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Rapid, simultaneous quantitation of mono and dioxygenated metabolites of arachidonic acid in human CSF and rat brain

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ABSTRACT

Currently, there are few biomarkers to predict the risk of symptomatic cerebral vasospasm (SV) in subarachnoid hemorrhage (SAH) patients. Mono and dioxygenated arachidonic acid metabolites, involved in the pathogenesis of ischemic injury, may serve as indicators of SV. This study developed a quantitative UPLC–MS/MS method to simultaneously measure hydroxyeicosatetraenoic acid (HETE), dihydroxyeicosatrienoic acid (DiHETrE), and epoxyeicosatrienoic acid (EET) metabolites of arachidonic acid in cerebrospinal fluid (CSF) samples of SAH patients. Additionally, we determined the recovery of these metabolites from polyvinylchloride (PVC) bags used for CSF collection. Linear calibration curves ranging from 0.208 to 33.3 ng/ml were validated. The inter-day and intra-day variance was less than 15% at most concentrations with extraction efficiency greater than 73%. The matrix did not affect the reproducibility and reliability of the assay. In CSF samples, peak concentrations of 8,9-DiHETrE, 20-HETE, 15-HETE, and 12-HETE ranged from 0.293 to 24.9 ng/ml. In rat brain cortical tissue samples, concentrations of 20-, 15-, 12-HETE, 8,9-EET, and 14,15-, 11,12-DiHETrE ranged from 0.57 to 23.99 pmol/g wet tissue. In rat cortical microsomal incubates, all 10 metabolites were measured with formation rates ranging from 0.03 to 7.77 pmol/mg/min. Furthermore, 12-HETE and EET metabolites were significantly altered by contact with PVC bags at all time points evaluated. These data demonstrate that the simultaneous measurement of these compounds in human CSF and rat brain can be achieved with a UPLC–MS/MS system and that this method is necessary for evaluation of these metabolites as potential quantitative biomarkers in future clinical trials.

1. Introduction

Cytochrome P450 (CYP) and lipoxygenase pathways catalyze the linear pathway of arachidonic acid metabolism to form various hydroxyeicosatetraenoic acid (HETEs) and epoxyeicosatrienoic acid (EETs) metabolites. EET metabolites are then metabolized by soluble epoxide hydrolase to produce dihydroxyeicosatrienoic acids (DiHETrEs) (Fig. 1) [1,2]. In brain tissue, these metabolites have been implicated in the pathogenesis of stroke. Previous studies have demonstrated that several of these metabolites are found in rodent stroke models [3–5], and in the cerebral spinal fluid of brain injured [6] and aneurysmal subarachnoid hemorrhage (SAH) patients [7]. These metabolites have been shown to regulate multiple microvascular processes including maintenance of vascular tone, promotion of new vessel growth, and altered regulation of cerebral blood flow (CBF). Specifically, 20-HETE has been shown to produce cerebral microvessel constriction [8], play a role in the autoregulation of CBF [9,10], regulate new blood vessel growth [11], and increase vascular remodeling [12]. Conversely, EET metabolites produce microvascular dilation [13,14], increase cerebral blood flow [15], and protect neurons [16] and astrocytes [17] from ischemic cell death *in vitro*. Although not as extensively studied, DiHETrEs, like the EETs, induce vasorelaxation in canine coronary arterioles [18], and in rat arterial smooth muscle cells [19]. Collectively, these studies demonstrate that CYP-mediated arachidonic acid metabolites produce multiple effects on the cerebral microvasculature and may be important mediators in the pathogenesis of cerebrovascular disease.

Due to the data associating CYP arachidonic acid metabolites to cerebrovascular pathogenesis, these metabolites have been suggested as possible biomarkers for symptomatic cerebral vasospasm

Abbreviations: SV, symptomatic vasospasm; SAH, subarachnoid hemorrhage; UPLC, ultra performance liquid chromatography; HETEs, hydroxyeicosatetraenoic acids; DiHETrEs, dihydroxyeicosatrienoic acids; EETs, epoxyeicosatrienoic acids; CSF, cerebrospinal fluid; PVC, polyvinylchloride; CBF, cerebral blood flow; CYP, cytochrome P450; MF, matrix factor.

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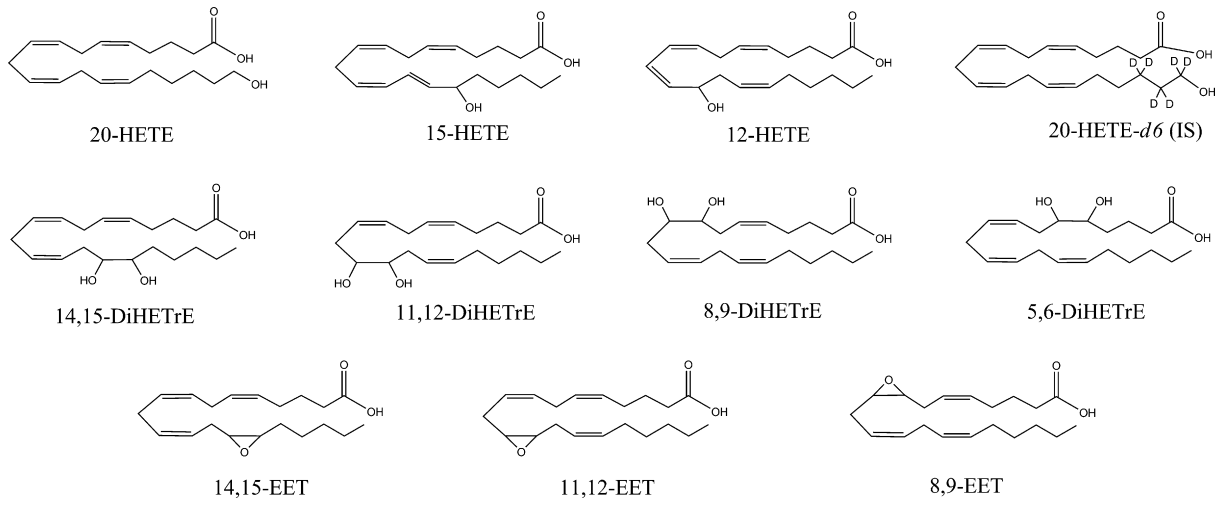

Fig. 1. Chemical structures of HETEs, EETs, DiHETrEs, and internal standard (IS).

(SV) after subarachnoid hemorrhage [7,20]. SV is unmet cerebral metabolic demand due to a vasospasm-induced decrease in cerebral blood flow resulting in a symptomatic deterioration of neurological function. This severe complication of subarachnoid hemorrhage occurs in almost one-third of SAH patients [21–23]. In SAH animal models, either inhibition of 20-HETE formation or prevention of EET degradation has been shown to be neuroprotective presumably by attenuating ischemic tissue damage associated with SV [24,25]. 20-HETE levels in cerebrospinal fluid (CSF) were found to be significantly higher in SAH patients compared to healthy controls [20]. The inhibition of CYP epoxygenase, which decreases EET formation, has also been shown to decrease CBF in rats via increased microvascular tone [26]. These monooxygenated arachidonic acid metabolites have been implicated as important mediators of cerebral regulation and may serve as useful biomarkers for SV.

These initial studies to determine the clinical importance of CYP arachidonic acid metabolites have created the need for sensitive, selective, and reproducible methods for measuring HETE, EET, and DiHETrE metabolites in human biological fluids. Multiple methods have been developed for the detection and quantification of these metabolites. Gas chromatography–mass spectrometry (MS) [27,28], liquid chromatography (LC)–mass spectrometry [29,30], LC–fluorescence detection [31], radioimmunoassays [32], electrophoresis [33], and enzyme immunoassays [34], have all aided in the quantitative analysis of these compounds from many different matrices. Although these assays are useful, limitations of these methods include high cost, limited sensitivity, cross-reactivity, and time-consuming analysis [35]. However, the most significant issue is the specificity for quantification of these highly similar isomeric metabolites in complex biological matrices. As a result, high performance liquid chromatography with tandem mass spectrometry (HPLC–MS/MS) has been successfully employed to quantify these highly similar isomeric metabolites.

While HPLC–MS/MS continues to be in the forefront in the investigation of these compounds from matrices including cell culture [36,37], tissue [38–40], and biological fluids [20,41], the limitation of flow rate applied to routine HPLC systems has resulted in extended run times for most of the reported methods to measure arachidonic acid metabolites. The shortest run time reported by these methods is 17 min, ultimately reducing data throughput [7]. A more efficient approach has been developed with the use of ultra performance liquid chromatography (UPLC), which uses high flow rates without the limitations of increased pressure or loss of performance. This new technique, developed in 2004, offers significant advantages over traditional HPLC methods, providing greater separation efficiency, improved sensitivity**,** and shortened run times [42,43]. UPLC systems, in conjunction with appropriate mobile phases and analytical columns, also offer benefits such as lower sample injection volumes and improved peak resolution [42,43].

Therefore, it was the purpose of this study to develop and validate a higher throughput UPLC–MS/MS method to simultaneously measure 10 arachidonic acid metabolites in CSF samples of SAH patients, rat cortical tissue, and rat cortical microsomal incubates. In addition, we investigated the effects of the matrix on the reproducibility and reliability of the data as recommended by the Food and Drug Administration (FDA) Quantitative Bioanalytical Methods Validation Report and other validation guidance documents [44,45]. Furthermore, we set out to determine the recovery of these metabolites from polyvinylchloride (PVC) bags, which is typically used for CSF collection in SAH patients. Development of this method is necessary for future evaluation of these metabolites as potential quantitative biomarkers in larger clinical trials.

2. Experimental

2.1. Chemicals and reagents

Stock standards of (±)14,15-dihydroxy-5Z, 8Z, 11Z-eicosatrienoic acid (14,15-DiHETrE), (±)11,12-dihydroxy-5Z, 8Z, 14Z-eicosatrienoic acid (11,12-DiHETrE), (±)8,9-dihydroxy-5Z, 8Z, 14Z-eicosatrienoic acid (8,9-DiHETrE), (±)5,6-dihydroxy-8Z, 11Z, 14Z-eicosatrienoic acid (5,6-DiHETrE), 20-hydroxy-5Z, 8Z, 11Z, 14Z-eicosatetraenoic acid (20-HETE), 20-hydroxy-5Z, 8Z, 11Z, 14Z-eicosatetraenoic-16,16,17,17,18,18-*d6* acid (20-HETE-*d6*), 12S-hydroxy-5Z, 8Z, 10E, 14Z-eicosatetraenoic acid (12-HETE), 15S-hydroxy-5Z, 8Z, 11Z, 13E-eicosatetraenoic acid (15-HETE), (±)14(15)-epoxy-5Z, 8Z, 11Z-eicosatrienoic acid (14,15-EET), (±)11(12)-epoxy-5Z, 8Z, 14Z-eicosatrienoic acid (11,12-EET), and (±)8(9)-epoxy-5Z, 11Z, 14Z-eicosatrienoic acid (8,9-EET) were purchased from Cayman Chemical Company (Ann Arbor, MI). High purity organic solvents were purchased from VWR (West Chester, PA) and all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO). Artificial cerebrospinal fluid (aCSF) was purchased from Tocris Bioscience (Ellisville, MO).

2.2. Animals

Male Sprague-Dawley rats (b.wt., 250–300 g) were obtained from Hilltop Laboratory Animals Inc. (Scottsdale, PA). The animals were maintained on a 12-h light/dark cycle and were given free access to pellets and water. Rats were placed under light anesthesia with 3:1 ketamine and xylazine (v/v) (Webster Veterinary Supply, Sterling, MA) or with the use of a spontaneous inhalational anesthetic system [SurgiVet (Smiths Medical, Waukesha, WI) V7216 Isotec 4] using isoflurane in conjunction with pure oxygen and nitrous oxide. Animals were sacrificed by decapitation, and brain cortical tissue was excised. All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

2.3. Chromatographic and mass spectrometric conditions

Liquid chromatography was performed using an Acquity ultra performance LC autosampler (Waters, Milford, MA). Separation of analytes was conducted on a UPLC BEH C-18, 1.7 μm (2.1 mm × 100 mm) reversed-phase column (Waters, Milford, MA) protected by a guard column (2.1 mm × 5 mm; Waters, Milford, MA) of the same packing material. Column temperature was maintained at 55 °C. Mobile phases, delivered at a flow rate of 0.5 ml/min, consisted of 0.005% acetic acid, 5% acetonitrile in deionized water (A) and 0.005% acetic acid in acetonitrile (B) at an initial mixture of 65:35 A and B, respectively. Mobile phase B increased from 35% to 70% in a linear gradient over 4 min, and again increased to 95% over 0.5 min where it remained for 0.3 min. This was followed by a linear return to initial conditions over 0.1 min with a 1.5 min pre-equilibration period prior to the next sample run. Total run time per sample was 6.4 min and all injection volumes were 7.5 μl.

Mass spectrometric analysis of analyte formation was performed using a TSQ Quantum Ultra (Thermo Fisher Scientific, San Jose, CA) triple quadrupole mass spectrometer coupled with heated electrospray ionization (HESI) operated in negative selective reaction monitoring (SRM) mode with unit resolutions at both Q1 and Q3 set at 0.70 full width at half maximum. Quantitation by SRM analysis on HETEs, EETs, and DiHETrEs was performed by monitoring their m/z transitions. The SRM conditions for these molecules and their retention times are shown in Table 1. Parameters were optimized to obtain the highest $[M-H]^-$ ion abundance and were as follows: capillary temperature, 400 °C, spray voltage, 3000 kV, and a source collision-induced dissociation set at 0 V. Sheath gas, auxiliary gas, and ion sweep gas pressures were set at 65, 55, and 3, respectively. Scan time was set at 0.01 s and collision gas pressure was set at 1.3 mTorr. Analytical data was acquired and analyzed using Xcaliber software version 2.0.6 (ThermoFinnigan, San Jose, CA).

Table 1
SRM conditions for the arachidonic acid metabolites and the deuterated internal standard in negative electrospray ionization mode.

	Precursor → Product (m/z)	Collision energy	Retention time (min)
20-HETE	319 → 245,289	18	3.69
15-HETE	319 → 219	14	4.08
12-HETE	319 → 179	16	4.28
14,15-EET	319 → 219	14	4.61
11,12-EET	319 → 167	15	4.77
8,9-EET	319 → 127	14	4.81
14,15-DiHETrE	337 → 207	18	3.22
11,12-DiHETrE	337 → 167	19	3.40
8,9-DiHETrE	337 → 127	25	3.57
5,6-DiHETrE	337 → 145	19	3.79
20-HETE-*d6*	319 → 251,295	18	3.69

2.4. Preparation of calibration standards and quality control samples

A stock solution of metabolites was prepared by combining 10 μg/ml of each metabolite in methyl acetate. This solution was further diluted with 80:20 methanol:deionized water to achieve a series of working solutions at 1 μg/ml, 100 ng/ml, and 25 ng/ml concentrations. A solution of 20-HETE-*d6*, used as the internal standard (IS), was prepared in 80:20 methanol:deionized water at a concentration of 1 μg/ml. Calibration standards and quality control (QC) samples were prepared from separate stock dilutions by spiking appropriate amounts of the working solutions into buffer. Calibrants were prepared at 0.208, 0.417, 0.833, 1.25, 1.67, 4.17, 8.33, 12.5, 16.67, and 33.33 ng/ml (12.5, 25, 50, 75, 100, 250, 500, 750, 1000, 2000 pg on column, respectively). QCs were prepared at 1.50, 7.50, and 20.83 ng/ml (90, 450, 1250 pg on column, respectively).

2.5. Sample analysis

2.5.1. CSF extraction

All protocols have been approved by the Institutional Review Board and informed consent was obtained from the human subjects or their representatives. Fresh CSF samples used in this study were collected from aneurysmal SAH patients via external ventricular devices (EVDs) at 12 h intervals throughout a 14-day period following a SAH. These samples were aliquotted into preservative-free cryostat vials and immediately stored at −80 °C until analysis. Varying volumes of sample from 0.11 to 1.0 ml were collected and used for analysis. Ideally, volumes of 1.0 ml of fresh CSF are analyzed for optimal metabolite determination, however, variables such as EVD clogging and variable CSF drainage rates contribute to the wide range of sample volumes that were collected. Prior to extraction CSF samples were diluted in a 0.12 M potassium phosphate buffer containing 5 mM magnesium chloride and 0.113 mM butylated hydroxytoluene (BHT).

2.5.2. Cortical tissue extraction

In a subsequent study, arachidonic acid metabolite concentrations were determined in brain cortical tissue samples of control Sprague-Dawley rats. Tissue procurement was carried out using methods previously outlined by Mu et al. [38]. Samples were homogenized in buffer as described above, centrifuged for 30 min at 10,000 rpm, and the supernatants were removed for analysis.

The metabolite concentrations in the above samples, as well as in the calibrants and quality controls were determined using a solid phase extraction (SPE) procedure. Briefly, diluted CSF and tissue supernatents were spiked with 12.5 ng of 20-HETE-*d6* as the IS. Samples were loaded onto Oasis HLB (30 mg) SPE cartridges (Waters, Milford, MA) that were conditioned and equilibrated with 1 ml of methanol and 1 ml of water, respectively. Columns were washed with three 1 ml volumes of 5% methanol and were eluted with 100% methanol. Extracts were dried under nitrogen gas at 37 °C and reconstituted in 125 μl of 80:20 methanol:deionized water. Using this method, CSF and tissue extract samples were concentrated 8-fold when 1 ml sample volumes were processed or less when processing smaller sample volumes.

2.5.3. Cortical microsomal incubations

Our method was also used in the quantitative analysis of arachidonic acid metabolites from rat cortical microsomal incubates. Rat brain cortical microsomes were prepared using the method described by Bolcato et al. [29]. Briefly, the incubations contained 300 μg of microsomal protein and 50 μM of arachidonic acid in a 1 ml volume of incubation buffer (0.12 M potassium phosphate buffer containing 5 mM magnesium chloride). Reactions were initiated by the addition of 1 mM NADPH with a subsequent 1 mM

concentration added 30 min afterwards. Incubations were carried out at 37 °C for 60 min and the reaction was stopped by placing the tubes on ice. To each sample, 12.5 ng of 20-HETE-*d6* was added as the internal standard. Microsomal incubations were extracted twice with 3 ml of diethyl ether, dried under nitrogen gas, and reconstituted in 80:20 methanol:deionized water in preparation for analysis.

2.6. Method validation

Validation of the SPE assay was performed by using ten standard concentrations of HETEs, EETs, and DiHETrEs, prepared in a 2 ml volume of the same phosphate buffer used for sample dilution, and extracted via SPE using the above method. The amount of metabolites in the standards ranged from 0.208 to 33.3 ng/ml (12.5–2000 pg on column). Three separate duplicate standard curves were prepared and analyzed over three consecutive days. Curves were calculated based on the peak area ratios between each metabolite to the IS and plotted against the amount of metabolite injected onto the column.

2.6.1. Precision and accuracy

Precision and accuracy of the method was determined by the analysis of quality control (QC) samples. Metabolites were spiked into buffer to yield low, medium, and high QCs, corresponding to 1.50, 7.50, and 20.83 ng/ml (90, 450, 1250 pg on column), respectively. Five samples at each level were analyzed for two days, followed by 10 replicates of each on the final day of validation. The lower limit of detection (LLOD) was determined by the minimum value with a signal-to-noise (S/N) ratio of $\geq$3:1. The lower limit of quantitation (LLOQ) was determined by the minimum value with accuracy and precision within $\pm$15% deviation of the nominal value. Accuracy was determined as the relative deviation in the calculated value (E) of a standard from that of its true value (T) expressed as a percentage. This accuracy, or relative standard error (RE%), was calculated using the equation $\mathrm{RE\%} = (E - T)/T \times 100$. Precision was evaluated and expressed as relative standard deviation (RSD%) of the mean concentrations using the equation $\mathrm{RSD\%} = \mathrm{SD}/M \times 100$. The criteria for acceptability of data included accuracy and precision within $\pm$15% deviation of the nominal value. The recovery efficiencies of the metabolites were determined at low and high concentrations (five to six independent samples each) at 1.50 and 20.83 ng/ml (90 and 1250 pg on column), respectively, by comparing the responses (area) of samples spiked before extraction to those spiked after extraction.

2.7. Metabolite stability and recovery determination

Stability of HETE, EET, and DiHETrE metabolites at room temperature and recovery from Medtronic Exacta drainage bags (Medtronic Neurosurgery, Goleta, CA) were measured over 12 h in order to determine the appropriateness of evaluating CSF collection bag samples for these metabolites. The duration of the experiment was chosen based on the time period between collection of CSF samples. For the evaluation of temperature stability, pooled bag CSF was aliquotted into silanized test tubes. One set of samples was spiked with metabolites at a concentration of 33.33 ng/ml while the other set of vials included control CSF with no metabolites for subtraction of basal CSF concentrations. All samples were kept at room temperature for up to 12 h. 500 μl aliquots were collected at baseline (time 0) and at 1, 6 and 12 h after start of incubation (n = 3 per time point). Sample aliquots were immediately frozen at −80 °C after room temperature incubation.

For evaluation of recovery from collection bags, CSF was pooled from SAH patients and aliquots of 18 ml were added into 1L Medtronic Exacta drainage bags made of PVC. One bag was spiked with 33.33 ng/ml of metabolites while the other bag included control CSF with no metabolite addition. 500 μl aliquots were collected at baseline (time 0) and at 1, 6 and 12 h after start of incubation (n = 4 per time point). Sample aliquots were immediately frozen at −80 °C after room temperature incubation.

At the time of sample processing, frozen samples from the above two experiments were thawed and processed using SPE and concentrations of metabolites were determined by UPLC–MS/MS as described previously. Concentration of metabolites measured in the control CSF was subtracted from the concentration of metabolites measured in the spiked CSF. Results were expressed as a percentage of initial concentration.

It is noteworthy that a direct measurement of 5,6-EET has not been attempted in most studies because, unlike the other EET regioisomers, 5,6-EET has been reported to be chemically unstable in physiological buffer solution and alkaline aqueous solution [33,46,47]. Also, 5,6-EET is hydrolyzed to 5,6-DiHETrE and its lactone under neutral and acidic conditions [33,46]. Therefore, a measurement of 5,6-EET in human CSF was excluded from our analysis.

2.8. Evaluation of matrix effects

Matrix effects were evaluated using 2 ml volume of the same phosphate buffer used for processing human CSF samples, rat cortical tissue extracts, and rat brain microsomal incubations and also artificial cerebrospinal fluid (aCSF) spiked with 0.113 mM BHT. Both matrices were extracted via SPE using the method described above and the eluate was spiked with low and high concentrations of HETEs, EETs, and DiHETrEs at 1.5 and 20.83 ng/ml, respectively, and *d6*-20-HETE internal standard at 12.5 ng/ml. Neat samples containing the same concentrations of HETEs, EETs, DiHETrEs and 20-HETE-*d6* internal standard were prepared in 80:20 methanol:deionized water. Samples were analyzed using UPLC–MS/MS as described above. The internal standard-normalized matrix factor (IS-normalized MF) for each sample was calculated based on the area ratio (analyte/internal standard) of the post-extraction spiked samples to the neat samples as described by Viswanathan et al. [45]. IS-normalized MF results are expressed as average ± standard deviation with coefficient of variation (CV) (n = 6). Values less than 1.00 indicate ion suppression and values greater than 1.00 indicate ion enhancement [44].

2.9. Statistical analysis

Statistical analysis was completed using GraphPad Prism software, version 4.03 (GraphPad Software, La Jolla, CA). In the metabolite stability and recovery studies, the percentage of initial concentration at each time point was compared using repeated measures one-way analysis of variance with Dunnett's *post hoc* test for each metabolite. In the matrix effects studies, IS-normalized MF values were compared using unpaired t-test (2 tailed). For all statistical tests, a $p < 0.05$ was considered significant.

3. Results

3.1. Development of UPLC–MS/MS method

Acetonitrile:water mobile phases with acetic acid were determined to provide the optimal chromatographic conditions and optimal sensitivity for CYP arachidonic acid metabolite detection. Using these parameters we saw an increase in sensitivity with a 2-fold increase in peak area, and a 3-fold increase in S/N as compared to methanol (data not shown).

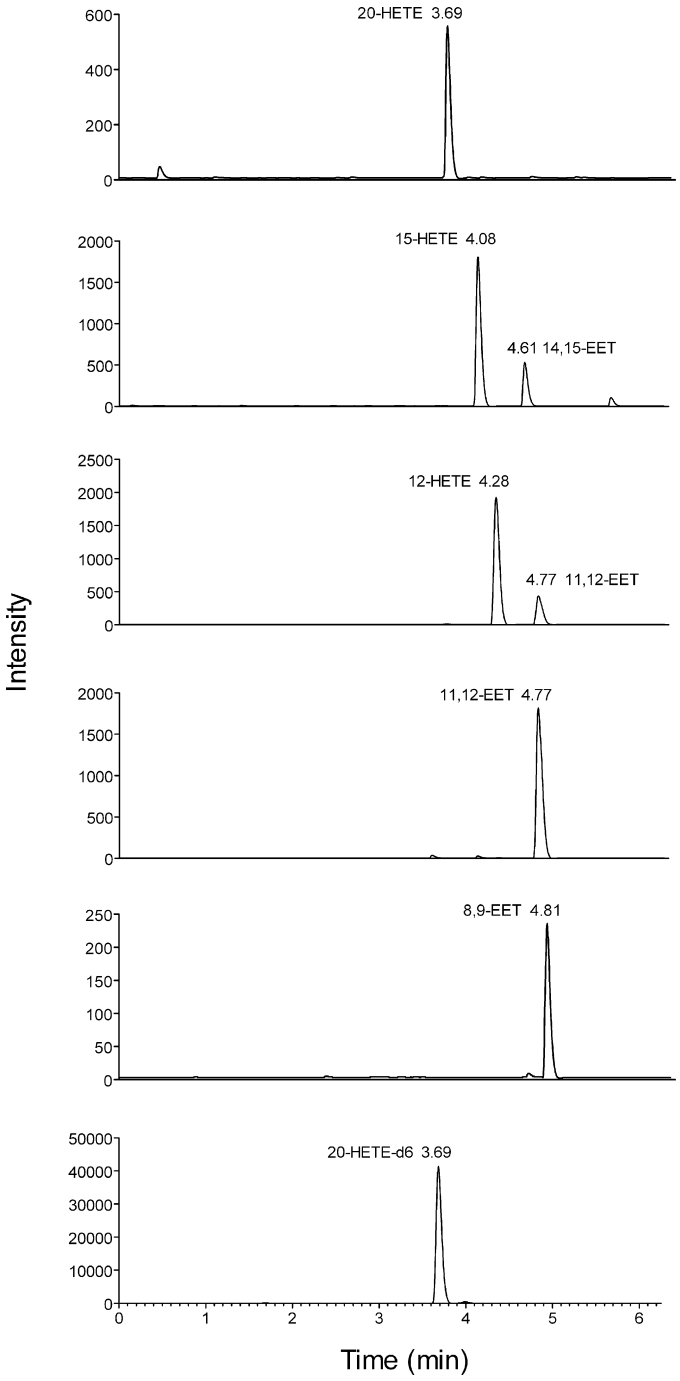

Fig. 2. Chromatographic profiles, corresponding to 25 pg on column, depicting the separation of HETEs and EETs using a UPLC tandem MS/MS triple quadrupole mass spectrometer. Resolution of the extracted standard mixture was performed on a reversed-phase Aquity BEH (Waters, Milford, MA) C-18 column (2.1 mm × 100 mm; 1.7 μm particle size). Metabolites were eluted at a flow rate of 0.5 ml/min over 6.4 min with a gradient from 35% acetonitrile containing 0.005% acetic acid to 95% acetonitrile containing 0.005% acetic acid.

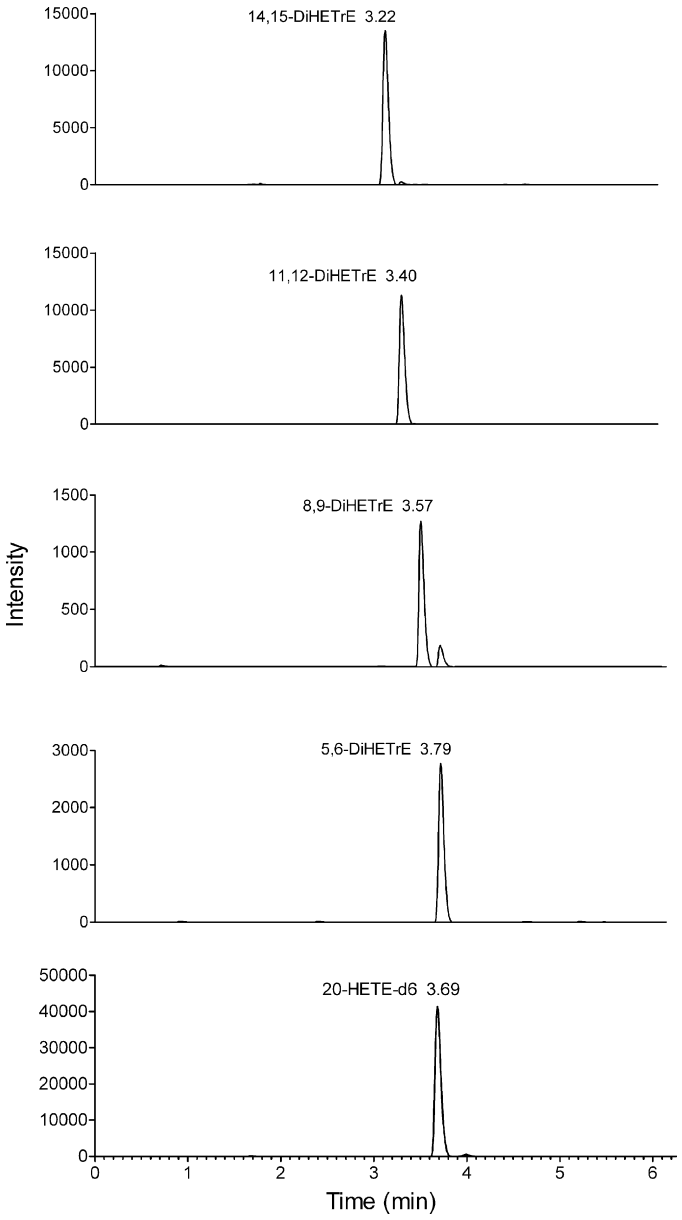

Fig. 3. Chromatographic profiles, corresponding to 25 pg on column, depicting the separation of DiHETrEs using a UPLC tandem MS/MS triple quadrupole mass spectrometer. Resolution of the extracted standard mixture was performed on a reversed-phase Aquity BEH (Waters, Milford, MA) C-18 column (2.1 mm × 100 mm; 1.7 μm particle size). Metabolites were eluted at a flow rate of 0.5 ml/min over 6.4 min with a gradient from 35% acetonitrile containing 0.005% acetic acid to 95% acetonitrile containing 0.005% acetic acid.

Under these experimental conditions, the representative chromatograms of a standard calibrant at 0.208 ng/ml (25 pg on column) of HETEs and EETs, and DiHETrEs are depicted in Figs. 2 and 3. The elution sequence was identified as 14,15-DiHETrE, 11,12-DiHETrE, 8,9-DiHETrE, 20-HETE, 5,6-DiHETrE, 15-HETE, 12-HETE, 14,15-EET, 11,12-EET, and 8,9-EET as determined by comparison to injections of individual compounds and product fragments.

3.2. Linearity, accuracy, and precision

Ten calibration standards over a range of 0.208–33.3 ng/ml were used to construct the curves for all metabolites. The weighting factor $1/Y$ typically provided the best fit of the plot as determined by visual inspection, correlation coefficient, and analysis of the residuals. Over 93% of the calibration standards fell within 15% deviation of back-calculated amounts from nominal spiked amounts for all levels. The remaining standards fell within 20% RSD and the correlation coefficients (R^2) were >0.99 for each metabolite.

The intra- and inter-day accuracy and precision for the assay were evaluated at three levels: 1.50, 7.50, and 20.83 ng/ml (90, 450, 1250 pg on column, respectively), using the QC samples within the three validation runs. Ten replicates at each concentration within a

Table 2
Accuracy and precision of the assay, represented by quality controls, for 10 arachidonic acid metabolites extracted in buffer.

	Spiked amount (pg on column)	Intra-assay concentration (mean ± std dev) (pg on column)	RE%	RSD%	Inter-assay concentration (mean ± std dev) (pg on column)	RE%	RSD%
20-HETE	90	86.94 ± 12.5	−3.40	14.38	82.81 ± 8.55	−7.99	10.32
	450	479.81 ± 17.76	6.62	3.70	466.49 ± 18.54	3.67	3.98
	1250	1303.41 ± 39.31	4.27	3.02	1311.37 ± 55.07	4.91	4.20
15-HETE	90	93.70 ± 7.15	4.11	7.63	89.92 ± 8.00	−0.09	8.9
	450	475.24 ± 17.67	5.61	3.72	468.74 ± 22.17	4.16	4.73
	1250	1315.00 ± 56.09	5.20	4.27	1300.70 ± 60.83	4.06	4.68
12-HETE	90	93.77 ± 3.77	4.19	4.02	91.49 ± 4.81	1.65	5.26
	450	458.95 ± 27.51	1.99	5.99	456.19 ± 23.72	1.38	5.20
	1250	1328.22 ± 57.48	6.26	4.33	1293.69 ± 53.67	3.50	4.15
14,15-EET	90	90.90 ± 5.52	1.00	6.08	90.91 ± 5.10	1.01	5.61
	450	464.51 ± 14.59	3.23	3.14	454.51 ± 21.11	1.00	4.64
	1250	1261.26 ± 53.60	0.90	4.25	1258.24 ± 60.54	0.66	4.81
11,12-EET	90	92.07 ± 8.07	2.31	8.77	93.02 ± 6.02	3.35	6.69
	450	470.24 ± 19.81	4.50	4.21	464.44 ± 29.67	3.26	6.39
	1250	1335.41 ± 73.77	6.83	5.52	1271.88 ± 75.94	1.75	5.97
8,9-EET	90	91.54 ± 8.11	1.72	8.86	83.85 ± 8.52	−6.84	10.16
	450	468.64 ± 24.40	4.14	5.21	456.38 ± 39.35	1.42	8.62
	1250	1326.40 ± 65.55	6.11	4.94	1299.05 ± 72.41	3.92	5.57
14,15-DiHETrE	90	91.74 ± 4.23	1.93	4.61	92.17 ± 4.97	2.42	5.39
	450	483.59 ± 18.45	7.46	3.82	472.74 ± 18.05	5.06	3.82
	1250	1281.76 ± 43.98	2.54	3.43	1278.34 ± 55.41	2.27	4.33
11,12-DiHETrE	90	92.03 ± 6.02	2.25	6.54	91.97 ± 4.67	2.20	5.07
	450	477.99 ± 16.92	6.22	3.54	470.92 ± 16.78	4.66	3.56
	1250	1277.72 ± 34.56	2.22	2.7	1271.23 ± 35.35	1.70	2.78
8,9-DiHETrE	90	90.50 ± 6.89	0.56	7.62	90.61 ± 6.14	0.68	6.78
	450	476.17 ± 18.95	5.82	3.98	470.50 ± 17.95	4.55	3.82
	1250	1274.85 ± 34.60	1.99	2.71	1258.13 ± 35.61	0.65	2.83
5,6-DiHETrE	90	89.69 ± 4.99	−0.34	5.56	91.79 ± 2.74	1.98	2.98
	450	476.89 ± 19.79	5.98	4.15	463.42 ± 14.67	2.98	3.17
	1250	1256.58 ± 40.26	0.53	3.20	1268.37 ± 44.23	1.47	3.49

Intra-assay relative standard deviation ranged from 2.70 to 14.38% and inter-day relative standard deviation ranged from 2.78 to 10.32%.

single day of validation were used to determine the intra-day reproducibility. Inter-day reproducibility was determined over three separate days using $n = 5$ at these concentrations. Calculated values of the QCs were generated using the equation of linear regression obtained from the calibration curves run within the same sequence. Results were evaluated and are given in Table 2. The %RSD for all analytes fell within 15%, indicating good reproducibility of the assay. The LLOQ was determined to be 0.208 ng/ml for all metabolites evaluated.

3.3. Recovery of analytes

The recovery efficiencies for all metabolites were determined at two different levels, at 1.50 and 20.83 ng/ml (90 and 1250 pg on column) (Table 3). The recovery ranged from 73 to 94%, with the EETs having the lowest recovery, however, all demonstrated good reproducibility with an %RSD below 16.75.

3.4. Analysis of human CSF samples

The method was applied to establish arachidonic acid metabolite concentrations in fresh human CSF samples from eight SAH patients. Samples taken at different time points from each patient were analyzed and concentrations were determined by using the equation of linear regression obtained from the calibration curves. Because these compounds are found endogenously in human CSF, it was not possible to conduct this validation using blank samples of this biological matrix.

Table 3
Recovery efficiency of metabolites in buffer.

	Spiked amount (pg on column)	Extraction efficiency %	RSD%
20-HETE	90	83.76	14.86
	1250	85.97	5.12
15-HETE	90	83.32	10.52
	1250	87.07	5.75
12-HETE	90	73.67	8.63
	1250	85.81	3.59
14,15-EET	90	89.35	12.18
	1250	87.12	6.78
11,12-EET	90	76.55	11.27
	1250	83.80	5.40
8,9-EET	90	72.73	16.75
	1250	80.51	5.51
14,15-DiHETrE	90	89.03	8.34
	1250	92.84	4.66
11,12-DiHETrE	90	90.98	6.04
	1250	94.11	4.27
8,9-DiHETrE	90	87.59	5.72
	1250	92.19	4.81
5,6-DiHETrE	90	86.62	9.18
	1250	89.40	4.67

Recovery efficiencies ranged from 73 to 94% for all metabolites with relative standard deviations below 16.75%.

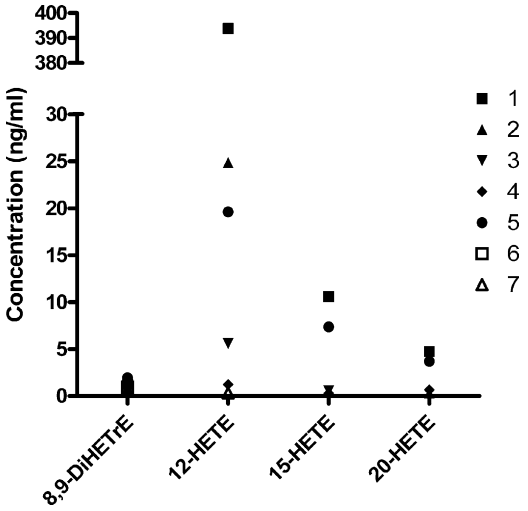

Fig. 4. Concentrations of arachidonic acid metabolites detected in fresh human CSF. Concentrations given are the highest concentrations seen in that patient over 14 days. Concentrations from seven patients ranged from 0.293 to 24.9 ng/ml for detected metabolites, with an outlier concentration at 394 ng/ml falling above the limit of quantitation for 12-HETE.

Peaks of 8,9-DiHETrE, 20-HETE, 15-HETE, and 12-HETE were found in quantitative amounts in all but one patient. Concentrations from seven patients ranged from 0.293 to 24.9 ng/ml for all metabolites, with an outlier concentration at 394 ng/ml falling above the LOQ for 12-HETE. Peaks of 14,15-DiHETrE, 11,12-DiHETrE, and 5,6-DiHETrE were found in amounts above LOD but below LOQ. No detectible amounts of 14,15-EET, 11,12-EET, or 8,9-EET were seen in these samples. All peaks were verified by comparing their m/z fragment and retention time with that of an authentic standard. The peak concentrations over the 14-day collection period are reported in Fig. 4. These results indicate that multiple arachidonic acid metabolites in CSF from subarachnoid hemorrhage patients can be quantified using this method and the metabolites with the highest concentrations measured were 12-, 15- and 20-HETE.

3.5. Analysis of cortical tissue samples

Rat brain cortex tissue samples ($n=4$) were analyzed from control animals to determine arachidonic acid metabolite concentrations using the standard calibration curves of each metabolite and results are shown in Fig. 5. Quantitative amounts of 14,15-, 11,12-DiHETrE, 20-, 15-, 12-HETE, and 8,9-EET were measured in these tissue samples and values ranged from 0.57 to 23.99 pmol/g wet tissue. The highest levels measured were 23.99 ± 4.03, 20.93 ± 2.42, and 13.74 ± 5.76 pmol/g wet tissue of 15-, 20- and 12-HETE, respectively. Detectible amounts of 8,9-DiHETrE and 5,6-DiHETrE were also seen in these samples. These results indicate that multiple arachidonic acid metabolites in rat brain cortex can be quantified using this method.

3.6. Analysis of cortical microsomal incubates

Microsomes isolated from rat brain cortex ($n=4$) of control animals were incubated with arachidonic acid to evaluate the formation rate of arachidonic acid metabolites. Quantitative amounts of all 10 arachidonic acid metabolites were detected and formation rates ranged from 0.03 to 7.77 pmol/mg/min as shown in Fig. 6. The highest arachidonic acid metabolite formation rates measured were 7.77 ± 0.67, 3.44 ± 0.39, 2.65 ± 0.27 and 2.52 ± 0.34 pmol/mg/min of 20-HETE, 14,15-, 11,12- and 8,9-EET, respectively. These results indicate that multiple arachidonic acid metabolites from rat cortical microsomal incubates can be quantified using this method. Furthermore, the results demonstrate that 20-HETE is a major cytochrome P450 metabolite of arachidonic acid.

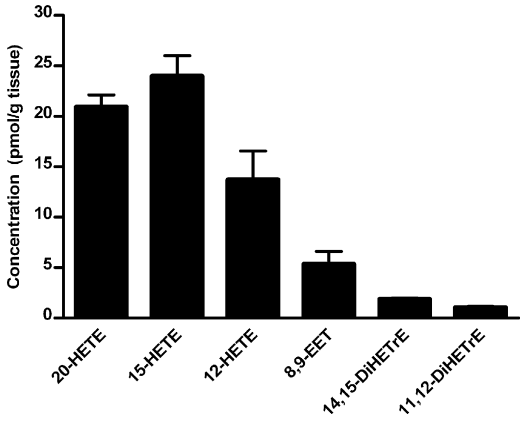

Fig. 5. Concentrations of arachidonic acid metabolites detected in rat brain cortical tissue. Quantitative amounts of 14,15-, 11,12-DiHETrE, 20-, 15-, 12-HETE, and 8,9-EET were measured in these tissue samples. The highest levels measured were 20.9 ± 2.4, 24.0 ± 4.0, and 13.7 ± 5.7 pmol/g tissue of 20-, 15- and 12-HETE, respectively. Detectible amounts of 8,9-DiHETrE and 5,6-DiHETrE were also seen in these samples.

3.7. Analysis of matrix effects

The effects of the phosphate buffer and aCSF matrix on the reproducibility of the assay to measure low and high concentrations of metabolites were determined. Table 4 shows the coefficient of variation (CV) of the peak area of each metabolite and IS and area ratio (analyte/IS) of the post-extraction spike in buffer and aCSF along with the CV of neat samples prepared in 80:20 methanol:deionized water ($n=6$). All of the CV values in Table 4 were below 15%, which demonstrated good precision and reliability of the assay. Most of the CV values (80%) for the area and area ratio of high metabolite concentrations were lower than the CV values for the low metabolite concentration in both aCSF and buffer. This indicates less variability in the assay at high concentrations of metabolites. Also, the CV of the area ratio was larger than the CV of the area of the metabolites and internal standard for most samples (86%) spiked post-extraction. This indicates that there was no compensating

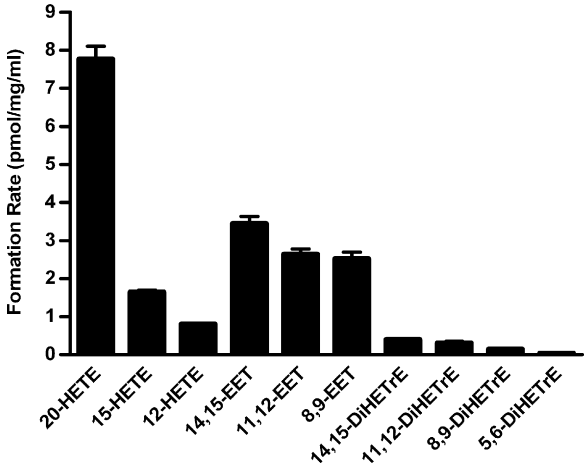

Fig. 6. Formation rate of arachidonic acid metabolites in rat cortical microsomal incubates. Quantitative amounts of all 10 arachidonic acid metabolites were detected and results are shown in Fig. 6. The highest arachidonic acid metabolite formation rates measured were 7.77 ± 0.67, 3.44 ± 0.39, 2.65 ± 0.27 and 2.52 ± 0.34 pmol/mg/min of 20-HETE, 14,15-, 11,12- and 8,9-EET, respectively.

Table 4
Assessment of matrix effects of the phosphate buffer and artificial cerebrospinal fluid (aCSF) on the reproducibility of the assay to measure low and high concentrations of metabolites.

Metabolite	Precision (CV%)											
	Low concentration (1.50 ng/ml)						High concentration (20.83 ng/ml)					
	Neat		Buffer		ACSF		Neat		Buffer		ACSF	
	Area	AR	Area	AR	Area	AR	Area	AR	Area	AR	Area	AR
20-HETE	9.5	10.7	4.3	4.9	6.4	7.3	1.9	2.8	2.8	2.8	2.2	4.5
15-HETE	5.2	4.9	6.8	7.4	4.5	5.3	3.5	3.6	1.1	2.2	1.7	3.2
12-HETE	5.9	6.6	3.4	4.7	8.8	10.0	5.3	6.7	4.8	3.7	2.4	3.3
14,15-EET	6.8	5.5	10.0	10.2	7.9	7.5	4.2	4.2	4.5	5.4	4.6	3.8
11,12-EET	4.0	5.7	4.6	5.1	7.6	5.0	2.6	3.4	4.4	3.9	2.1	3.6
8,9-EET	10.7	8.5	5.9	5.5	14.9	14.8	6.0	6.2	3.4	4.4	4.3	5.2
14,15-DiHETE	3.7	4.6	1.6	2.1	3.9	3.7	4.6	5.0	4.5	4.5	2.1	4.2
11,12-DiHETE	3.7	5.5	3.9	4.4	5.4	5.3	5.8	5.2	4.4	5.3	2.9	5.1
8,9-DiHETE	12.1	9.6	10.4	10.4	11.8	10.3	6.1	5.8	6.9	7.6	4.1	6.3
5,6-DiHETE	5.2	5.3	5.4	5.7	4.6	4.0	9.2	9.0	5.5	5.7	2.9	5.9
20-HETE-*d6*	4.0	–	2.4	–	1.5	–	1.9	–	1.9	–	3.7	–

Data are represented as coefficient of variation (CV%) of the peak area of each metabolite and IS and area ratio (analyte/IS) of the post-extraction spike in phosphate buffer and aCSF along with the CV of neat samples prepared in 80:20 methanol:deionized water ($n = 6$). The samples were spiked with low and high concentrations of HETE, EETs, and DiHETrEs at 1.5 ng/ml and 20.83 ng/ml, respectively. Results indicate good reproducibility and reliability of the assay to measure low and high concentrations of metabolites in both phosphate buffer and aCSF.

Table 5
Comparison of the internal standard-normalized matrix factor (IS-normalized MF) of the phosphate buffer and artificial cerebrospinal fluid (aCSF) at low and high concentrations of metabolites.

Metabolite	Internal standard-normalized matrix factor							
	Low concentration (1.5 ng/ml)				High concentration (20.83 ng/ml)			
	Buffer		aCSF		Buffer		aCSF	
	Value	CV	Value	CV	Value	CV	Value	CV
20-HETE	0.99 ± 0.08	8.6%	0.99 ± 0.14	13.8%	0.98 ± 0.03	3.3%	0.97 ± 0.04	4.6%
15-HETE	0.98 ± 0.06	6.2%	0.96 ± 0.03	3.6%	0.99 ± 0.06	5.9%	1.02 ± 0.06	6.0%
12-HETE	1.00 ± 0.09	9.3%	1.00 ± 0.14	14.0%	0.96 ± 0.08	8.1%	0.99 ± 0.07	6.8%
14,15-EET	1.13 ± 0.13	11.2%	1.07 ± 0.08	7.8%	1.06 ± 0.07	6.6%	1.09 ± 0.06	5.6%
11,12-EET	1.02 ± 0.09	8.6%	0.92 ± 0.10	11.2%	0.98 ± 0.06	6.0%	0.98 ± 0.04	4.4%
8,9-EET	1.07 ± 0.07	6.6%	0.97 ± 0.14	14.8%	1.01 ± 0.10	10.2%	0.91 ± 0.08	8.8%
14,15-DiHETE	1.05 ± 0.06	5.5%	1.07 ± 0.08	7.2%	0.98 ± 0.09	9.3%	1.07 ± 0.10	9.1%
11,12-DiHETE	1.00 ± 0.07	6.9%	0.97 ± 0.06	5.7%	0.98 ± 0.10	10.1%	1.08 ± 0.10	9.3%
8,9-DiHETE	0.97 ± 0.12	12.2%	0.92 ± 0.10	10.6%	1.00 ± 0.10	10.5%	1.10 ± 0.10	9.1%
5,6-DiHETE	0.94 ± 0.07	7.4%	1.01 ± 0.07	6.7%	0.99 ± 0.10	9.9%	1.07 ± 0.12	11.7%

Data are shown as the average IS-normalized MF values ± standard deviation (s.d.) and the CV for phosphate buffer and aCSF spiked with low and high concentrations of HETE, EETs, and DiHETrEs at 1.5 ng/ml and 20.83 ng/ml, respectively ($n = 6$). IS-normalized matrix factor values in phosphate buffer were compared to aCSF. Results indicate minimal effects of the phosphate buffer and aCSF matrix on the reproducibility and reliability of the assay and no difference in matrix effects of phosphate buffer and aCSF at low and high metabolite concentrations.

effect of the internal standard on the matrix effects as described by Matusweski et al. [44]. Table 5 shows the average IS-normalized MF values of the samples ± standard deviation (s.d.) and the CV ($n = 6$). The average IS-normalized MF values ranged from 0.92 to 1.13 with most of the values (68%) within 5% of 1.00. An IS-normalized MF (or absolute MF) of 1 signifies no matrix effects. A MF value less than 1 signifies ion suppression while a value greater than 1 signifies ion enhancement or analyte loss in the absence of matrix. An IS-normalized MF (or absolute MF) of 1 is not necessary for a reliable bioanalytical assay. However, a highly variable MF would indicate lack of reproducibility in the assay. All of the CV of the IS-normalized MF values were below 15%, which demonstrates minimal effects of the phosphate buffer and aCSF matrix on the precision and reliability of the assay at low and high metabolite concentrations. Also, the matrix effects, measured by IS-normalized MF values, were not significantly different in aCSF as compared to buffer for all metabolites.

3.8. Analysis of metabolite stability and recovery

Table 6 demonstrates that HETE, EET, and DiHETrE metabolites were not significantly altered from baseline for up to 12 h of room temperature incubation in silanized borosilicate glass. Table 7

Table 6
Comparison of arachidonic acid metabolites during 12-h incubation in silanized tubes.

Metabolite	$t = 1$ h % ± s.d.	$t = 6$ h % ± s.d.	$t = 12$ h % ± s.d.
20-HETE	97.5 ± 4.0%	93.1 ± 7.8%	95.1 ± 15.5%
15-HETE	99.4 ± 6.3%	85.4 ± 2.7%	90.7 ± 14.2%
12-HETE	96.7 ± 3.8%	87.8 ± 8.4%	92.1 ± 11.6%
14,15-EET	94.5 ± 2.3%	86.1 ± 7.0%	82.4 ± 10.8%
11,12-EET	96.6 ± 2.0%	87.6 ± 4.3%	87.0 ± 10.0%
8,9-EET	100.2 ± 10.3%	93.4 ± 13.7%	88.2 ± 6.4%
14,15-DiHETrE	94.3 ± 7.8%	102.4 ± 3.1%	109.6 ± 11.3%
11,12-DiHETrE	95.9 ± 2.7%	96.7 ± 2.6%	102.3 ± 12.5%
8,9-DiHETrE	95.8 ± 1.9%	93.9 ± 7.6%	94.6 ± 12.6%
5,6-DiHETrE	98.0 ± 5.2%	93.0 ± 4.3%	98.5 ± 10.5%

Data at each time point is presented as mean percentage of initial concentration ± s.d. ($n = 3$). The percentage of initial concentration at each time point was compared using repeated measures one-way analysis of variance (ANOVA) with Dunnett's *post hoc* test. Results indicate that incubation of CSF in silanized test tubes over 12 h does not significantly affect the measurable amount of the arachidonic acid metabolites.

Table 7
Comparison of arachidonic acid metabolites during 12-h incubation in Medtronic Exacta drainage bags.

Metabolite	$t = 1$ h % ± s.d.	$t = 6$ h % ± s.d.	$t = 12$ h % ± s.d.
20-HETE	95.3 ± 9.4%	94.8 ± 8.4%	91.3 ± 9.2%
15-HETE	87.2 ± 15.0%	90.3 ± 8.9%	70.9 ± 5.4%*
12-HETE	75.5 ± 14.0%*	76.7 ± 11.1%*	61.4 ± 5.6%**
14,15-EET	70.6 ± 20.6%*	71.1 ± 19.4%*	46.1 ± 10.1%**
11,12-EET	60.6 ± 20.1%*	65.9 ± 25.0%*	40.9 ± 19.3%**
8,9-EET	62.1 ± 20.2%*	68.1 ± 26.8%	48.4 ± 25.9%**
14,15-DiHETrE	103.1 ± 8.8%	89.7 ± 11.9%	94.4 ± 18%
11,12-DiHETrE	100.2 ± 3.2%	93.1 ± 7.7%	93.3 ± 5.2%
8,9-DiHETrE	101.1 ± 4.1%	97.9 ± 9.4%	95.6 ± 8.1%
5,6-DiHETrE	94.8 ± 5.8%	90.3 ± 9.1%	85.3 ± 9.4%

Data at each time point is presented as mean percentage of initial concentration ± s.d. ($n = 4$). The percentage of initial concentration at each time point was compared using repeated measures one-way analysis of variance (ANOVA) with Dunnett's *post hoc* test. Results indicate that incubation of CSF collected in drainage bags over 12 h significantly reduces the measurable amount of 15-HETE, 12-HETE, 14,15-EET, 11,12-EET, and 8,9-EET, while the measurable amount of 20-HETE, 14-15-DiHETrE, 11,12-DiHETrE, 8,9-DiHETrE, and 5,6-DiHETrE did not significantly change.

shows some of these metabolites were significantly reduced upon room temperature incubation in PVC bags. Specifically, the recovery of 12-HETE, 15-HETE, 8,9-EET, 11,12-EET and 14,15-EET was significantly altered by contact with the PVC collection bags over 12 h ($p < 0.05$). Only 12-HETE, 8,9-EET, 11,12-EET and 14,15-EET showed a significantly lower recovery as early as 1 h after addition to the PVC bag and a highly significant recovery loss at 6 and 12 h after addition to the PVC bag ($p < 0.01$). 20-HETE and all DiHETrE metabolites were not significantly altered at any of the time points evaluated. These results suggest that the use of CSF collection bag samples for assessment of EET metabolites and possibly 12-HETE will be confounded by recovery loss due to the PVC material in the tubing and collection bags.

4. Discussion

In this study we report a useful method to measure 10 arachidonic acid metabolites in various matrices including fresh CSF of SAH patients as well as rat cortical tissue and microsomal incubates. These compounds were simultaneously detected, identified, and quantified using a validated UPLC–MS/MS method. Furthermore, we determined that these metabolites are generally stable for 12 h at room temperature; however, significant recovery loss is observed for 12-HETE, 15-HETE and EET metabolites when incubated in PVC sampling bags. The largest degree of recovery loss was observed with the EET metabolites which were reduced below 50% of baseline at 12 h. Collectively, these results suggest that UPLC–MS/MS is an accurate and sensitive method for quantification of HETE, EET, and DiHETrE metabolites in human CSF and rat brain.

More specifically, this article describes the validation of linear calibration curves ranging from 0.208 to 33.3 ng/ml. The inter-day and intra-day variance was less than 15% at most concentrations with extraction efficiency greater than 73%. We demonstrated that the matrix effect of the phosphate buffer and aCSF did not significantly affect the reliability and reproducibility of the assay, the matrix effect of the phosphate buffer is not different from aCSF, and there was no change in UPLC–MS/MS response due to the phosphate buffer and aCSF matrix for all metabolites measured. Therefore, the phosphate buffer can serve as a surrogate matrix for aCSF.

Our results also demonstrate that HETE, EET, and DiHETrE metabolites are stable at room temperature in CSF for up to 12 h. However, some of these metabolites were susceptible to recovery loss when incubated in PVC bags. The degree of recovery loss was directly related to the lipophilicity of the individual metabolites. The most lipophilic class of metabolites are the EETs which demonstrated significant loss of recovery as early as 1 h of room temperature incubation in PVC bags. Lesser in magnitude, but statistically significant losses in recovery of 12-HETE and 15-HETE were observed at the 12 h time point, with negligible losses at earlier time points. Little to no significant losses were observed with 20-HETE or DiHETrE metabolites. Collectively, these results suggest that CSF drainage samples from bags kept at room temperature for up to 12 h are appropriate for quantification of 20-HETE, DiHETrE, and possibly 12-HETE and 15-HETE metabolites. EET metabolites would be expected to undergo significant recovery loss and potentially confound variability if collected from tubing or collection bags made from PVC.

Concentrations of HETE, EET, and DiHETE metabolites were measured in human CSF, rat brain microsomes, and rat brain tissue extracts. Metabolites measured in human CSF included 12-, 15-, and 20-HETE and 8,9-DiHETrE. Several of these metabolites have been previously detected in CSF. 20-HETE levels have been measured in CSF of rats [3], dogs [48], and humans following SAH [7,20]. In addition to 20-HETE, our lab has also measured 12-HETE in the CSF of SAH patients [7]. Metabolites measured in rat brain cortical tissue included 12-, 15-, and 20-HETE, 11,12- and 14,15-DiHETE and 8,9-EET. Several of these metabolites, to date, have been previously detected in rat brain or cortical tissue. Adesuyi et al. and Hambrect et al. report measurements of 12-HETE levels in rat brain [49,50]. Also, Yue et al. measured 12-HETE, 20-HETE, 14,15-EET and 11,12-EET in sham and traumatic brain injured (TBI) rats [31,39]. Furthermore, Tanaka et al. and Renic et al. measured 20-HETE levels in rat brain after ischemic injury, while only Renic et al. showed combined EET and DiHETrE levels [51,52]. The level of 20-HETE measured in rat brain cortical tissue and human CSF using our method was comparable to the results reported by Yue et al. [39] and Roman et al. [20], respectively.

The inability to detect some metabolites in human CSF or rat brain tissue may be due to low endogenous levels or loss of metabolites due to metabolism or other elimination pathways. EET metabolites were not detected in the CSF samples analyzed, however, a stable hydration product 8,9-DiHETrE was observed in low concentrations. Similarly, 11,12- and 14,15-EET were not measured in rat brain cortical tissue but their corresponding DiHETE metabolites were measured. Therefore, a direct measurement of the metabolites in CSF or brain tissue will be dependent on pathways of formation and elimination.

All ten metabolites were measured when arachidonic acid was incubated with rat brain cortical microsomes as shown in Fig. 6. The metabolites that showed the highest formation rates were 20-HETE, 14,15-, 11,12- and 8,9-EET, respectively. The 20-HETE formation rate shown in Fig. 6 is similar to the formation rates shown in similar arachidonic acid incubations with rat brain cortical microsomes [29,38]. Collectively, these studies show that our UPLC–MS/MS method can quantify 10 arachidonic acid metabolites in CSF, rat brain cortical tissue, and rat brain cortical microsomal incubates.

The results from our method are similar to other studies that have reported the detection of similar metabolites in different matrices. Yoshida et al. described the simultaneous measurement of several HETEs from plasma and liver tissue and reported concentrations of 12-HETE and 15-HETE as 18.9 and 58.0 ng/ml in plasma, and 2500 and 2000 ng/g in liver, respectively [53]. A direct methodological comparison with respect to sensitivity and LOQ cannot be determined from the information given. However, it should be noted that the concentration of 12-HETE and 15-HETE measured in plasma fell within our standard calibration range of 0.208–33.33 ng/ml. In addition, Newman et al. described a method

for measurement of epoxides, hydroxyeicosatetraenoic acids and hydroxyoctadecadienoc acids (HODEs) by HPLC–MS/MS in VLDL and HDL particles in rat plasma [54]. Newman et al. also simultaneously quantified cytochrome P450 metabolites in rat urine [54,55]. The LOQ from this method is reported as 0.17, 0.26–0.48, and 0.05–0.09 ng/ml for 20-HETE, EETs, and DiHETrEs, respectively. The LOQ for all metabolites measured using our method was 0.208 ng/ml, which is comparable to the values reported by Newman et al. [55]. Also, our UPLC–MS/MS method reports a shorter run time of 6.4 min as compared to the 47 and 31 min run times reported by the HPLC–MS/MS methods described by Yoshida et al. and Newman et al. [53,55]. One advantage of the method described in this article is the shorter run time and comparable LOQ for measurement of these metabolites in biological matrices.

Collectively, these data will aid in the quantitative methods in future clinical studies aimed at determining the utility of these metabolites as therapeutic targets and/or biomarkers for disease progression and complications.

5. Conclusion

A solid phase extraction procedure coupled with a UPLC–MS/MS method was validated and successfully applied for the simultaneous extraction of 10 arachidonic acid metabolites. This method was optimized to provide high resolution and sensitivity for the detection of these analytes in human CSF samples, rat brain cortical tissue, and rat cortical microsomal incubates with an analysis time of 6.4 min per sample. Quantitative amounts of 8,9-DiHETrE, 20-HETE, 15-HETE, and 12-HETE were found in CSF samples whereas 12-, 15-, and 20-HETE, 11,12- and 14,15-DiHETE and 8,9-EET were found in rat brain cortical tissue. Moreover, all 10 metabolites were detected in rat cortical microsomal incubates with 20-HETE showing the greatest formation rate. All quantitative amounts of arachidonic acid metabolites fell within the validated linear range of 0.208–33.3 ng/ml. This work also demonstrates that the simultaneous measurement of these compounds can be established with the reliability, reproducibility, and rapid analysis time governed by the use of a UPLC system. In addition, we determined that the effect of the phosphate buffer used in this assay does not significantly affect the reliability and reproducibility of the assay. Finally, we determined that these metabolites are stable at room temperature for up to 12 h. However, significant loss in EET metabolites, and to a lesser extent 12-HETE, occurs when incubated in PVC bags. The increased throughput and collection stability of these metabolites should help to propel the clinical investigation of the role of these metabolites in the pathogenesis of diseases of cerebrovascular origin.

Acknowledgements

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