PGs) and thromboxanes (TXs)), while the LOX pathway produces the leukotrienes (LTs) as well as some of the hydroxyeicosatetraenoic acids (HETEs). The CYP pathway mainly produces the epoxyeicosatrienoic acids (EETs), dihydroxyeicosatrienoic acids (DiHETEs) as well as some HETEs. AA and AA-derived eicosanoids are

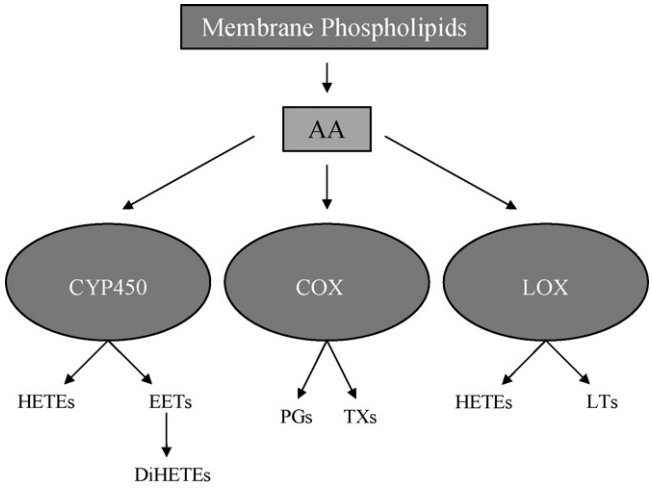

Fig. 2. Oxidative pathways of arachidonic acid. AA: arachidonic acid, CYP450: cytochrome P450, COX: cyclooxygenase, LOX: lipoxygenase, PG: prostaglandin, TX: thromboxane, LT: leukotriene, HETE: hydroxyeicosatetraenoic acid, EET: epoxyeicosatrienoic acid and DiHETE: dihydroxyeicosatrienoic acid.

potent pro- and anti-inflammatory mediators, the detection of which may provide insight into the development of inflammatory conditions. For example, prostaglandin E_2 (PGE_2) is involved in the regulation of renal water reabsorption [14], as well as in the development of fever, hyperalgesia, vasodilation, and increased vascular permeability, promoting an increased inflammatory response. Prostacyclin (PGI_2) is a vasodilator and potent inhibitor of platelet aggregation, while thromboxane A_2 (TXA_2) is a vasoconstrictor and potent promoter of platelet aggregation. The delicate balance between PGI_2 and TXA_2 synthesis is a primary concern in the development of CVD. Leukotriene B_4 (LTB_4), the major LOX product, is a very potent pro-inflammatory agent, promoting chemotaxis and vascular adhesion [15,16]. Leukotrienes C_4, D_4, and E_4 (LTC_4, LTD_4, and LTE_4, respectively) collectively referred to as the cysteinyl-LTs, are synthesized mainly during anaphylactic reactions and have been shown to increase vascular permeability and mucous secretion, contract smooth muscle cells and cause bronchoconstriction and vasoconstriction, which heavily implicates them in the pathogenesis of asthma and other inflammatory respiratory conditions [16–18]. The EETs have been shown to be potent anti-inflammatory agents that can act to reduce leukocyte adhesion to endothelial surfaces [19], while the HETEs, especially 20-HETE have been implicated in the development of hypertension in rats, possibly through renal vasoconstriction and/or sodium retention mechanisms [20,21].

1.3. Analysis of eicosanoids

Current analytical techniques for the separation of eicosanoids in standards as well as biological matrices include high-performance liquid chromatography with both UV (HPLC-UV) [22–25] and fluorescence (HPLC-FL) [26–29] detection, capillary electrophoresis with UV detection (CE-UV) [30] and gas chromatography–mass spectrometry (GC–MS) [31–33]. The above techniques have significant disadvantages that limit their effectiveness in bioanalytical applications. UV detection requires active chromophores that many of the eicosanoids lack. In addition, HPLC-UV methods typically lack the sensitivity necessary to quantitate endogenous levels of eicosanoids. Furthermore, methods that employ fluorescence or GC–MS analyses involve complex and time-consuming sample purification and derivitization steps that tend to complicate the sample preparation and introduce errors. An increasingly popular choice for the analysis of eicosanoids is HPLC coupled to mass spectrometry (either single or tandem, LC–MS or LC–MS–MS, respectively). While several researchers have developed methods that separate and quantitate low levels of endogenous eicosanoids [34–38], to the best of our knowledge, there are no currently published methods that separate the major products of all three metabolic pathways simultaneously. Takabatake et al. have developed a method for the separation and quantitation of all the prostaglandins in human synovial fluid using LC–MS–MS [39]. Nithipatikom et al. have developed two methods utilizing LC–MS to determine DiHETE and EET levels in bovine coronary artery endothelial cells and canine plasma [40] and prostaglandin levels in

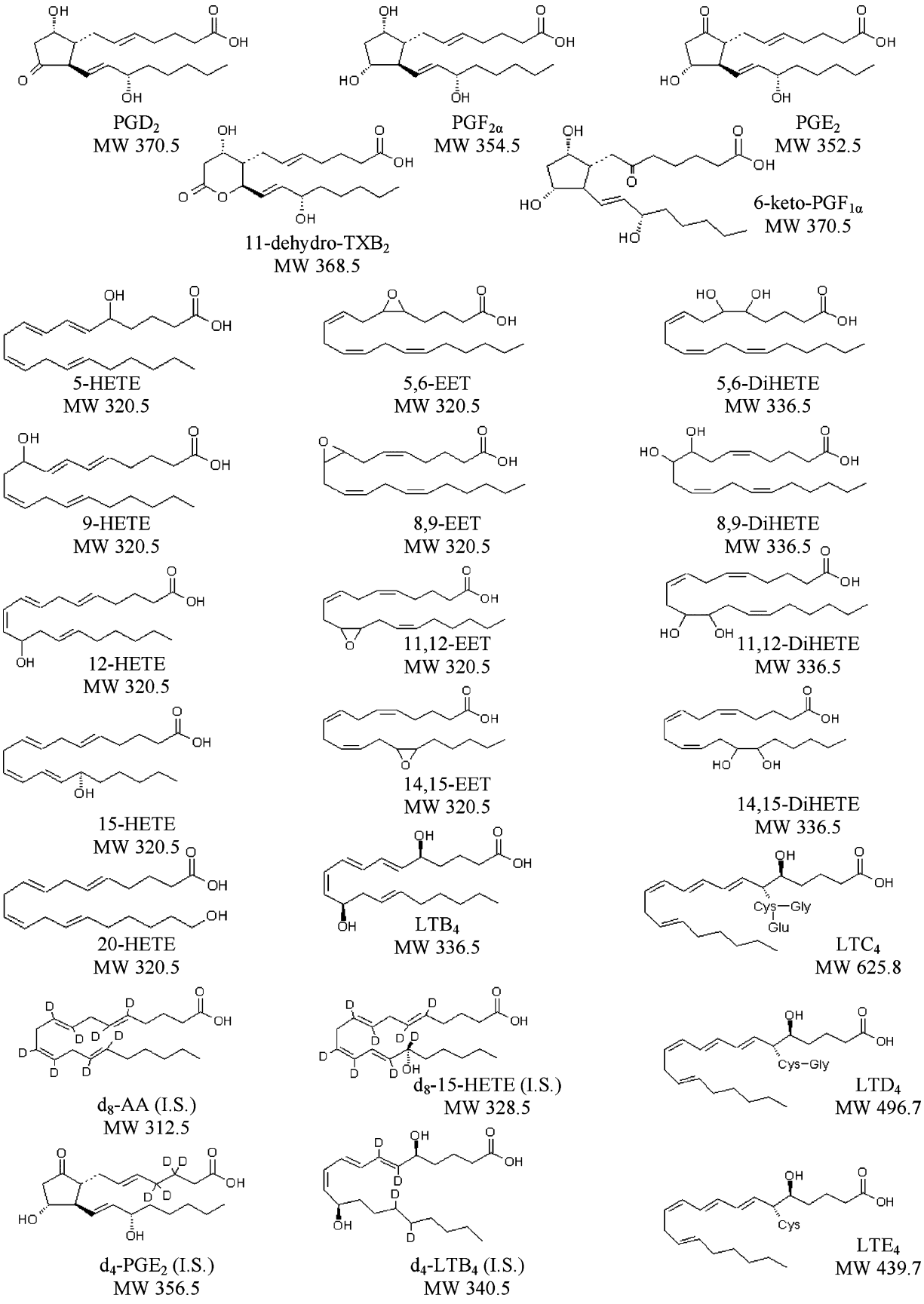

Fig. 3. Structures of all bioactive eicosanoids and deuterated internal standards. PG: prostaglandin, LT: leukotriene, TX: thromboxane, HETE: hydroxyeicosatetraenoic acid, EET: epoxyeicosatrienoic acid, DiHETE: dihydroxyeicosatrienoic acid and AA: arachidonic acid.

bovine coronary artery endothelial cells and human prostate cancer cells [41]. Our lab has previously reported a validated LC–MS method for analysis of PGs, HETEs, DiHETEs, EETs, and AA in rat brain tissue [42]. However, this method neglected to include the LTs, which have been shown to be vital to the inflammatory response. In this paper, we report a method for the simultaneous analysis of all the major metabolic products of AA (PGs, LTs, HETEs, DiHETEs and EETs) through all three enzymatic pathways (COX, LOX and CYP) using LC–ESI-MS. The structures of the eicosanoids studied as well as the deuterated internal standards used can be found in Fig. 3.

2. Experimental

2.1. Chemicals and materials

The prostaglandins (PGE_2, $PGF_{2\alpha}$, PGD_2, 6-keto-$PGF_{1\alpha}$, PGI_2, TXB_2 and 11-dehydro-TXB_2), leukotrienes (LTB_4, LTC_4, LTD_4 and LTE_4), and AA were purchased from Biomol (Plymouth Meeting, PA, USA). The HETEs (5-HETE, 9-HETE, 12-HETE, 15-HETE and 20-HETE), EETs (5,6-EET, 8,9-EET, 11,12-EET, 14 and 15-EET), DiHETEs (5,6-DiHETE, 8,9-DiHETE, 11,12-DiHETE and 14,15-DiHETE), and deuterated eicosanoids used as internal standards (3,3,4,4-$^2H_4$-PGE_2, 5,6,8,9,11,12,14,15-$^2H_8$-15-HETE, 6,7,14,15-$^2H_4$-LTB_4 and 5,6,8,9,11,12,14,15-$^2H_8$-AA) were purchased from Cayman Chemical (Ann Arbor, MI, USA). 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).

2.2. LC–MS method

The HPLC used was an Agilent 1100 series HPLC (Agilent Technologies, Santa Clara, CA, USA) with a binary pump, in-line degasser, and a thermostated autosampler. The HPLC was coupled to an Agilent G1946 mass selective detector (MSD). The separation was performed on a Symmetry C18 4.6 mm × 250 mm column with a 5-μm particle size (Waters Corp., Milford, MA, USA). The mobile phase used was a mixture of acetonitrile with 0.1% formic acid (v/v) (B) and water with 0.1% formic acid (v/v) (A) and the flow rate was set at 1 mL/min. Gradient elution was employed and was used as follows: 40% B for 10 min, 40–65% B from 10 to 25 min, hold 65% B from 25 to 40 min, 65–90% B from 40 to 45 min, hold 90% B from 45 to 55 min, 5 min re-equilibration at 40% B from 55 to 60 min. The injection volume was 20 μL. Electrospray ionization (ESI) in the negative ion mode was used as the ionization source. Nitrogen was used as the nebulizer gas and was maintained at a flow of 12.0 L/min with a nebulizer pressure of 35 psi. The gas temperature was set at 350 °C and the capillary voltage was 3000 V. The fragmentor voltage was set at 120 V and the gain was 2.0. Chromatograms were obtained using selective ion monitoring divided into four time segments as seen in Table 1.

Table 1
Ions monitored for the compounds analyzed in respective time ranges

Time (min)	*m/z*	Ions monitored
0.00	351	PGE_2, PGD_2
	353	$PGF_{2\alpha}$
	355	d_4-PGE_2
	367	11-dehydro-TXB_2
	369	6-keto-PGF1α
	495	LTD_4
12.00	438	LTE_4
	624	LTC_4
20.00	319	HETEs, EETs
	327	d_8-15-HETE
	335	LTB_4
	337	DiHETEs
	339	d_4-LTB_4
49.00	303	AA
	311	d_8-AA

2.3. Sample preparation

Prostaglandin and 11-dehydro-TXB_2 stock solutions were prepared by dissolving solid samples in 1-mL methanol to create a stock mass concentration of 0.5 and 0.1 mg/mL, respectively. All other eicosanoids were supplied dissolved in other suitable solvents. The stock mass concentrations were as follows: leukotrienes (0.05 mg/mL), HETEs, EETs, and DiHETEs (0.1 mg/mL) and AA (100 mg/mL). Working standard solutions of all eicosanoids were prepared by serial dilution in methanol from stock solutions to create the necessary concentrations. All solutions were stored at −80 °C when not in use.

2.4. Recovery samples for method validation

Accuracy was determined by the recalculating of each sample based on the weighted calibration curve equation. Four sample preparations in methanol at two different mass concentrations (50 and 250 pg/μL for PGs, HETEs, and DiHETEs, 100 and 500 pg/μL for LTs and EETs, 10 and 100 ng/μL for AA) corresponding to the lower and upper levels of the linear range were used. Each sample was injected in triplicate. Recovery rates were calculated by $([C_{\text{calculated}}]/[C_{\text{nominal}}] \times 100)$. Precision was determined by calculating the relative standard deviation (R.S.D.) for all injections at each concentration analyzed. Instrument precision, or system suitability, was determined by analyzing 10 injections of an eicosanoid standard. Repeatability, or intra-assay precision, was determined using the four samples at two different mass concentrations prepared for the accuracy analysis injected in triplicate. To obtain inter-assay, or intermediate, precision, two samples of different mass concentrations were analyzed on 5 consecutive days.

2.5. Rat kidney sample preparation

Approximately 100 mg of spontaneously hypertensive rat kidney tissue was placed in a 2-mL polypropylene microcen-

trifuge vial with 200 μL of methanol with 0.01 M BHT and 5 μL of formic acid. The tissue was homogenized with a Tissue Terror homogenizer (BioSpec Products Inc., Bartlesville, OK). The homogenized mixture was then centrifuged at 14000 rpm at 0 °C for 15 min. The supernatant was collected and diluted with water to make a final volume of 2 mL. An Oasis solid phase extraction cartridge (Waters Corp., Milford, MA) was sequentially preconditioned with 2 mL 0.1% formic acid (v/v), 2-mL methanol, and 2-mL ethyl acetate. The diluted supernatant was loaded on the column and washed with 2 mL 0.1% formic acid (v/v) and 2 mL 10% methanol with 0.1% formic acid (v/v). The eicosanoids were eluted with 1.5-mL ethyl acetate with 0.01 M BHT and 0.5-mL methanol with 0.2% formic acid and 0.01 M BHT. 10 μL of an internal standard solution (d_4-PGE_2: 2.5 ng/μL, d_8-15-HETE: 500 pg/μL, d_4-LTB_4: 1 ng/μL, d_8-AA: 50 μg/μL) was added and the samples were evaporated to dryness under nitrogen. Evaporated samples were reconstituted with 100-μL methanol and subject to LC–MS analysis.

3. Results

3.1. Method development and validation

The direct injection of authentic standards resulted in a chromatogram with sharp, well defined peaks and baseline separation except for the co-eluting LTE_4 and LTC_4 (14 min), 5,6-DiHETE and 20-HETE (29 min), and the four deuterated internal standards and their non-deuterated counterparts. However, the co-eluting compounds have significantly different masses and are completely resolved when selective ion monitoring is used. Fig. 4 shows a representative total ion chromatogram for the separation of all 23 eicosanoids and four internal standards in methanol. Peak selectivity was demonstrated by injections of individual compounds in methanol. Blanks were analyzed and found to show no discernable interferences near the test compounds. Quantitation was performed using deuterated internal standards. Response ratios were determined by A_{sample}/A_{IS}. The prostaglandins and 11-dehydro-TXB_2 were quantitated using d_4-PGE_2. The leukotrienes were quantitated using d_4-LTB_4. The DiHETEs, HETEs, and EETs were quantitated using d_8-15-HETE. AA was quantitated using d_8-AA.

3.1.1. Sensitivity

The sensitivity of the method is described in the terms of the limit of detection (LOD) and limit of quantitation (LOQ). The LOD was defined here as the concentration (in terms of on-column eicosanoid levels in pg) at which the peak response was three times that of the noise (3 S/N) while the LOQ was defined as the concentration with a peak response corresponding to 10 times that of the noise (10 S/N). The validation results for all 23 eicosanoids can be found in Table 2. Most of the eicosanoids exhibited LOQs in the pg range.

3.1.2. Linearity

The linear range was established for all 23 compounds and shown to span up to four orders of magnitude. An examination of the residual plot showed a general increase in residuals with concentration for all compounds studied. This effect is common in bioanalytical methods and can be counteracted by applying a weighting factor to the data and recalculating the line equation [43,44]. Weighting factors applied included $1/y$, $1/\sqrt{y}$, $1/y^2$, $1/x$, $1/\sqrt{x}$, and $1/x^2$. The best weighting factor was chosen based on the plots of percent relative error (%RE) versus concentration that provided the best random distribution around the x-axis as well as the lowest sum of %RE across the entire concentration range (data not shown). Based on these criteria, $1/x$ was selected as the weighting factor for the calibration curves. The weighted line equations can be found in Table 2.

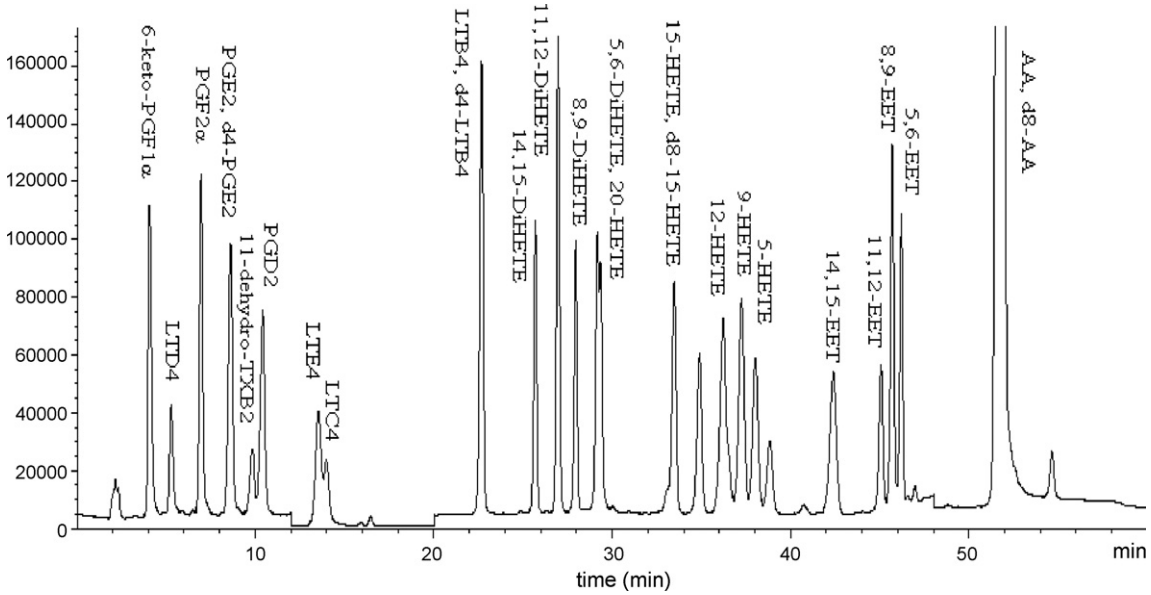

Fig. 4. Representative LC–MS chromatogram of 23 eicosanoids and 4 internal standards using SIM as defined in Table 1. Samples prepared in methanol and injected on chromatographic system described in text.

Table 2
Validation results: sensitivity and linearity

Compound	LOD (pg)	LOQ (pg)	Linear range (pg)	Line equation	R
6-Keto-$PGF_{1\alpha}$	1.6	5.5	5.5–10000	$y = 0.00813x - 0.00310$	0.99
$PGF_{2\alpha}$	4.3	14.4	14.4–10000	$y = 0.00770x - 0.00052$	0.99
PGE_2	7.6	25.3	25.3–10000	$y = 0.00453x - 0.00088$	0.99
11-Dehydro-TXB_2	15.7	52.5	52.5–20000	$y = 0.00211x - 0.00139$	0.99
PGD_2	6.2	20.6	20.6–10000	$y = 0.00652x - 0.00075$	0.99
LTB_4	4.7	15.7	15.7–20000	$y = 0.01260x + 0.00340$	0.99
LTC_4	9.8	32.6	32.6–20000	$y = 0.00405x - 0.00429$	0.98
LTD_4	18.0	60.2	60.2–20000	$y = 0.00428x - 0.00569$	0.99
LTE_4	9.3	30.9	30.9–20000	$y = 0.00703x - 0.00263$	0.99
14,15-DiHETE	10.0	20.0	20.0–10000	$y = 0.03508x + 0.01481$	0.99
11,12-DiHETE	10.0	20.0	20.0–10000	$y = 0.05862x + 0.01753$	0.99
8,9-DiHETE	10.0	20.0	20.0–10000	$y = 0.03148x + 0.00834$	0.99
5,6-DiHETE	10.0	20.0	20.0–10000	$y = 0.00347x + 0.00487$	0.99
20-HETE	7.0	23.2	23.2–10000	$y = 0.03111x + 0.09990$	0.99
15-HETE	7.4	24.7	24.7–10000	$y = 0.04507x + 0.00351$	0.99
12-HETE	8.8	29.3	29.3–10000	$y = 0.04647x - 0.00996$	0.99
9-HETE	9.4	31.3	31.3–10000	$y = 0.04149x - 0.00535$	0.99
5-HETE	12.9	43.0	43.0–10000	$y = 0.03055x - 0.00252$	0.99
14,15-EET	25.4	84.7	84.7–20000	$y = 0.02245x - 0.07548$	0.99
11,12-EET	174.0	223.8	223.8–20000	$y = 0.01258x - 0.07796$	0.99
8,9-EET	10.6	35.4	35.4–20000	$y = 0.02465x - 0.01773$	0.99
5,6-EET	16.0	53.2	53.2–20000	$y = 0.01721x - 0.03984$	0.99
AA	39.8	132.0	132.0–5000000	$y = 0.14126x + 0.03345$	0.98

3.1.3. Accuracy and precision

Recovery for all compounds fell within 78–115% for all eicosanoids with the greatest loss exhibited in the thermally labile and unstable compounds like the cys-LTs and the EETs (Table 3). System suitability was assessed and all compounds were shown to have R.S.D. values $\leq$17% with most compounds <5% (Table 4). Additionally, inter-assay precision analyzed on 5 consecutive days showed similar results with R.S.D. values $\leq$12% (Table 4). Similar to the recovery data, the unstable compounds (LTC_4, LTD_4, and 5,6-EET) exhibited more variability throughout the experiment.

Table 3
Validation results: accuracy

	Level 1		Level 2	
Compound	Concentration (pg/μL)	Recovery (%)	Concentration (pg/μL)	Recovery (%)
6-Keto-$PGF_{1\alpha}$	50	96.6	250	105.5
$PGF_{2\alpha}$	50	96.2	250	107.3
PGE_2	50	85.5	250	98.5
11-Dehydro-TXB_2	50	99.1	250	109.1
PGD_2	50	91.3	250	102.4
LTB_4	100	102.9	500	100.2
LTC_4	100	94.1	500	95.9
LTD_4	100	84.1	500	100.3
LTE_4	100	97.1	500	101.5
14,15-DiHETE	50	106.7	250	109.6
11,12-DiHETE	50	114.5	250	104.7
8,9-DiHETE	50	107.5	250	110.7
5,6-DiHETE	50	102.0	250	105.6
20-HETE	50	106.5	250	112.0
15-HETE	50	88.8	250	101.3
12-HETE	50	89.7	250	105.3
9-HETE	50	92.1	250	99.7
5-HETE	50	92.1	250	100.2
14,15-EET	100	95.4	500	102.2
11,12-EET	100	78.2	500	100.0
8,9-EET	100	101.2	500	105.3
5,6-EET	100	108.6	500	110.9
AA	1 ng/μL	120	100 ng/μL	98.7

Table 4
Validation results: precision

Compound	Level 1			Level 2		
	Concentration (pg/μL)	Intra-assay precision %R.S.D. ($n = 10$)	Inter-assay precision %R.S.D. ($n = 15$)	Concentration (pg/μL)	Intra-assay precision %R.S.D. ($n = 10$)	Inter-assay precision %R.S.D. ($n = 15$)
6-Keto-$PGF_{1\alpha}$	50	3.44	3.20	250	0.64	2.18
$PGF_{2\alpha}$	50	3.37	1.22	250	0.55	0.82
PGE_2	50	3.14	1.21	250	0.45	1.06
11-Dehydro-TXB_2	50	4.64	2.66	250	0.95	1.30
PGD_2	50	4.14	1.71	250	0.85	1.80
LTB_4	100	1.76	0.68	500	1.83	1.01
LTC_4	100	17.11	7.65	500	16.94	11.21
LTD_4	100	6.68	9.27	500	5.41	10.35
LTE_4	100	6.55	3.66	500	6.52	2.02
14,15-DiHETE	50	1.64	3.04	250	1.68	3.07
11,12-DiHETE	50	3.28	3.16	250	1.41	2.59
8,9-DiHETE	50	3.5	2.69	250	0.92	2.30
5,6-DiHETE	50	3.95	2.41	250	1.09	2.24
20-HETE	50	4.12	3.29	250	0.95	4.09
15-HETE	50	2.35	2.25	250	2.05	1.59
12-HETE	50	1.65	1.45	250	2.25	1.81
9-HETE	50	3.69	3.13	250	1.97	3.24
5-HETE	50	4.64	2.73	250	1.66	3.34
14,15-EET	100	3.48	2.24	500	3.60	4.45
11,12-EET	100	2.23	4.00	500	3.04	3.94
8,9-EET	100	1.33	4.47	500	0.83	6.57
5,6-EET	100	2.1	6.76	500	1.07	9.37
AA	1 ng/μL	12.5	2.85	100 ng/μL	15.3	6.67

3.2. Application of method to rat kidney

The method was applied to spontaneously hypertensive rat (SHR) kidneys to investigate the biological applications of the method. The selected ion chromatograms (SIC) of the compounds that were found in SHR kidney are shown in Fig. 5. Hypertensive rats were chosen based on the fact that the range and distribution of eicosanoids was expected to be the greatest. The kidney analysis revealed significant levels of several eicosanoids, including $PGF_{2\alpha}$, PGE_2, PGD_2, 11-dehydro-TXB_2, 14,15-DiHETE, 11,12-DiHETE, 8,9-DiHETE, 20-HETE, 15-HETE, 12-HETE, and AA, and trace levels of 9-HETE. 5-HETE, 8,9-EET, and 5,6-EET.

4. Discussion

The choice of compounds to study is directly influenced by the inherent stability of the compounds *in vivo*. Prostacyclin (PGI_2) and thromboxane (TXA_2) exhibit very short half-lives and are hydrolytically degraded into biologically inactive metabolites. In addition, chromatographic peaks for these compounds appeared very broad and distorted. It is therefore common in bioanalytical methods to substitute 6-keto-$PGF_{1\alpha}$ and 11-dehydro-TXB_2 for PGI_2 and TXA_2, respectively. These compounds have been proven to be reliable markers of *in vivo* synthesis of PGI_2 and TXA_2 and were used in the development and validation of this method [45,46].

An interesting trend was revealed during the analysis of the HETEs. The HETEs are derived from the CYP450 enzyme via the allylic oxidase and $\omega/\omega - 1$ hydrogenase pathways. Standards are readily available for the major HETEs including the ones used in this method (20-, 15-, 12-, 9-, and 5-HETEs). However, some of the HETEs that do not have readily available standards are found *in vivo* and can have significant biological activity, especially the $\omega - 1$ hydrogenase product 19-HETE [47]. The lack of standards makes it difficult to include these compounds in the development of methods. There is, however, a quadratic relationship between the retention times of the HETEs and the position of OH substitution (Fig. 6). The relationship applies only to the instrumental conditions described above, specifically the isocratic plateau of 65% acetonitrile with 0.1% formic acid (v/v) between 25 and 40 min. The HETE retention time increases as OH substitution moves farther away from the aliphatic ω terminal and closer to the carboxylic acid moiety at the α terminal. There is no way to quantitate these compounds without reliable standards, but qualitative analysis is possible. Qualitative analysis of HETEs can provide useful insight into the changes in eicosanoid synthesis under specific disease conditions. This relationship has not been previously reported, but is in agreement with the data presented by Eling [22] and Yue [42]. Additionally, there are no readily available deuterated standards for the EETs and DiHETEs. Therefore, d_4-15-HETE was used to quantify these compounds due to their structural similarities.

An extensive search of the literature revealed that there is currently no reported method for the separation of all of the metabolites of arachidonic acid. This is possibly due to the complex chromatographic difficulties introduced with the addition of the leukotrienes. HPLC analysis of LTs produces notoriously poor peak shapes due to the protonated amino acid moieties on

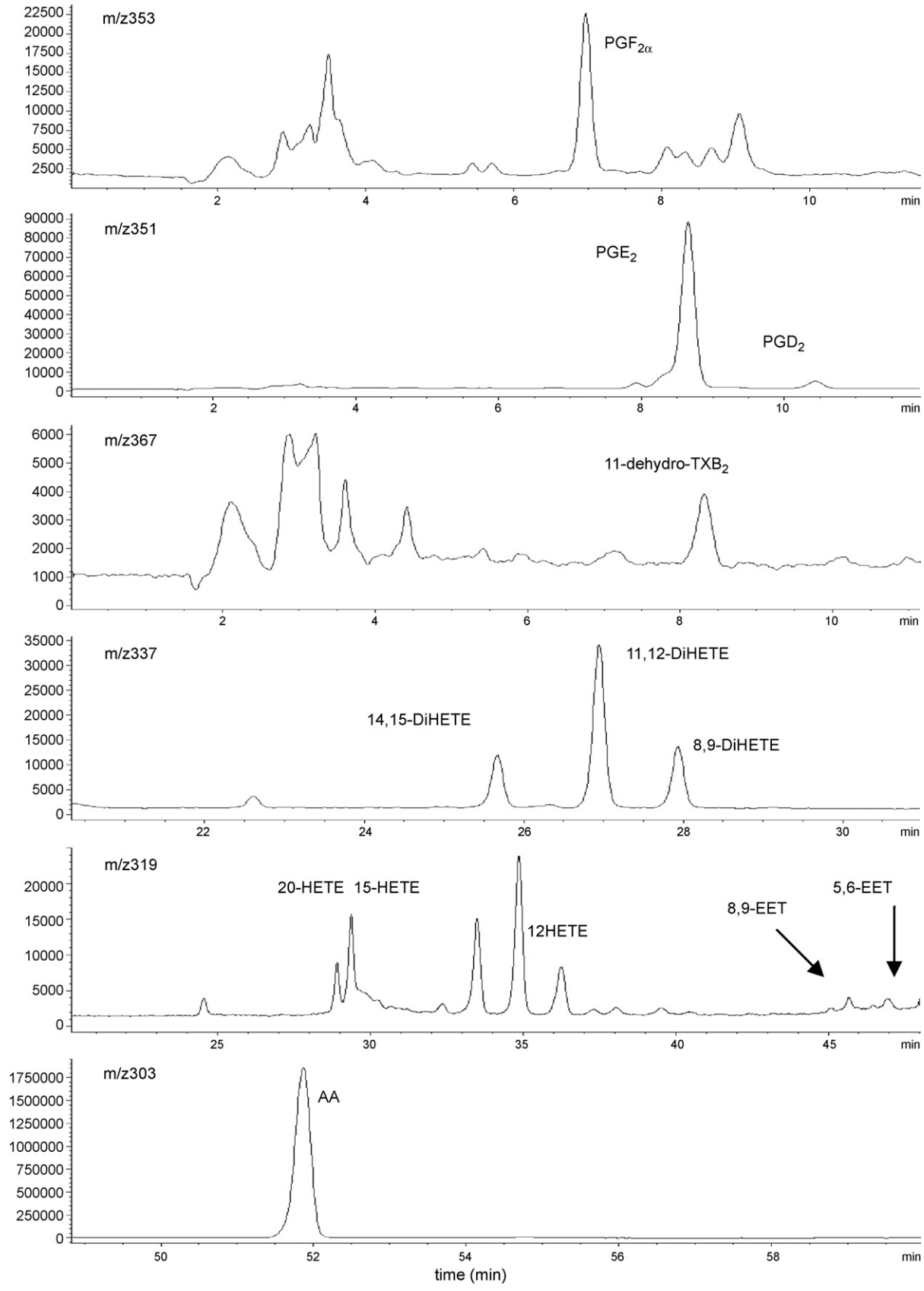

Fig. 5. Selected ion chromatograms of eicosanoids found in SHR kidney. Tissue samples prepared and injected using sample preparation described in the text.

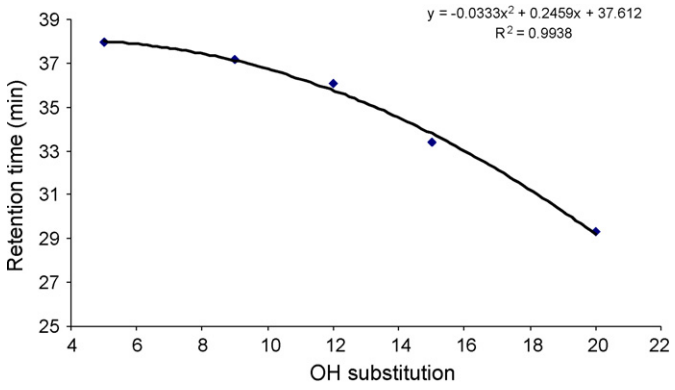

Fig. 6. Relationship between retention time and position of OH substitution for selective HETEs. Retention time increases as OH substitution moves closer to the carboxylic acid moiety at the α terminal.

the cys-LTs. The positively charged amino acids tend to have a strong ionic interaction with underivitized residual silanols on the HPLC column stationary phase. To avoid this problem, the method employed a column with the residual silanols endcapped with trimethylsilane. The protonated amino acid moieties on the cysteinyl-LTs also alter the relative polarity of the compound. This changes the elution of the LTs relative to one another. As a result, LTD_4 elutes with the PGs and LTB_4 elutes with the DiHETEs while LTC_4 and LTE_4 co-elute at approximately 14 min (Fig. 4). Changes in mobile phase configuration created little resolution between LTC_4 and LTE_4 and actually proceeded at the cost of a significant increase in total run time. The use of mass selective detection in the analysis allows co-eluting compounds to be resolved free of interferences as long as the compounds have a significant mass differential. An additional hurdle facing researchers is the inherent instability of the cys-LTs both *in vivo* and *ex vivo*, especially LTC_4 and LTD_4. The ubiquitous presence of peptidase enzymes act to catalyze the cleavage of amino acid residues on LTC_4 and LTD_4 *in vivo* while autooxidative degradation contributes to *ex vivo* instability. Therefore, BHT was used in all sample preparations as an antioxidant to prevent oxidative loss. However, a relatively large variability is still seen in the cys-LTs, especially LTC_4 (Table 4). This is most likely due to the previously mentioned instability of LTC_4 and its penchant for adsorption on exposed surfaces of vials, SPE cartridges and components of the chromatographic system [48]. Kita et al. showed that there is significant sample loss associated with LTC_4 when stainless steel tubing is used in the chromatographic system versus polyether-ether-ketone (PEEK)-based tubing [49]. The variability observed with LTC_4 is consistent with previously published methods.

This method is the first reported method to use LC–MS to simultaneously analyze all the major eicosanoids derived from all three metabolic pathways. The method is linear over several orders of magnitude and sensitive enough to quantitate endogenous levels of eicosanoids. Application of this method to SHR kidney tissues provided insight into which eicosanoids are present in the kidney, an organ commonly targeted for hypertension. The eicosanoids were extracted from the kidney in a single solid phase extraction procedure, creating a time efficient method whereby the entire eicosanoid profile can be obtained in one analysis. Hypertensive animals were used in this study in order to demonstrate the bioanalytical applicability of the method. The eicosanoid distribution was expected to be the greatest in the hypertensive animal due to more extensive inflammatory damage to the kidney. A direct comparison of hypertensive and normotensive animals to determine the role of inflammation in hypertension is the subject of a future manuscript.

The eicosanoids are important mediators of inflammation derived from arachidonic acid in response to inflammatory stimuli. Unlike the cytokines and other protein-based biomarkers which are generally synthesized hepatically, the eicosanoids are synthesized at the site of the injury to control and regulate inflammation locally. The eicosanoids have wide ranging physiological functions including acting to increase and/or reduce the inflammatory response. Therefore, it is important to be able to measure the activity of the eicosanoids as a whole in order to obtain a snapshot of the entire eicosanoid profile. Monitoring the eicosanoid profile and identifying trends that correlate to specific disease states can help aid in the development of improved therapeutic protocols for the treatment and prevention of CVD.

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