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Determination of bioactive eicosanoids in brain tissue by a sensitive reversed-phase liquid chromatographic method with fluorescence detection

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Abstract

Arachidonic acid (AA) is metabolized to prostaglandins (PGs) via cyclooxygenases (COX) catalysis, and to epoxyeicosatrienoic acids (EETs), dihydroxyeicosatrienoic acids (DiHETrEs), and hydroxyeicosatetraenoic acids (HETEs) via cytochrome P450 (CYP450) enzymes. A reliable and robust fluorescence based HPLC method for these eicosanoids was developed. A new selective reverse-phase solid phase extraction (SPE) procedure was developed for PG, DiHETrEs, HETE, and EETs of interest from rat cortical brain tissue. The eicosanoids were derivatized with 2-(2,3-naphthalimino)ethyl-trifluoromethanesulphonate (NE-OTf), followed by separation and quantification at high sensitivity using reverse-phase HPLC with fluorescent detection, and further identified via LC/MS. The derivatization was studied and optimized to obtain reproducible reactions. Various PGs, DiHETrEs, HETEs, EETs, and AA were sensitively detected and baseline resolved simultaneously. LC/MS under positive electrospray ionization selected ion monitoring (SIM) mode was developed to further identify the peaks of these eicosanoids in cortical brain tissue. The method was applied in the traumatic brain injured rat brain.

Keyword: Eicosanoids

1. Introduction

Eicosanoids are defined as the biological oxidation products of arachidonic acid and some other C20 polyene acids that give rise to a wide variety of products of remarkable physiological activity [1]. Arachidonic acid (AA) can be metabolized to many bioactive eicosanoids (Fig. 1) [2], including oxidation to prostaglandins (PGs) via cyclooxygenases (COX), and to epoxyeicosatrienoic acids (EETs), dihydroxyeicosatrienoic acids (DiHETrEs), and hydroxyeicosatetraenoic acids (HETEs) via cytochrome P450 (CYP450) enzymes. Representative structures are shown in Fig. 2. These compounds occur in trace concentrations in many organisms and tissues, including the mammalian brain, and play important and diverse roles as physiologic and pathophysiologic mediators.

Rofecoxib (Merck), Celecoxib and Valdecoxib (Pfizer/Pharmacia), three FDA approved, highly specific cyclooxygenase-2 (COX2) inhibitors, are being used to treat peripheral inflammation, and may prove beneficial to the injured brain as well. COX2 becomes overexpressed and PG levels rise in the brain early after traumatic brain injury (TBI) and persists for days [3]. Overexpression of COX2 in brain tissue can have deleterious effects since the by-products of its enzymatic reactions are free radicals that are injurious when abundant to neural membranes, tight junctions of the blood brain barrier and vessels [4,5]. Therefore, blocking COX2 enzyme activity may improve functional recovery. Several investigations, including our own work with traumatic brain injury [6], have demonstrated the neuroprotective effect of

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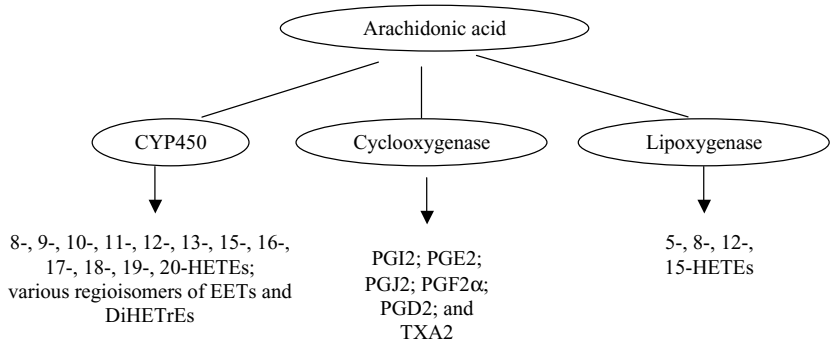
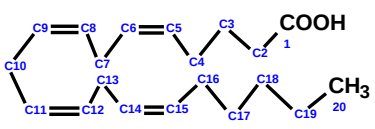


Fig. 1. Selective pathways for the metabolism of arachidonic acid.

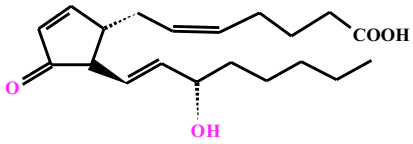
this class of compounds [7,8]. In a rat model of traumatic brain injury, DFU [5,5-dimethyl-3(3-fluorophenyl)-4(4-methylsulphonyl)phenyl-2(5H)-furanone] (Merck & Co.), a highly specific COX2 enzyme inhibitor, reduced central inflammation and markedly improved functional recovery at 3 day post-injury, compared to vehicle-treated, sham operated littermate controls [6]. However, the neuroprotective mechanisms of COX2 inhibitor are not well understood.

COX2 inhibitors prevent the formation of PGs from AA, yet this may comprise only part of their beneficial effects. We are testing the hypothesis that selected eicosanoid metabolites of AA, formed in the presence of these inhibitors, are neuroprotective. The CYP450 derived EETs and HETEs are prime candidates for neuroprotective eicosanoids. Specific HETEs (e.g., 12- and 15-HETE) have been shown to limit glutamate-mediated neurotoxicity in cultured neurons [9]. Specific EETs (e.g., 11,12-EET) have been found to inhibit NFκB activation and reduce adhesion molecule expression in vivo and in vitro in endothelial cells [10]. Other studies have shown that EETs selectively inhibit the proliferation of specific cell types in vitro without toxicity or apoptosis [11]. Thus, these eicosanoids may protect the injured brain by reducing the penetration and proliferation of inflammatory cells. We set out to determine whether specific isomers of the CYP450 eicosanoids were amplified in the brain after traumatic brain injury, in the presence of a COX2 inhibitor.

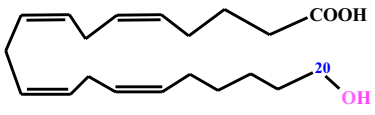
To facilitate investigation of this hypothesis, a quantitative assay of eicosanoids is necessary. Currently, GC/MS [2,12–15], LC/MS [16], capillary electrophoresis/UV [15,17], and fluorescence detected HPLC [15,18–23] have been used to measure some of these eicosanoids. GC/MS with negative ion chemical ionization has been the most commonly used technique and can give specific mass information of the peaks. However, it is not suitable for labile compounds, and needs TLC steps to purify target compounds before analysis. The reported concentration range of those eicosanoids by GC/MS method are 0.5–5 ng/ml. An LC/MS method with negative electrospray ionization mode



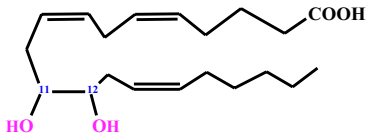
AA



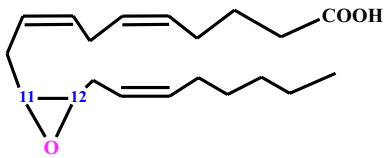
PGJ2



20-HETE



11,12-DiHETrE



11,12-EET

Fig. 2. The representative structures of eicosanoids derived from arachidonic acid.

was reported by Nithipatikom [16] to analyze 20-HETEs, DiHETrES and EETs in endothelial cells, plasma, astrocytes and microsome samples at tens of pg level. However the instrument is expensive and the sensitivity offered is strongly dependent on the method of detection. More recently, a capillary electrophoresis with on-line UV detection method was reported to analyze the DiHETrEs and EETs from murine liver at tens of pg levels [17]. The need for highly sensitive and low cost methods for analyzing labile bioactive eicosanoids has recently driven the development of fluorescence detected based HPLC methods. Many fluorescent reagents can be used to derivatize the free carboxyl group of fatty acids to reach the pg detection limit [15,18,19,21–24]. Maier [18] and Nithipatikom [19] have used 2-(2,3-naphthalimino)ethyl-trifluoromethanesulphonate (NE-OTf) as the fluorescent reagent to determine 20-HETE in urine in the ng range and EETs in endothelial cells in the pg range individually.

Here we present a sensitive, reliable and robust fluorescence detected HPLC method (FD-HPLC) for measuring eicosanoids from biological samples. A new selective solid phase extraction (SPE) procedure was developed to extract PGs, DiHETrEs, HETEs and EETs of interest from cortical brain tissue, followed by derivatization with NE-OTf, separation by a new reverse-phase FD-HPLC method, and further confirmation via LC/MS.

The method development required (i) developing a new SPE procedure, (ii) qualifying and quantitatively validating the dye derivatization protocols and (iii) developing and validated a quantitative HPLC method.

The overall method developments included five stages described as follows.

1. Fluorescent derivatization was studied and optimized to obtain consistent performance of reactions (Section 3.1).
2. The FD-HPLC method was developed and optimized to sensitively detect and separate PGs, DiHETrEs, HETEs, EETs, and AA simultaneously (Section 3.2).
3. The SPE procedure coupling with FD-HPLC method was developed and optimized in terms of reproducibility, selectivity and pH effect (Section 3.3).
4. The LC/MS method under positive electrospray ionization selected ion monitoring (SIM) mode was developed to further confirm the fluorescent derivatization and identify the peaks in biological samples (Section 3.4).
5. The method was applied for detection of eicosanoid metabolites appearing in injured rat brains to facilitate future studies with injured rat brain treated with DFU (Section 3.5).

2. Experimental

2.1. Standards and chemicals

Eicosanoid standards 14,15-DiHETrE, 11,12-DiHETrE, 8,9-DiHETrE, 20-HETE, 15-HETE, 12-HETE and 11,12-EET were obtained from Cayman Chemical Company, and PGF2α, PGE2, PGD2, PGJ2, 14,15-EET, 8,9-EET, and AA were obtained from BIOMOL Research Laboratories Inc., tridecanoic acid (TA) was obtained from Sigma. Wit2 [20-hydroxyecoisa (5Z), (12Z) dienoic acid] was a gift from J.R. Falck. The fluorescent dye, 2-(2,3-naphthalimino)ethyl-trifluoromethanesulphonate (NE-OTf) was obtained from Molecular Probe Inc.

HPLC grade acetone and hexane, anhydrous acetonitrile, formic acid (96%), and *N*,*N*-diiospropylethylamine were obtained from Aldrich. HPLC grade acetonitrile, water and methanol were obtained from Fisher Scientific. SPE cartridge Oasis®HLB (1 cm^3/30 mg, 30 μm) was purchased from Waters Corporation.

2.2. Biological samples

Male Sprague–Dawley rats, each weighing 300–400 g, were studied. A lateral cortical impact model of TBI model was utilized (this model generates moderate levels of head injury including the temporary loss of strength and coordination in the limbs contralateral to the injury and significant retrograde amnesia and learning deficits). Animals were pre-anesthetized using 2% isoflurane, then given oxygen with 0.75% isoflurane through a facemask on a stereotactic frame. The cranium was exposed and a lateral craniectomy was made over the somatosensory cortex using a 6 mm trephine. The exposed dura was subjected to a 5 mm diameter piston impact (4 m/s velocity, 3.0 mm depth, 100 ms duration). Animals were sacrificed by decapitation at either 6 or 24 h post-injury. Brains were rapidly removed, snap frozen on dry ice and stored at −80 °C. Thick sections (300 μm) were cut on a cryostat at −8 °C.

2.3. Sample preparation

Up to 20 mg cortical brain tissue was dissected from a thick section inside a cryostat at −15 °C and added to a 2 ml micro-centrifuge tube with 200 μl methanol and 0.4 μl 96% formic acid on ice. The brain tissue in tube surround with crushed ice was homogenized using a micro-ultrasonic cell disrupter at 30 amplitudes (2 mm probe, highest power 100 amplitudes, Kontes) followed by centrifuging at 14,000 rpm for 10 min at 0 °C. The supernatant was transferred to a fresh tube and diluted to 10% methanol by adding 1.8 ml water. The diluted supernatant was loaded onto 1 ml Oasis®HLB SPE cartridge on a vacuum manifold. The SPE cartridge has been pre-cleaned and conditioned with 1 ml methanol, 1 ml acetone, 2 ml hexane, 1 ml acetone, 1 ml methanol and 2 ml water by low feeding speed in sequence. The cartridge was washed with 3 ml water, 1 ml 10% methanol and allowed to dry under argon at reduced pressure for 10 min. The eicosanoid metabolites were collected by elution with 2 ml anhydrous acetonitrile. The acetonitrile solution was then evaporated to dryness under the stream of argon (4.8 grade).

2.4. Fluorescent derivatization reaction

The standards or samples were dissolved in 136 μl anhydrous acetonitrile. 4 μl *N,N*-diiospropylethylamine catalyst (dried with molecular sieves 5 Å) was added to the solution. Freshly prepared 10 μl NE-OTf (2 mg/ml) in anhydrous acetonitrile was added and vortexed lightly for 2 s. The tubes were then placed in a desiccator and kept at 4 °C for 30 min. The reactions were terminated using argon to evaporate the solution to dryness. Samples were stored at −80 °C.

2.5. Fluorescent detected HPLC

The standards and samples were dissolved in 40μl methanol and analyzed by Jasco HPLC System. The HPLC system consisted of a Rheodyne manual injector with a 20 μl loop, two Jasco PU-980 HPLC pumps, a Jasco CO-965 column oven, and a Jasco FP-920 fluorescence detector. The injection volume was 8 μl. Chromatographic separations were performed on a HPLC Symmetry® C18 column (4.6 mm × 250 mm, dp 5 μm, Waters Corp.) at 30 °C. Mobile phase A consisted of 0.5% formic acid in water, and mobile phase B consisted of 0.5% formic acid in acetonitrile. A flow rate of 1 ml/min was used to deliver the mobile phase A and B gradient as follows: 50–65% B in 40 min; 65–100% B in 80 min; 100% B for 20 min. The fluorescence detector (FD) was set at 100 gain with excitation wavelength 260 nm and emission wavelength 396 nm. Data acquisitions utilized Borwin 1.50.08 version software.

2.6. System suitability testing

A standard solution (8 μl) containing 14 different eicosanoids at 12.5 ng/ml was injected five times continuously, followed by injection of the blank (derivatization reaction without any added eicosanoids). The retention time, asymmetry, number of theoretical plates and relative standard deviation (R.S.D.) were evaluated.

2.7. Limit of detection and limit of derivatization reaction

The limit of detection and quantitation (LOD and LOQ) were evaluated by injecting a 20% aliquot of progressive dilutions of the original solution (2.5 ng/μl) for each standard and signal to noise ratio higher than 3 for LOD and higher than 10 for LOQ. The limit of derivatization reaction [24] was studied by injecting of a 20% aliquot of original reaction mixtures containing decreasing amounts of standards without any dilution directly onto the HPLC column; and the limit was defined as the lowest amount yielding a signal to noise ratio higher than 4.

2.8. Calibration curve

Reaction mixtures of standards of interest varied from 70 to 2000 pg were derivatized individually with NE-OTf. The resultant samples were reconstituted in 40 μl methanol followed by injection of 8 μl into the HPLC.

2.9. Recovery

The recoveries at 1 ng were obtained by calculating the area ratio of each standard through and without the SPE procedure.

2.10. LC/MS confirmation

The Agilent 1100 Series liquid chromatograph/mass selective detector (LC/MSD) system was used for peak confirmation. The HPLC system consisted of a binary pump, a thermostatted autosampler and a column thermostat. Chromatographic separations were the same as the FD-HPLC method. The autosampler was set at 4 °C and the injection volume was 5 μl.

The HPLC column effluent was pumped to a VL mode quadrupole mass spectrometer (MSD) with atmospheric electrospray ionization source. The detection was under the selected ion monitoring (SIM) positive mode, where ions with *m/z* of 646Da (PGF2α); 644Da (PGD2 and PGE2); 626Da (PGJ2); 631Da (DiHETrEs); 612Da (HETEs and EETs); and 596Da (AA) were monitored. The data acquisitions were accomplished using Chem Station A.07.01 software. The following mass detector parameters were used for all experiments: gas temperature 350 °C; drying gas flow rate 10 l/min; nebulizer pressure 40 psig; fragmentor voltage 70 V; and capillary voltage 4000 V.

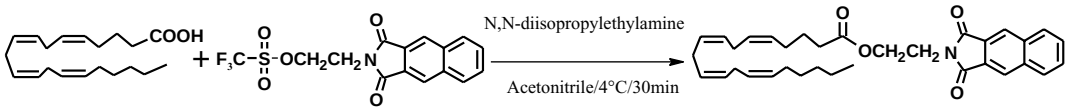
3. Results and discussion

3.1. Optimization of fluorescent derivatization reaction

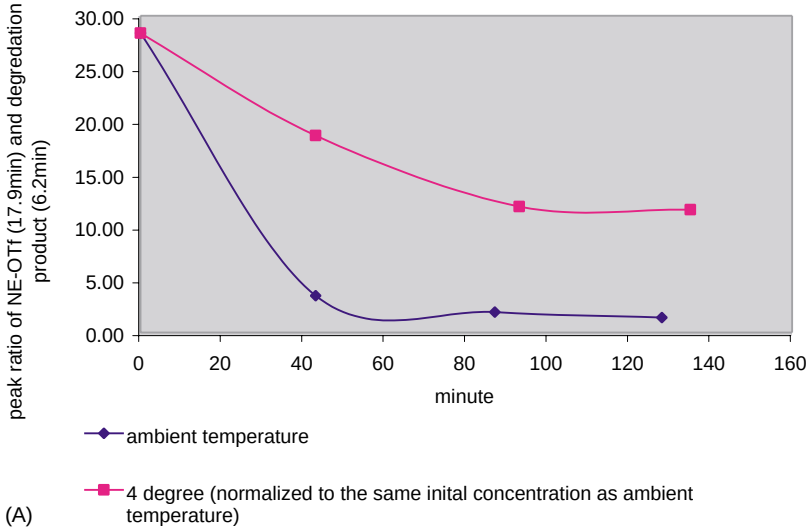
In preliminary experiments, we attempted to use a previously published method to derivatize standards, however, the resulting chromatograms of labeled eicosanoids were not reproducible, and interfering peaks from side reactions and degradation products were seen in the chromatograms [18]. The chromatogram of a blank sample with only fluorescent reagent and catalyst showed production of substantial impurities. Under these conditions, the derivatization was questionable for accurate quantification of real eicosanoid peaks via FD-HPLC.

To obtain consistent performance of reactions, we investigated the stability of the reagents and parameters including the ratio of NE-OTf and *N,N*-diisopropylethylamine, temperature, reaction time and sequence of reagents addition. The optimized reaction conditions for reproducible and controlled measurements are presented in Scheme 1.

Fig. 3A shows the stability of NE-OTf in fresh anhydrous acetonitrile (36.4 mM) at ambient temperature (20 °C) and 4 °C via HPLC monitoring (HPLC Symmetry® C18 column 4.6 mm × 250 mm, dp 5 μm at 25 °C. Flow rate was



Scheme 1. Reaction of AA with NE-OTf.



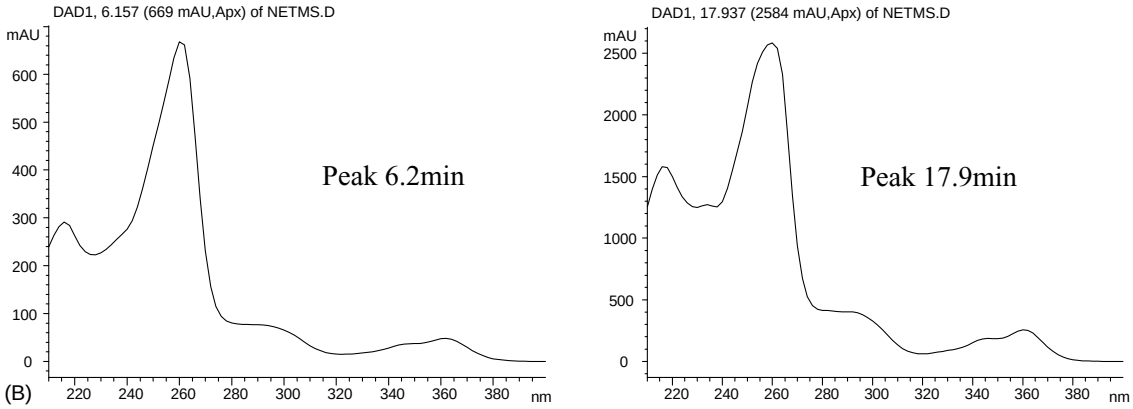


Fig. 3. (A) Stability of NE-OTf in fresh anhydrous acetonitrile (36.4 mM) at ambient temperature and 4 °C via HPLC monitoring. (B) On-line UV spectra of peak 6.2 min and peak 17.9 min.

1 ml/min. Mobile phase A was water and B was acetonitrile: 40–100% B in 35 min. UV detector was set at 360 nm. The instrument was HP1100). The degradation of NE-OTf occurred immediately after mixing with the rate of degradation at 4 °C being much slower than at ambient temperature. Two major peaks at 17.9 and 6.2 min were detected immediately after preparation of NE-OTf in anhydrous acetonitrile. The on-line UV spectra of these two peaks were shown in Fig. 3B, which indicates they have the same chromophore. The masses of these two peaks were obtained by LC/MS under electrospray positive & negative scan mode using a 85% acetonitrile with 0.1% formic acid aqueous mobile phase at a flow rate of 1 ml/min. The peak at 17.9 min was NE-OTf with a $[M+H]^+$ at *m/z* 374, a $[M+Na]^+$ at *m/z* 396, characterized positive fragmented ion at *m/z* 224.1, and characterized negative fragmented ion at *m/z* 149 (Fig. 4). The peak at 6.2 min was a hydrolysis product correspondent to 2-(2-hydroxyethyl)-1H-benzo[5]isoindole-1,3(2H)-dione, which has a $[M + H]^+$ at *m/z* 242.1, a $[M + Na]^+$ at *m/z* 264.1 and characterized positive fragmented ion at *m/z* 224.1 (Fig. 4).

The degradation rates of NE-OTf in fresh anhydrous organic solvents including tetrahydrofuran and dichloromethane and in 50/50 acetonitrile/water were also

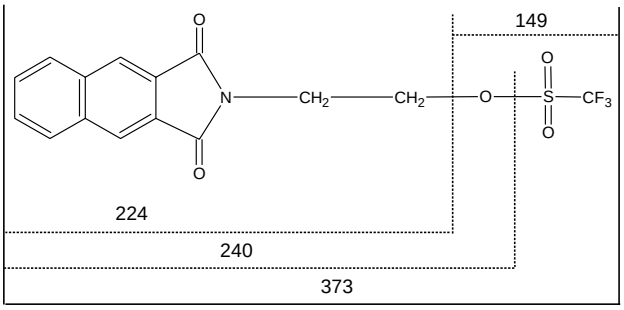


Fig. 4. Proposed degradation and fragmentation for NE-OTf.

compared. The NE-OTf decomposed fastest in 50/50 acetonitrile/water solution followed by acetonitrile, tetrahydrofuran, and then dichloromethane. These studies indicate that the NE-OTf is unstable even in fresh anhydrous solvent, and very sensitive to both polarity of the solvent and moisture. Based on this, to reduce the side reactions and to create a controlled reaction system, the reaction was optimized using anhydrous acetonitrile, treating the catalyst *N*,*N*-diisopropylethylamine with molecular sieves of 5 Å, using NE-OTf stored at −80 °C over desiccant, making fresh NE-OTf solution immediately before each reaction, and adding it to the reaction mixture last, rapidly vortexing for a fixed and minimum time, running the reaction inside the desiccator at 4 °C.

The solubility of NE-OTf in icy acetonitrile was 6 mg/ml. To ensure that NE-OTf was dissolved immediately in acetonitrile at low temperature, the concentration of NE-OTf solution was modified from Maier et al. [18], who used 36.4 mM (13.6 mg/ml) to 5.36 mM (2 mg/ml). The reactions at 4 °C and at ambient temperature for 30 and 60 min were compared. The efficiency of the reaction at 4 °C was higher than at ambient temperature. In addition, the reactions with excess NE-OTf for 10, 30, and 60 min at 4 °C were monitored by FD-HPLC. No obvious labeled eicosanoid peaks were observed at 10 min, and peak intensities of labeled eicosanoids at 30 min were higher than those at 60 min over 4%. We determined that the reaction reached equilibrium at approximately 30 min and that even with the excess dye used in these experiments, dye decomposition measureably reduces fluorescence response after 45 min. Therefore, the reactions were completed at 30 min, and were terminated using argon to evaporate acetonitrile and *N*,*N*-diisopropylethylamine to dryness. Samples were then stored at −80°. The reactions with different ratios of NE-OTf and *N*,*N*-diisopropylethylamine were also studied. The observation shows that the an excess amount of catalyst *N*,*N*-diisopropylethylamine with respect to NE-OTf was essential for a high yield reaction, which is in agreement with Yasaka et al. [25] who use 18-crown-6 as the catalyst. The final reaction condition using 10 μl NE-OTf (0.0536 μmol), 4 μl *N*,*N*-diisopropylethylamine (22.9 μmol), and additional 136 μl anhydrous acetonitrile at 4 °C for 30 min offers a very clean background, and high and reproducible efficiency for PG, DiHETrE, HETE, EET and AA standards.

3.2. Optimization of fluorescent detected HPLC

Representative chromatograms for separation of 16 eicosanoid standards by reversed-phase FD-HPLC are presented in Fig. 5. With the aim to separate PGs, DiHETrEs, HETEs, EETs and AA simultaneously, special effort was devoted to optimize the mobile phase by trying different organic solvents, varying acidic modifiers, various isocratic and diverse gradients. The same mobile phase of FD-HPLC was used for LC/MS for further confirmation, thus, the mobile phase should be volatile to be compatible with LC/MS.

In preliminary experiments, an isocratic method [18] using methanol: water:acetic acid with 82:18:0.1 (v/v) at a rate of 1.3 ml/min was attempted in our lab. However, some of the regioisomers of eicosanoids were only partially separated. Also, the PGs could not be assayed due to coelution with unreacted dye, introducing a very intense background in the beginning of the chromatogram.

Manual injector with 20 μl loop was used for analysis, thus, a sample volume of 8 μl was injected for best run-to-run reproducibility [26]. Considering the 250 mm column length, a standard flow rate of 1 ml/min was utilized throughout the method development process. First, aqueous methanol mobile phase containing 0.1% acetic acid with various isocratic and gradient conditions were attempted. Baseline separations were achieved among the DiHETrEs, HETEs, EETs and AA, however only partial separations were obtained between PGD2, PGE2 and PGF2α. The elution sequence was PGF2α, followed by PGE2, PGD2, PGJ2, 14,15-DiHETrE, 11,12-DiHETrE, 8,9-DiHETrE, 20-HETE, 15-HETE, 12-HETE, 14,15-EET, 11,12-EET, 8,9-EET, and AA. The critical pair resolution for PGE2 and PGD2 was only 0.99, and increasing acetic acid modifier concentration to 0.5% did not improve the resolution. Formic acid (0.5%) provided a better separation, but did not yield baseline separation. Aqueous acetonitrile and aqueous acetonitrile/methanol (50/50) mobile phases containing 0.5% formic acid with diverse isocratic and gradient conditions were further explored. Baseline separation for PGJ2, 14,15-DiHETrE, 11,12-DiHETrE, 8,9-DiHETrE, 20-HETE, 15-HETE, 12-HETE, 14,15-EET, 11,12-EET, 8,9-EET and AA was achieved, but a better resolution of critical band pairs for PGE2 and PGD2 was not obtained. However, the aqueous acetonitrile conditions provided several advantages over aqueous methanol conditions. First, the time taken to elute unreacted dye in the beginning of the chromatogram was relative independent of the amount of dye used for the reaction. Second, the eicosanoid peak shapes were sharper and offered better sensitivity. Considering that our major focus was to detect DiHETrEs, HETEs and EETs, the aqueous acetonitrile mobile phase containing 0.5% formic acid was chosen for routine analysis, and the aqueous methanol mobile phase was used to confirm the analysis. (A: water with

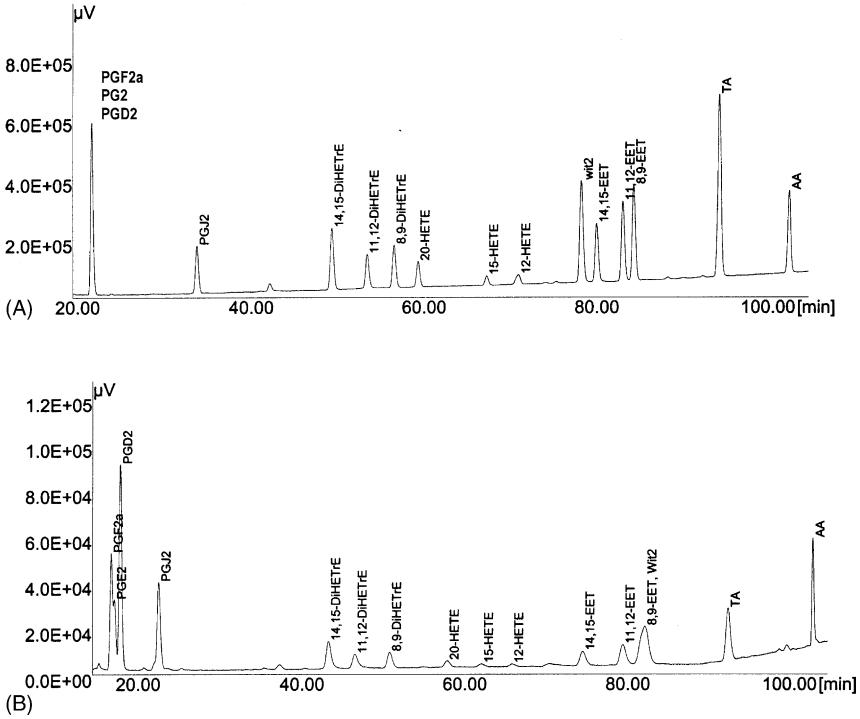


Fig. 5. Fluorescent detected HPLC chromatogram of 16 eicosanoid standards (100 pg for each standard). (A) aqueous acetonitrile condition (A: water with 0.5% formic acid; B: acetonitrile with 0.5% formic acid. Gradient: 50–65% B in 40 min; 65–100% B in 80 min; 100% B for 20 min. Flow rate: 1 ml/min. Excitation: 260 nm; emission: 396 nm). (B) Aqueous methanol condition (A: water with 0.5% formic acid; B: methanol with 0.5% formic acid. Gradient: 70–75% B in 5 min; 75–85% B in 80 min; 85–100% B in 20 min; 100% B for 20 min. Flow rate: 1 ml/min. Excitation: 260 nm; emission: 396 nm).

0.5% formic acid; B: methanol with 0.5% formic acid. Gradient: 70–75% B in 5 min; 75–85% B in 80 min; 85–100% B in 20 min; 100% B for 20 min. Flow rate: 1 ml/min) (Fig. 5B). Both the acetonitrile and methanol HPLC procedures were free from impurity interference by comparison to the blank, and the resolution between regioisomers of DiHETrE, and HETEs and EETs were all higher than 1.88. During method development, retention and selectivity were affected when room temperature shifted. For ease of control and considering the thermal stability of those compounds, an oven temperature of 30 °C was used.

To optimize the sensitivity of the fluorescent detection, the emission spectra of peaks were obtained by on-line scan. The maximum UV absorbance, 260 nm, was utilized as the excitation wavelength. The maximum emission for the background of unreacted dye was 406–486 nm, for PGJ2 was 400 nm, for DiHETrEs was 398–402 nm, for HETEs was 396–398 nm, for EETs was 396 nm, and for AA was 390 nm. Therefore, the excitation wavelength 260 nm and emission wavelength 396 nm were used for all FD-HPLC experiments. Fig. 6 shows the representative on-line scan fluorescent emission spectra with excitation wavelength 260 nm.

The results of system suitability testing for five injections at 100 pg level of each standard were very reproducible (Table 1). The variation from injection to injection was low. The R.S.D. for retention time was 0.02–0.20%. Resolution R.S.D. was <4.2%. Asymmetry R.S.D. ranged from 1.13 to 5.32%. Plate number R.S.D. was between 0.74 and 3.97%, except for 15-HETE, which was 16.78%. Area R.S.D. was <5%, except 15-HETE was 8.56%. The resolutions of these eicosanoid peaks ranged from 1.88 to 23.95. Since this range was so great, coelution of other non-eicosanoids is not a complication. The asymmetries of these peaks ranged from 0.94 to 1.13, indicating satisfactory peak shape. The plate numbers were all higher than 30,000 and the separation was efficient. In addition, this method remained unaffected when formic acid concentration varied from 0.1 to 0.5% and the mobile phase used from day to day. Overall, the method was robust.

Fig. 7 shows representative calibration curves generated by coupling standard derivatization reactions with FD-HPLC in the range of 70–2000 pg. The linearity indicates that our optimized fluorescent derivatization reaction following by FD-HPLC is a sound quantitative method, a prerequisite for developing a reliable assay for eicosanoids in biological samples.

The LOD and LOQ were evaluated by the progressive dilution of the original derivatization reaction containing 100 ng of each standard (2.5 ng/μl) (Table 2). LODs ranged from 2 to 20 pg, while LOQs ranged from 20 to 70 pg, respectively. Since all samples were derivatized for HPLC detection, it was necessary to assay the minimum amount of eicosanoids that could be detected via derivatization. Therefore the limit of derivatization reaction was studied by using representative PG, HETE, DiHETrE and EET in decreasing amount. The limit of derivatization reaction is the point at

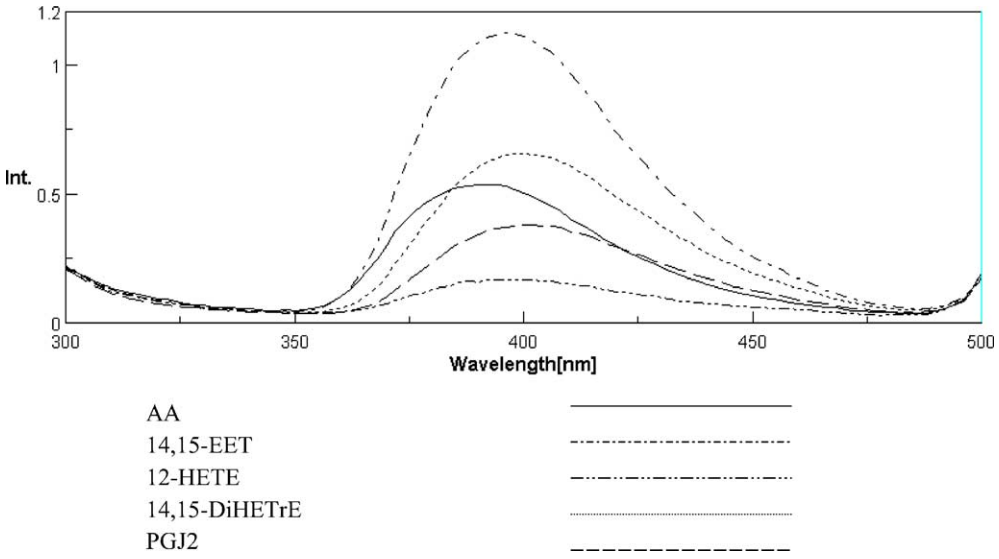


Fig. 6. The HPLC on-line scan fluorescent emission spectra of representative eicosanoids with excitation wavelength 260 nm.

Table 1
System suitability testing of FD-HPLC method at 100 pg of each standard for five injections

	RT (min)		Resolution		Asymmetry		Plate		Area	
	Mean	R.S.D. (%)	Mean	R.S.D. (%)	Mean	R.S.D. (%)	Mean	R.S.D. (%)	Mean	R.S.D. (%)
PGD2	22.11	0.20	N/A	N/A	1.13	1.13	30787	0.74	992071	3.56
PGJ2	34.24	0.15	21.76	0.62	1.07	3.13	49915	1.87	331032	4.74
14,15-DiHETrE	49.81	0.09	23.95	0.79	1.10	2.49	83282	1.43	483922	3.30
11,12-DiHETrE	53.92	0.08	5.94	0.90	1.12	3.73	95790	1.68	270776	3.69
8,9-DiHETrE	57.05	0.07	4.46	0.57	1.11	3.04	102880	1.20	342627	3.35
20-HETE	59.82	0.06	3.95	0.63	1.10	1.10	117878	1.75	200128	2.47
15-HETE	67.81	0.07	11.20	4.19	1.00	5.19	138773	16.78	79460	8.56
12-HETE	71.48	0.04	4.23	3.59	0.94	5.32	80531	3.93	109296	3.66
14,15-EET	80.51	0.04	2.54	0.57	1.09	2.97	228880	0.84	435515	2.98
11,12-EET	83.52	0.04	4.51	0.49	1.10	2.00	251592	1.51	586148	3.78
8,9-EET	84.77	0.03	1.88	1.04	1.11	1.89	259424	1.10	701061	3.38
AA	102.63	0.02	12.62	0.17	1.07	2.40	474813	0.75	543673	3.86

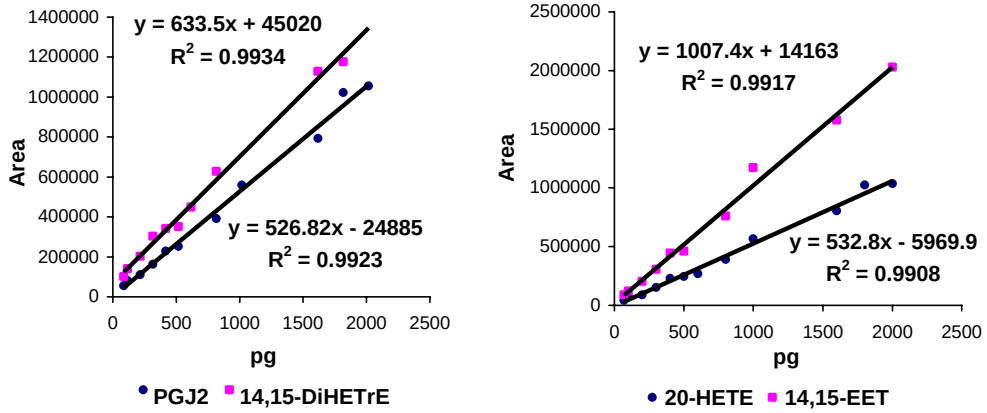


Fig. 7. Representative calibration curves generated by coupling derivatization reaction and FD-HPLC. Reaction mixtures of standards ranged from 70 to 2000 pg were derivatized individually with NE-OTf. The resultant samples were reconstituted in 40 μl methanol followed by injection of 8 μl in to HPLC.

Table 2
LOD and LOQ (diluted from 2.5 ng/μl solution) and limit of derivatization reaction

FD-HPLC compound	LOD (diluted from 2.5 ng/μl)[a] (pg)	LOQ (diluted from 2.5 ng/μl)[b] (pg)	Limit of derivatization reaction[c]	
			Sample concentration (pg/μl)	Injected amount (pg)
PGJ2	2	20	2.5	20
11,12-DiHETrE	5	20	2.5	20
15-HETE	20	70	8.75	70
14,15-EET	2	10	0.625	5

[a] Signal to noise ratio higher than 3.
[b] Signal to noise ratio higher than 10.
[c] Signal to noise ratio higher than 4.

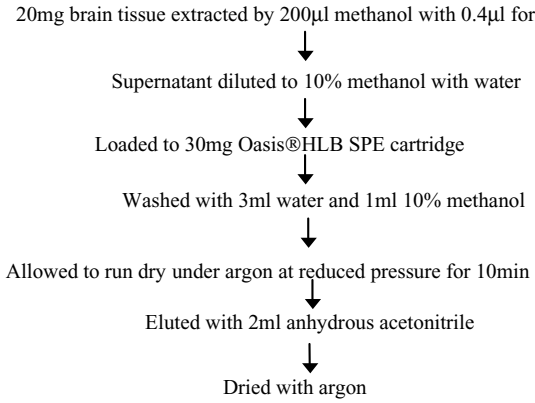
which the FD-HPLC signal to noise ratio higher than 4 for a given reaction concentration reconstituted in 40 μl methanol for HPLC analysis, and ranged from 0.625 to 2.5 pg/μl. Data in Table 2 indicate that the sensitivity of the method was dependent not only on the sensitivity of detection but also on the yield of the derivatization reaction.

3.3. Optimization of sample preparation

To date, few specific sample preparation procedures have been reported for eicosanoids other than AA in brain tissue. Yasaka et al. [25] used methanol to extract AA from mouse cerebrum, then dried the supernatant followed by derivatization without SPE clean up. In preliminary experiments, a liquid–liquid extraction method was attempted by using methanol, ethanol, or acetone to extract eicosanoids from brain tissue for derivatization. However, large amounts of fats were coextracted, preventing the complete solubilization in acetonitrile for subsequent steps. Therefore, a liquid–liquid extraction method was impractical for our purpose and a more powerful approach SPE procedure was developed. Eicosanoids are very lipophilic and avidly bind to proteins [2] and other fats. Additionally, brain tissue has a high moisture and fat content. Therefore the solvent chosen should efficiently extract lipid and disrupt protein binding. Here, alcohol and acetone were good candidates, as they (1) dissolve eicosanoids; (2) permeate through wet and fatty brain tissue matrix; (3) break down tissue membranes to release eicosanoids; and (4) facilitate protein precipitation. In addition, the use of acidic conditions not only ensured the free carboxylic acid form of eicosanoids for higher efficiency, but also reduced protein binding. Methanol, ethanol, or acetone, together with phosphoric acid or formic acid yielded similar extraction efficiency. Considering the ease of evaporating the solvent before reaction and compatibility with subsequent SPE step, methanol and formic acid were adopted for extraction.

With the purpose of developing a robust SPE procedure for clean up of the methanol extractions of the brain tissue, the choices of SPE cartridge were carefully considered and evaluated. The derivatization was sensitive to moisture, thus the SPE cartridge must be dried before elution with anhydrous organic solvent. However, the cartridge running dry between washing and eluting steps usually causes irreproducibility. In preliminary experiments, 1 cm^3 C18 Sep-Pak® cartridges for urine samples [18] was used, but the process was not reproducible and eicosanoid retention was compromised. Consequently, 1 cm^3 Oasis® HLB cartridges were employed as they were stable even in wet and dry recycling conditions and offered both good retention of eicosanoids as well as and reproducibility.

The conditions for extraction and SPE procedures were optimized by comparing the samples spiked before SPE and after SPE with representative HETEs, DiHETrEs, EETs and AA. Pre-cleaning steps of cartridge were optimized to reduce extractable contaminants from sorbent and plastic reservoir by using 1 ml methanol, 1 ml acetone, 2 ml hexane, 1 ml acetone, and 1 ml methanol, sequentially, with low feeding speed. The ratio of methanol and water for loading and washing steps were optimized to retain eicosanoids on the cartridge and allow pass through or wash off of undesirable co-extracted matrix interference. Anhydrous acetonitrile is a stronger mobile phase than methanol and elutes eicosanoids more efficiently. Our new sample preparation procedure for cortical brain tissue is shown in Scheme 2. The linearity of the method coupling the sample preparation with derivatization and FD-HPLC was evaluated by analyzing samples spiked with representative standards at 0.5, 2, and 10 ng levels (Fig. 8). The correlation coefficients (R^2) were all higher than 0.99, which indicated that the extraction and SPE procedures coupling with derivatization method developed in our lab were suitable for quantitative purposes. The extrac-



Scheme 2. Sample preparation procedure for cortical brain tissue.

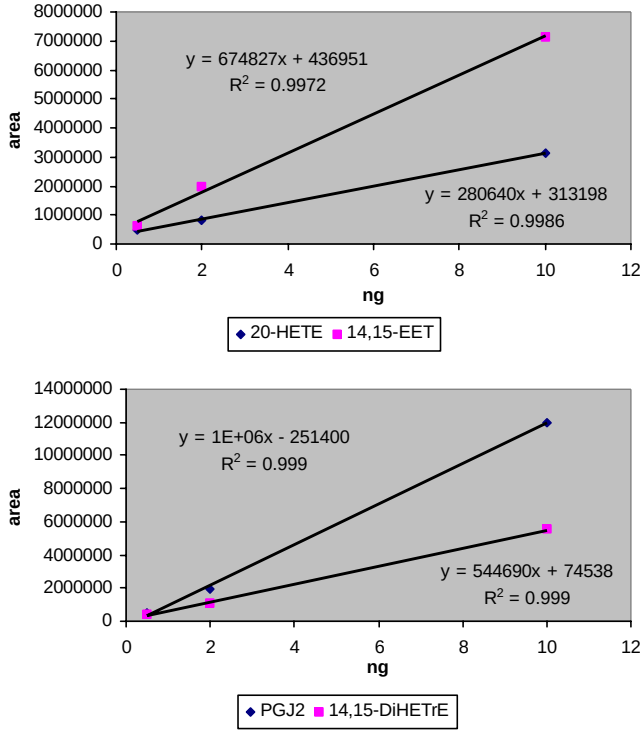


Fig. 8. Coupling the sample preparation with derivatization and FD-HPLC. The representative standards at 0.5, 2 and 10 ng levels were spiked into cortical brain tissue, followed by extraction and SPE, derivatization and FD-HPLC analysis.

tion efficiency at 0.5 and 2 ng level were evaluated by comparison of the analyte response of samples spiked before extraction and SPE with the response of samples spiked after extraction and SPE (Table 3).

3.4. LC/MS confirmation

There were two purposes for this experiment. The first was to confirm that the reaction has produced properly labeled eicosanoids. The second was to correlate the retention time of peaks to their structures. The same chromatography conditions as FD-HPLC were used for LC/MS. The

Table 3
The extraction efficiency of representative eicosanoids (by comparison of the analyte response of samples spiked before extraction and SPE with the response of samples spiked after extraction and SPE)

	PGJ2 (%)	14,15-DiHETrE (%)	20-HETE (%)	14,15-EET (%)
0.5 ng	92.3	106.0	117.2	96.7
2 ng	94.1	67.7	72.7	79.5

Table 4
Correlation of FD-HPLC with LC/MS

	PGJ2	14,15-DiHETrE	12-HETE	14,15-EET	AA
RT (min)					
FD/HPLC	34.24	49.81	71.48	80.51	102.63
LC/MS	34.63	50.08	71.68	80.61	102.36
Ion mass of the peak (Da)	626.4	630.4	612.4	612.4	596.4

retention times for standards were similar for both methods (Table 4). Both atmospheric chemical ionization (APCI) and atmospheric electrospray ionization (API-ES) under positive and negative scan mode were attempted to obtain signals on mass detector. The API-ES under positive mode offered the best detection. The masses of the most intense pseudo molecular ions for labeled standards were found to be $[M + 69]^+$, adduct of formic acid and sodium ion. The representative mass spectra under scan mode are shown in Fig. 9. For further confirmation of peaks in cortical brain tissue, the selected ion monitoring (SIM) positive mode was used for best sensitivity.

3.5. Analysis of cortical brain tissue samples

Rat cerebral cortex from sham and brain injured animals were analyzed by the FD-HPLC method and further confirmed via LC/MS. Peaks of 15-HETE, 12-HETE, 14,15-EET, 11,12-EET, 8.9-EET and AA were detected in both sham and injured cortex. Unidentified eicosanoids with the same mass as PGDF2α and PGD2 (19.14 and

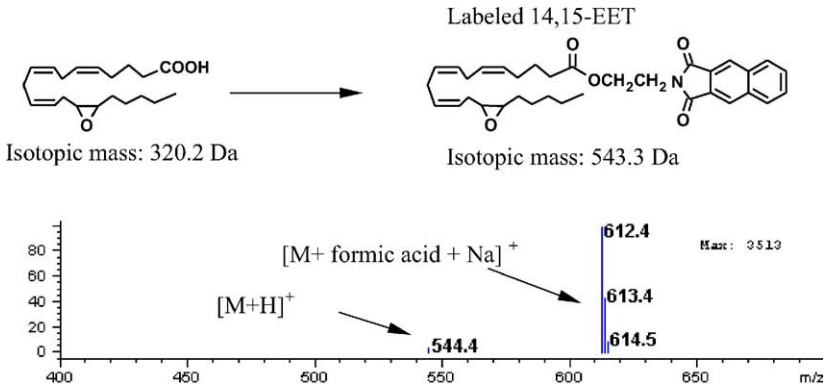


Fig. 9. MS spectra of 80.61 min peak in LC/MS.

Table 5
The levels of 14,15-EET in sham and injured rat brains at 6 and 24 h post-injury, both contra-lateral and ipsi-lateral to the injury site

Animal number	Concentration (pg/mg wet weight)	Average concentration (pg/mg wet weight)	R.S.D. (%)
Contra lateral			
6 h			
Sham 407	173.9	157.8	14.4
Sham 408	141.8		
Injured 406	73.2	263.2	102.1
Injured 734	453.1		
24 h			
Sham 413	453.1	349.5	38.2
Sham 414	255.2		
Injured 1224	87.4	96.3	13.2
Injured 1225	105.3		
Ipsi lateral			
6 h			
Sham 408	109.9	115.8	7.2
Sham 431	121.7		
Injured 406	87.9	71.9	31.5
Injured 742	55.8		
24 h			
Sham 397	216.4	277.5	31.1
Sham 414	338.6		
Injured 1224	234.4	267.0	17.2
Injured 1225	299.5		

25.67 min, respectively) were detected, as well as DiHETrE (47.22 min), and EETs (82.00, 83.53, and 86.46 min). Future LC/MS/MS experiments will be necessary to elucidate the structures of these unknown peaks.

Table 5 shows the levels of 14,15-EET being determined in sham and injured rat brains at 6 and 24 h post-injury, both contra-lateral and ipsi-lateral to the injury site. The amount of 14,15-EET in sham and injured cerebral cortex was determined to be from 71.9 to 349.5 pg/mg wet weight using the recovery observed at 1 ng level and the calibration curve shown in Fig. 7. The total recovery of 14,15-EET was $33+3\%$ ($n = 3$) obtained by adjusting for the SPE recovery and background contributions.

4. Conclusion

This study is first to report to identify trace brain bioactive eicosanoids including PGs, DiHETrEs, HETEs, EETs, and AA via a single FD-HPLC assay. A new selective and reproducible SPE sample preparation procedure with minimum steps was developed for cortical brain tissue. Due to the complexity of the brain tissue matrix and lipophilicity of eicosanoids, in some ways, sample preparation stage for quantifying eicosanoids is more challenging than in other liquid biological fluid (plasma, urine and microdialysis), and is most critical for successful analysis. This SPE procedure should be applicable to any biological fluids and tissues with only slight modification. Furthermore, the LC/MS was first used to further identify peaks in real biological samples. Overall, the methodology described in this paper provides a quantitative way to study eicosanoids and will help elucidate the mechanism of eicosanoid metabolites in health and disease in various biological tissues.

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