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Rapid simultaneous analysis of cyclooxygenase, lipoxygenase and cytochrome P-450 metabolites of arachidonic and linoleic acids using high performance liquid chromatography/mass spectrometry in tandem mode

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ABSTRACT

Eicosanoids are oxidized arachidonate-derived lipid products generated by cyclooxygenase, lipoxygenase and cytochrome P-450 pathways. They are involved in diverse processes in health and disease and they are highly bioactive. Gas chromatography and enzyme immunoassays were used to quantify these mediators in the past. However, the recent availability of high-sensitivity liquid chromatography–mass spectrometry has provided a new approach for quantification that minimizes the sample size and the required preparation. This paper describes a rapid and simple technique for the simultaneous quantitative analysis of prostaglandin (PG) E_2 and PGJ_2; leukotrienes (LT) B_4 and D_4; 5-, 12-, 15- and 20-hydroxyeicosatetraenoic acids (HETEs); 13-hydroxyoctadecadienoic acid (13-HODE); 5,6-, 8,9-, 11,12- and 14,15-epoxyeicosatrienoic acids (EETs); and 11,12- and 14,15-dihydroxieicosatrienoic acids (DHETs) in cell culture supernatants and urine. We simultaneously analyzed 14 arachidonic acid metabolites representative from the three pathways, together with 13-HODE, a linoleic-derived product. Solid phase extraction was used for the sample preparation. The recoveries obtained ranged from 25% to 100%, depending on the metabolites. The LC/MS/MS method used the gradient on a C_{18} column and electrospray ionization in negative ion detection mode. The method was optimized for sensitivity and for separation within 20 min. The linear ranges of the calibration curves were 0.1–200 ng/ml for PGE_2, PGJ_2, LTB_4, 5-HETE, 12-HETE, 15-HETE, 13-HODE, 11,12-EET, 11,12-DHET and 14,15-DHET, and 1–200 ng/ml for LTD_4, 20-HETE, 5,6-EET, 8,9-EET and 14,15-EET. The advantages of this method include minimal sample preparation, high sensitivity and elimination of the problem associated with thermal instability in gas chromatography analysis.

1. Introduction

Eicosanoids are lipid peroxidation products of 20-carbon polyunsaturated fatty acids as arachidonic acid (AA). They are generated in biological systems by three enzymatic pathways: the cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) pathways.

COX catalyzes the conversion of AA to prostaglandin (PG) H_2, the immediate precursor of prostanoids (prostaglandins, prostacyclins and thromboxanes) [1]. The main LOXs in mammalian cells are considered to be 5-, 12-, and 15-LOX. Thus, AA is metabolized by 5-LOX to yield 5-hydroxyeicosatrienoic acid (5-HETE) and leukotriene A_4 (LTA_4), the precursor of LTB_4 and cysteinyl LTs such as LTC_4, LTD_4 and LTE_4. On the other hand, 12- and 15-LOX produce 12- and 15-HETEs and lipoxins [1]. LOXs such as 15-LOX can also convert the abundant linoleic acid that is present in biomembranes to 13-hydroxyoctadecadienoic acid (13-HODE) [2]. CYPs metabolize AA by one or more of the following reactions: bis-allylic oxidation (lipoxygenase-like reaction) to generate 5-, 8-, 9-, 11-, 12- and 15-HETE; ω/ω-1 hydroxylation, which yields 16-, 17-, 18-, 19- and 20-HETEs; or olefin epoxidation to produce 5,6-, 8,9-, 11,12- and 14,15-epoxyeicosatrienoic acids (EETs). Finally, the cytosolic epoxide hydrolases rapidly catalyze the enzymatic hydration of EETs and transform them into dihydroxyeicosatrienoic acids (DHETs)

Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; CYP, cytochrome P450; DHET, dihydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatrienoic acid; 13-HODE, 13-hydroxyoctadecadienoic acid; LLOQ, lower limit of quantification; LOD, limit of detection; LOX, lipoxygenase; LT, leukotriene; PG, prostaglandin.

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[3]. Thus, AA and other polyunsaturated fatty acids are the precursors of a large number of biologically active molecules that are collectively named eicosanoids.

Eicosanoids take part in many physiological and pathophysiological processes in practically every organ, tissue and cell [4,5]. Lipidomics is an emerging area of lipid research that studies the profiles and complete composition of lipids in parallel with their functional role in any given tissue or system [6,7]. The study of eicosanoid profiles in conjunction with other metabolic profiles, enzyme activities, protein and gene expression can offer valuable insights to tissue and organ function in health and disease. GC/MS and GS/MS/MS [8], LC/fluorescence [9] and LC/MS methods have been used to measure some of these eicosanoids at low concentrations. Recently, LC/MS has been widely used in bioanalytical work, since it is a powerful analytical technique that combines the resolving power of liquid chromatography with the detection specificity of mass spectrometry.

In this study, we present a rapid method for the simultaneous qualitative and quantitative assay of PGs, LTs, HETEs, EETs, DHETs and 13-HODE. This method can be used to study the profiles of eicosanoid families in different cellular systems and fluids. Consequently, it can assist lipidomic or systems biology applications that aim to explore physiological/pathophysiological states, novel biomarkers or the development of therapeutic approaches.

2. Materials and methods

2.1. Materials

RPMI 1640 medium, fetal bovine serum (FBS), penicillin, streptomycin, and trypsin-EDTA were purchased from Invitrogen (Paisley, UK). LTD_4, PGE_2, PGJ_2, PGJ_2-d_4, LTB_4, LTB_4-d_4, 14,15-DHET, 11,12-DHET, 5-HETE, 5-HETE-d_8, 12-HETE, 12-HETE-d_8, 15-HETE, 15-HETE-d_8, 20-HETE, 20-HETE-d_6, 13-HODE, 13-HODE-d_{11}, 5,6-EET, 5,6-EET-d_{11}, 8,9-EET, 11,12-EET, 14,15-EET and 14,15-EET-d_{11} were purchased from Cayman Chemical (Ann Arbor, MI).

2.2. Cell culture

Murine 3T6 fibroblasts (ATCC CCL96) were cultured in RPMI 1640 containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were harvested with trypsin/EDTA and seeded in 6-well plates (tissue-culture cluster 6; Costar Cambridge, MA, USA) for experimental purposes. Cultures were maintained in a temperature- and humidity-controlled incubator at 37 °C with 95% air–5% CO_2.

2.3. Liquid chromatography/tandem mass spectrometry measurements of eicosanoids

To simultaneously separate 15 eicosanoids and 8 deuterated internal standards, we used a liquid chromatograph Perkin Elmer series 200 (Norwalk, CT) equipped with a quarternary pump and thermostated autosampler. In addition, a triple quadrupole mass spectrometer API3000 (ABSciex, Concord, Ontario, Canada) equipped with a TurboIonSpray source operating in negative ion mode was used to obtain the MS/MS data. Eicosanoids were separated using a Polarity dC_{18} 150 mm × 2.1 mm i.d. 5 μm column. Ten microliters of sample was injected into the column. The mobile phase consisted of water/acetonitrile with 0.1% formic. The flow rate was 0.3 ml/min. Gradient conditions were used and optimized to obtain the best separation in less than 30 min as follows (t[min], % acetonitrile) = (0,60), (15,75), (16,60), (30,60). MS conditions were: nebulizer gas (N_2, arbitrary units) 10, curtain gas (N_2, arbitrary units) 12, collision-activated dissociation (CAD) gas (N_2, arbitrary units) 4, and auxiliary gas (nitrogen), which was heated to 400 °C and introduced into the source at 6000 cm^3 min^{-1}. Ion spray voltage was −4500 V. The declustering potential (DP) and collision energy (CE) were optimized for each compound in infusion experiments. The focusing potential (FP) of −200 V, entrance potential (EP) of -10 V and collision cell exit potential (CXP) of −15 V were common for all the multiple reaction monitoring (MRM) transitions. The triple quadrupole mass spectrometer was calibrated with the TurboIonSpray source using a test mixture solution of poly(propyleneglycol) obtained from ABSciex to ensure that mass accuracy specifications and sensitivity were achieved over the entire mass range. All the MS and MS/MS parameters were optimized in infusion experiments and full scan data acquisition was performed by scanning from m/z 100–800 in profile mode and using a cycle time of 2 s with a step size of 0.1 μ and a pause between each scan of 2 ms. In product ion scan experiments MS/MS product ions were produced by CAD of selected precursor ions in the collision cell of the triple quadrupole mass spectrometer and mass analyzed using the second analyzer of the instrument. Multiple MRM acquisition was done monitoring each transition with a dwell time of 500 ms. The MRM mode was required because many compounds could present the same nominal molecular mass, but the combination of the parent mass and unique fragment ions was used to selectively monitor eicosanoids in the samples. MRM transitions in negative ionization mode for each eicosanoid were chosen on the basis of specificity and sufficient sensitivity. Here, Q1 is set on the specific patent m/z (Q1 is not scanning) the collision energy is set to produce the optimal diagnostic charged fragment of that parent ion, and Q3 is set to the specific m/z of that fragment. Only ions with this exact transition will be detected. Moreover, the lower limit of quantification's (LLOQ) and the limit of detection's (LOD) precision, and accuracy achieved with this technique are difficult to match with other methodologies. Thus, the MRM is the method of choice for quantitative measurements, due to the higher selectivity and sensitivity in LC–MS/MS. However, MRM method is not specific because each eicosanoid present several m/z transitions. Thus, transitions with the best signal/noise ratio were chosen (Table 1), and were used to measure LTD_4, PGE_2, LTB_4, 14,15-DHET, 11,12-DHET, 20-HETE, PGJ_2, 13-HODE, 15-HETE, 12-HETE, 5-HETE, 14,15-EET, 11,12-EET, 8,9-EET and 5,6-EET, respectively. This was the elution sequence in our experimental conditions. The results were in agreement with the values in the available literature [10–12].

2.4. Solid-phase extraction of eicosanoids

Samples were acidified (2 M HCl, 10 min, 4 °C) and centrifuged (4000 × g, 10 min, 4 °C). Eicosanoids were extracted using a solid phase extraction method. First, the cell culture supernatant (5 ml) was applied to a Sep-Pack C18 cartridge (Waters Corp., Milford, MA) that had been preconditioned with ethanol and water. Then, the cartridges were washed with water (10 ml), 15% ethanol aq. solution (10 ml) and hexane (10 ml). Finally, eicosanoids were eluted with 10 ml of ethyl acetate. Urine samples were collected from volunteers and extracted as described for the cell culture supernatant. The eluates were evaporated under a stream of nitrogen and the residue was dissolved in 150 μl mobile phase water/acetonitrile (4:6, v/v) containing 0.1% formic acid. It was then transferred to an insert in the sample vial and was ready for LC/MS analysis or frozen at −80 °C. All extraction procedures were carried out under conditions of minimal light to reduce the potential for photodegradation of the eicosanoids.

2.5. Calibration and validation

A stock solution of eicosanoids was prepared by mixing 1 μg/ml of each metabolite in ethyl acetate. This solution was further diluted

Table 1
MRM transitions, retention times, the lower limit of quantification (LLOQ) and the limit of detection (LOD) of eicosanoids and deuterated internal standards.

	MS and LC parameters		Retention time (min)	LOD (ng/ml)	LLOQ (ng/ml)
	Q1 *m/z*	Q3 *m/z*			
Eicosanoid					
LTD_4	495.5	177.4	1.07	0.006	0.021
LTB_4	335.4	195.1	3.17	0.004	0.010
PGE_2	351.3	315.3	1.78	0.001	0.004
PGJ_2	315.1	271.5	6.03	0.007	0.025
14,15-DHET	337.3	207.2	4.10	0.001	0.003
11,12-DHET	337.3	167.4	4.53	0.005	0.015
5-HETE	318.9	115.2	8.70	0.003	0.011
12-HETE	319.5	179.0	8.25	0.026	0.087
15-HETE	319.2	219.5	7.31	0.006	0.018
20-HETE	319.3	301.2	5.70	0.026	0.087
13-HODE	295.3	277.1	6.79	0.005	0.015
5,6-EET	319.5	163.4	12.35	0.095	0.316
8,9-EET	319.5	154.9	11.84	0.063	0.214
11,12-EET	319.2	167.5	11.23	0.050	0.168
14,15-EET	319.5	301.3	10.59	0.026	0.087
Deuterated eicosanoid					
PGJ_2-d_4	319.4	275.4	5.98	0.015	0.048
LTB_4-d_4	339.5	197.0	3.13	0.004	0.014
5-HETE-d_8	327.5	116.1	9.06	0.023	0.076
12-HETE-d_8	327.5	308.2	8.67	0.050	0.165
15-HETE-d_8	327.5	226.1	7.41	0.017	0.059
13-HODE-d_{11}	299.3	197.9	6.69	0.024	0.080
5,6-EET-d_{11}	330.3	268.1	12.05	0.195	0.650
14,15-EET-d_{11}	330.3	202.4	10.39	0.396	1.321

to prepare a curve calibration. These standard curves were typically constructed over the range of 0.1–200 ng/ml for each analyte. The ratios of LC–MS/MS peak areas of the analyte/standard were calculated and were used to construct calibration curves of peak area ratio against analyte concentration using unweighted linear regression analysis. The linearity and reproducibility of the assay method were assessed in five replicate analyses.

The selectivity of the method was also investigated using a standard mixture of eicosanoids. The degree of interference was assessed by inspection of the individual MRM chromatograms, and no significant interfering peaks from the standard mixture were found at the retention times, in the MRM channels of any of the eicosanoids. There also were no interfering peaks from the cell supernanat at the retention time and in the MRM channel of the eicosanoids.

Standard curves were analyzed over different experiments and accuracy and precision were evaluated at two levels (25 and 100 ng/ml) and given in Table 2. Accuracy was determined as the relative deviation in the calculated value (C) of a standard from that of its true values (T) expressed as a percentage. This accuracy, or relative standard error (RE%), was calculated using the equation $RE\% = (C - T)/T \times 100$. Precision was expressed as the relative standard deviation (RSD%) of the mean using the equation $RSD\% = SD/M \times 100$. The criteria for acceptability of data included accuracy and precision within ±15% deviation of the nominal value.

The recovery of solid-phase extraction was first evaluated after adding eicosanoids standards mixture to cell culture medium that did not contain appreciable amount of these eicosanoids. Moreover, deuterated eicosanoids were added to cell culture supernatants to confirm the extraction efficiency in these samples which contains eicosanoids released by cells.

2.6. Enzyme immunoassay of eicosanoids

PGE_2, LTB_4 and 12-HETE were extracted from the cell culture supernatant medium through C_{18} reverse-phase extraction columns in ethyl acetate. Next, the organic phase was evaporated in a stream of nitrogen. Eicosanoids were then measured using PGE_2 and LTB_4 enzyme immunoassay kits (Cayman Chemical Co., Ann Arbor, MI), and 12-HETE was measured using an Assay Designs enzyme immunoassay (Ann Arbor, MI). These measurements were made according to the manufacturers' protocols.

Table 2
Intra- and inter-assay accuracy (RE%) and precision (RSD%) for each eicosanoid assayed.

Eicosanoid	ng/ml	Intra-assay		Inter-assay	
		RE%	RSD%	RE%	RSD%
LTD_4	25	−4.93	6.09	4.38	8.15
	100	4.48	3.38	−7.81	18.67
LTB_4	25	−2.76	6.19	−5.15	7.99
	100	2.69	4.30	4.72	1.43
PGE_2	25	−4.99	4.74	−0.26	3.18
	100	−0.53	7.78	1.84	2.12
PGJ_2	25	−3.90	8.24	0.61	3.33
	100	−2.14	6.43	2.79	0.24
14,15-DHET	25	−3.57	2.14	−0.51	7.17
	100	3.00	2.97	4.19	2.49
11,12-DHET	25	−3.90	2.03	2.08	6.84
	100	−0.89	5.07	0.95	1.71
5-HETE	25	2.74	5.72	3.35	1.45
	100	4.79	3.91	−1.17	1.96
12-HETE	25	−7.88	6.36	6.74	10.30
	100	1.40	4.43	−0.59	2.06
15-HETE	25	−6.88	2.93	3.34	6.31
	100	0.96	0.88	−0.14	0.26
20-HETE	25	3.38	6.96	1.52	3.57
	100	−0.93	9.24	1.10	5.46
13-HODE	25	0.79	4.50	1.88	4.50
	100	1.50	4.64	1.97	1.45
5,6-EET	25	−2.80	2.42	−1.42	7.70
	100	−1.68	2.56	26.35	12.36
8,9-EET	25	3.73	14.98	4.51	13.81
	100	9.31	17.86	0.10	7.88
11,12-EET	25	−11.15	21.74	3.01	4.18
	100	4.88	8.06	4.99	2.19
14,15-EET	25	−15.91	1.06	7.36	5.85
	100	0.17	2.04	1.42	2.22

Curve standard points were tested five times in the same assay to assess intra-assay accuracy/precision whereas these curve standard points were also tested in five separate assays to assess inter-assay accuracy/precision.

3. Results

We carried out a rapid and efficient extraction method followed by LC–MS/MS analysis to determine 15 eicosanoids in cell culture supernatants. The differences in functional groups and their positions are sufficient for a good chromatographic separation by a reverse phase C_{18} HPLC column using water-acetonitrile mobile phase.

Standard calibration lines were typically constructed for each analyte and a good linear response was achieved for all compounds. The response from the LC–MS method was linear between 0.1 and 200 ng/ml for PGE_2, PGJ_2, LTB_4, 12-HETE, 15-HETE, 13-HODE, 11,12-EET, 11,12-DHET and 14,15-DHET and between 1 and 200 ng/ml for LTD_4, 20-HETE, 5,6-EET, 8,9-EET and 14,15-EET. For the determination of the sensitivity of the method, we determined the LLOQ and LOD for each eicosanoid. The culture medium was spiked with external standard specimens in a concentration range of 0.01–200 ng/ml and he LLOQ was determined as the concentration with a signal-to-noise (S/N) ratio >10, whereas the LOD was calculated using an S/N ratio of 3. Values for the LLOQ and LOD for each eicosanoid are summarized in Table 1 together with the retention time for all eicosanoids.

The intra- and inter-day accuracy and precision for the assay were evaluated at 25 and 100 ng/ml (250 and 1000 pg on column, respectively). Five replicates were used to determine the intra-day reproducibility whereas inter-day reproducibility was determined over five separate assays performed in different days. The intraday precision range was within a RSD% of 0.88–21.74, and the inter-day precision range was between 0.24 and 18.67, being the RSD% obtained from almost all the eicosanoids lower than 10% thus indicating an acceptable level of reproducibility (Table 2).

Eicosanoids are very lipophilic and avidly bind to proteins and lipid material in a biological matrix. Acidic conditions resulted in the conversion of eicosanoids to the free carboxylic acid form for higher extraction efficiency but also reduced protein binding. The ratio of ethanol to water for loading and walking steps was found to be critical and was optimized to retain eicosanoids on the column while allowing undesirable contaminants. To assess the recovery of the extraction method, culture medium was spiked with standard eicosanoids. Similar extraction recoveries were obtained for each eicosanoid between 0.1 and 50 ng/ml (data not shown). To assay the influence of cells as well as products released by cells on eicosanoid extraction recovery, supernatant cell cultures were spiked with mixed deuterated eicosanoids at 0.5 ng/ml (2.5 ng/sample for each eicosanoid) and analyzed. The recoveries of eicosanoids added to culture medium (0.1 ng/ml) and deuterated eicosanoids added to the samples are summarized in Table 3. They ranged from 25% to 100% depending on the metabolites. We can observe a lower recovery in the presence of cells as a consequence of eicosanoid release by the cell, their incorporation into membranes or their metabolism. These last recoveries were used to calculate the final eicosanoid concentrations in the samples.

This method was applied to study the AA metabolites synthesized by 3T6 fibroblasts. Representative extracted ion chromatograms of the standards and the biological sample extract area shown in Fig. 1. The analysis of eicosanoids in 3T6 fibroblast cell culture supernatant showed the presence of PGE_2, LTB_4 and 13-HODE, 5-, 12, and 15-HETE but not detectable amounts of LTD_4 and the CYP-450 pathway metabolites such as EETs and DHETs (Fig. 1B, Table 4). In contrast, LTD_4 and 11,12-EET were detected and quantified in human urine samples (Table 4). All peaks were verified by comparing their m/z fragment and retention time with that of a standard. Our results also show that 10% FBS increases PGE_2, LTB_4, 13-HODE, 5-, 12-, and 15-HETE concentrations in 3T6 fibroblast cell culture supernanat (5, 0.5, 3, 3, 2 and 4-fold, respectively, Table 4), and the metabolites with the highest concentrations measured were 13-HODE and 5-, 12- and 15-HETEs. Moreover, PGE_2, LTB_4 and 12-HETE concentrations in cell medium in the presence and absence of FBS were measured by enzyme immunoassay and by LC–MS/MS, and similar results were obtained with both techniques (Table 4). The low concentration of PGE_2 and LTB_4 in the samples together with their low extraction recoveries lead to a low concentration of these eicosanoids in the sample finally injected and consequently a complex elution profile (Fig. 1). However, peaks corresponding to PGE_2 and LTB_4 decreased when cells were incubated without FBS whereas the background did no change, thus confirming that these peaks correspond to these two eicosanoids. Moreover, peaks corresponding to PGE_2 and LTB_4 were reduced by COX and 5-LOX inhibitors, respectively (data not shown).

Table 3
Recovery of eicosanoids and deuterated eicosanoids in complete cell culture media using LC–MS/MS.

	Extraction efficiency
Eicosanoid	
LTD_4	37.6 ± 4.3
LTB_4	39.9 ± 8.6
PGE_2	39.1 ± 0.9
PGJ_2	64.2 ± 3.3
14,15-DHET	22.5 ± 4.1
11,12-DHET	23.6 ± 1.5
5-HETE	99.9 ± 2.9
12-HETE	90.8 ± 0.7
15-HETE	97.7 ± 0.3
20-HETE	63.6 ± 10.8
13-HODE	79.5 ± 0.9
5,6-EET	58.2 ± 7.5
8,9-EET	74.7 ± 2.3
11,12-EET	70.7 ± 13.5
14,15-EET	69.3 ± 8.5
Deuterated eicosanoid	
PGJ_2-d_4	42.3 ± 0.4
LTB_4-d_4	22.6 ± 0.3
5-HETE-d_8	91.9 ± 8.1
12-HETE-d_8	96.1 ± 2.4
15-HETE-d_8	40.8 ± 2.9
13-HODE-d_{11}	38.1 ± 3.8
5,6-EET-d_{11}	21.2 ± 1.4
14,15-EET-d_{11}	41.2 ± 2.4

Eicosanoid mixture was added to 5 ml complete cell medium to reach 0.1 ng/ml of each eicosanoid and extraction was performed as described in Section 2. Deuterated eicosanoids (0.5 ng/ml) were added to the supernatant of cell cultures before extraction as internal standard of the extraction procedure. Data is the mean of 4 determinations ± SE.

4. Discussion

LC–MS/MS is a useful, high-throughput tool for the quantification of AA metabolites. In contrast, most traditional methods of analyses such as LC–UV or GC/MS require extensive sample preparation and long analysis times. For instance, GC/MS requires two derivatization steps. In contrast, eicosanoid immunoassays, while highly sensitive, only allow the measurement of one metabolite at a time, which significantly lowers the sample throughput. In this study we report an LC–MS/MS method for the simultaneous separation and quantification of 14 arachidonic acid metabolites including PGE_2 and PGJ_2 which are representative of COX pathway metabolites; LTB_4, LTD_4 and 5-HETE metabolites of the 5-LOX pathway; 12- and 15-HETE produced by 12/15 LOX or CYP-450; and EETs, DHETs and 20-HETE as metabolites synthesized exclusively by CYP-450s. These eicosanoids were analyzed in less than 15 min together with 13-HODE, a linoleic metabolite produced by 15-LOX [13] and eight deuterated eicosanoids. Thus, this rapid analysis is suitable for large sample numbers.

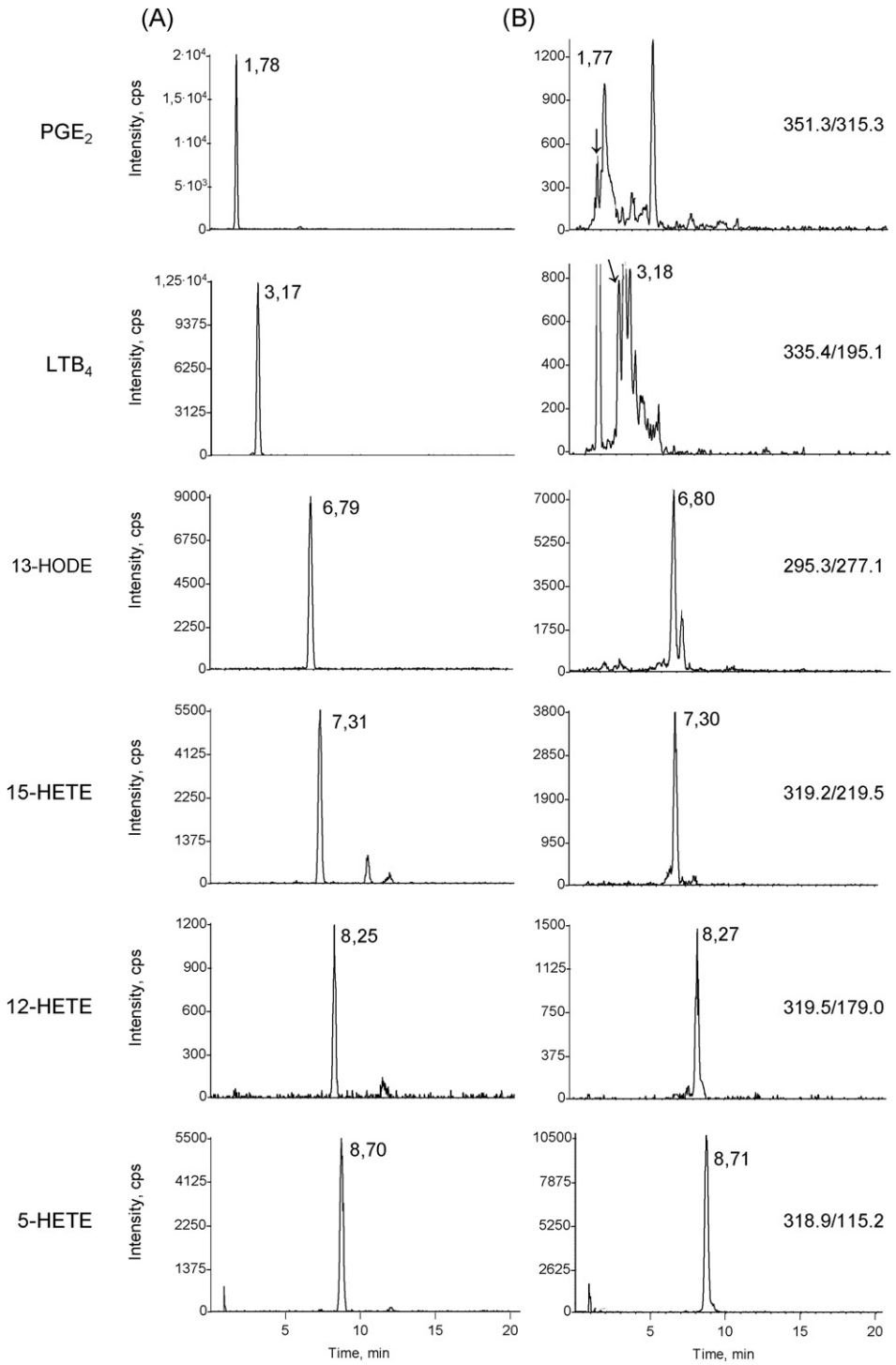

Fig. 1. Chromatographic profiles corresponding to 0.25 ng eicosanoid standard on column (A), and eicosanoids from 3T6 fibroblast cell culture media (B). Extraction and LC–MS/MS conditions were described in Section 2. The retention time and the *m/z* transition for each eicosanoid are included.

Several authors have reported LC–MS/MS protocols to analyze eicosanoids such as prostanoids [14,15], prostanoids and HETEs [16,17], prostanoids/HETEs/HODEs [18], EETs and DHETs [19], HETEs/EETs/DHETs [11,20] and prostanoids/leukotrienes/HETEs/HODEs [21]. Recently, Yue et al. [10] reported a simultaneous analysis of prostanoids/HETEs/EETs/DHETs. Interestingly, our LC–MS/MS protocol is suitable for simultaneously analyzing representative metabolites from the three AA pathways such as prostanoids, leukotrienes, HETEs, EETs and DHETs, together with linoleic metabolites such as 13-HODE. Interestingly, the overall chromatographic run time was relatively short compared with previously published methods.

Sample preparation was quick and simple as it only required purification through a reverse-phase C_{18} solid-phase extraction column. The problem of thermal stability for HETE analysis under GC/MS conditions was avoided and the method permitted multicomponent analysis. A sample volume of 5 ml was needed to

Table 4
Concentration of eicosanoids (ng/ml) in fibroblast supernatant cultures and urine measured by LC–MS/MS or EIA.

	Cells without FBS		Cells in 10% FBS		Urine
	HPLC–MS	EIA	HPLC–MS	EIA	HPLC–MS
Eicosanoid					
LTD_4	n.d.		n.d.		0.09 ± 0.02
LTB_4	0.21 ± 0.07	0.18 ± 0.03	0.31 ± 0.05	0.28 ± 0.01	n.d.
PGE_2	0.36 ± 0.09	0.31 ± 0.04	1.56 ± 0.39	1.16 ± 0.02	n.d.
PGJ_2	n.d.		n.d.		n.d.
14,15-DHET	n.d.		n.d.		n.d.
11,12-DHET	n.d.		n.d.		n.d.
5-HETE	1.05 ± 0.01		2.74 ± 0.24		n.d.
12-HETE	0.90 ± 0.20	1.09 ± 0.16	2.16 ± 0.14	2.32 ± 0.42	n.d.
15-HETE	0.52 ± 0.03		2.17 ± 0.32		n.d.
20-HETE	n.d.		n.d.		n.d.
13-HODE	0.94 ± 0.03		2.96 ± 0.22		0.11 ± 0.11
5,6-EET	n.d.		n.d.		n.d.
8,9-EET	n.d.		n.d.		n.d.
11,12-EET	n.d.		n.d.		0.39 ± 0.18
14,15-EET	n.d.		n.d.		n.d.

Data is the mean of 4 determinations ± SE. n.d. not detected because concentrations were lower than LOD.

quantify the eicosanoids with higher LOD's and LLOQ's values and/or lower extraction recoveries. However, a lower volume could be used to quantify some of these analytes.

The standard calibration curves were linear over a large concentration range for all compounds with good correlation coefficients (data not shown). Under the described conditions, between 0.1 and 1 ng/ml of LTD_4, LTB_4, PGE_2, PGJ_2, 11,12-DHET, 14,15-DHET, 13-HODE, and 5-, 12-, 15- and 20-HETE were detected, and the LLOQ for these eicosanoids were in the low nanomolar range (<5 ng/ml). However, EETs had higher LOD and LLOQ values than the above mentioned metabolites. These values were comparable to those reported by other authors [15,21] and were in the range of concentrations in biological samples that are around 0.1 μM (10–30 ng/ml) [22,23]. Thus, a suitable simultaneous quantification can be carried out in biological samples of these eicosanoids.

Eicosanoid profiling was performed in cell culture medium and using the assay method described. Fibroblast culture media has appreciable concentrations of PGE_2, LTB_4, 5-HETE, 12-HETE, 15-HETE and 13-HODE but not of PGJ_2, LTD_4, 20-HETE, EETs and DHETs. The precision of the method in the analysis of the identified compounds in replicates indicated an acceptable level of reproducibility. Moreover, the addition of 10% FBS increased the concentration of these metabolites in the cell culture medium as due to fibroblast metabolism, as FBS did not present appreciable amounts of these compounds (data not shown). Although we were unable to find any previous references to the simultaneous measurement of all these metabolites in fibroblast cultures, our findings are in agreement with previous reports on the presence of PGE_2, LTB_4 and 12-HETE in 3T6 fibroblast media [24] as well as with 5-HETE [25], 15-HETE [26] and 13-HODE [27] production by fibroblasts. Furthermore, PGE_2, LTB_4 and 12-HETE concentrations measured by LC–MS were similar to values obtained using enzyme immunoassays, findings that contribute to confirm the quality of the results obtained by this method. Although we could not detected the presence of PGJ_2, LTD_4, 20-HETE or EETs/DHETs in fibroblast culture media, appreciable concentrations of LTD_4 and 11,12-EET were observed in urine. These findings suggest that the COX pathway and the LOX pathway in particular are involved in 3T6 fibroblast metabolism of polyunsaturated fatty acids, whereas the CYP pathway does not have a relevant effect.

In conclusion, the validated method presented here described a sensitive and rapid LC–MS/MS assay for detecting eicosanoids in cell culture media. This method was also successfully used to determine eicosanoids in other biological samples, such as urine. The method has some advantages over others, including enzyme immunoassays, LC with fluorescence detection and GC–MS, because it can determine several compounds simultaneously, does not require sample derivatization, is extremely sensitive, and does not have high background interference from derivatizing reagents. Thus, this assay provides a quantitative technique to study the role of eicosanoids in physiological and pathophysiological processes.

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