Published in final edited form as:

Methods Mol Biol. 2018 ; 1730: 123–133. doi:10.1007/978-1-4939-7592-1_10.

LC-MS/MS Analysis of the Epoxides and Diols Derived from the Endocannabinoid Arachidonoyl Ethanolamide

Amy A. Rand, Patrick O. Helmer, Bora Inceoglu, Bruce D. Hammock, and Christophe Morisseau

Abstract

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is a useful tool to characterize the behavior of natural lipids within biological matrices. We report a LC-MS/MS method developed specifically to analyze CYP products of the arachidonoyl ethanolamide (anandamide, AEA), the epoxyeicosatrienoic acid ethanolamides (EET-EAs) and their hydrolyzed metabolites, and the dihydroxyeicosatrienoic acid ethanolamides (DHET-EAs). This method was used to measure EET-EA biotransformation to DHET-EAs by two human epoxide hydrolases: the soluble EH (sEH) and the microsomal EH (mEH). In general, sEH and mEH substrate preference was similar, based on k_{cat}/K_M . The 14,15-EET-EA and 11,12-EET-EA were the most efficiently hydrolyzed, followed by 8,9-EET-EA and 5,6-EET-EA. The method was also used to detect endogenous levels of these lipids in mouse tissues, although levels were below the instrumental detection limit (0.1–3.4 nM). Because both AEA and EETs are biologically active, the method described herein will be invaluable in revealing the role(s) of EET-EAs in vivo.

Keywords

Liquid chromatography mass spectrometry; Anandamide; CYP450; Epoxide hydrolase; EET-EAs; DHET-EAs; Limit of -detection; Enzyme kinetics; Bioactivity

1 Introduction

The endocannabinoid arachidonoyl ethanolamide (anandamide, AEA) is a natural lipid-signaling molecule found in most tissues that mediates neurological, immune, and cardiovascular functions by primarily targeting cannabinoid receptors [1]. While it is primarily metabolized by fatty acid amide hydrolase (FAAH) to arachidonic acid, it is also metabolized through several cytochrome P450s (CYP450), including CYP3A4, CYP2B6, CYP2D6, CYP4F2, CYP2J2, and CYP4X1 [2–4]. AEA is converted to monooxygenated products in mouse liver and brain microsomes [5]. These products include the epoxyeicosatrienoic acid ethanolamides (EET-EAs), which were detected, upon incubation with AEA, in human liver microsomes and brain mitochondrial fractions [6], as well as in bovine and porcine heart microsomes [4].

While the biological function of EET-EAs needs to be further explored, it is anticipated that they have important physiological roles due to their structural similarity to the

epoxyeicosatrienoic acids (EETs) [7]. EETs are formed from CYP oxidation of arachidonic acid and have far-reaching physiological effects involved in pain, inflammation, and kidney and cardiovascular diseases [8, 9]. The 5,6-EET-EA is a potent agonist for the cannabinoid receptor 2 [10], which may mediate brain and liver pathological outcomes such as neurodegeneration and fibrosis, respectively, by triggering anti-inflammatory responses [2]. While endogenous levels of EET-EAs are uncertain and may be produced in an activity-dependent manner, they are transformed by epoxide hydrolases (EHs) to their corresponding dihydroxyeicosatrienoic acid ethanolamides (DHET-EAs) [6, 10]. The biological activity of DHET-EAs has not yet been reported but might be similar to the less bioactive diols from EETs [11]. sEH inhibitors, which enhance levels of EETs and other epoxy fatty acids, have similar activities to cannabinoid receptor 2 agonists [12].

Analysis of EET-EAs and DHET-EAs is important to further our understanding of their biological activity, metabolic fate, and distribution within tissues. Here, we use two sample preparation methods for analysis of EET-EAs and DHET-EAs by LC-MS/MS. We report a sample dilution LC-MS/MS method to examine the hydrolysis of EET-EAs to DHET-EAs by two human epoxide hydrolases: the soluble EH (sEH) and the microsomal EH (mEH). While the EET-EAs had similar turnover with both sEH and mEH (Table 1), enzyme efficiency for each substrate (k_{cat}/K_M) varied; the 14,15-EET-EA and 11,12-EET-EA were the most efficiently hydrolyzed followed by 8,9-EET-EA and 5,6-EET-EA (Fig. 1 and Table 1). We also use solid-phase extraction (SPE), paired with the reported LC-MS/MS method, to detect endogenous levels of EET-EAs and DHET-EAs in mouse liver, kidney, spleen, and brain tissue. Although levels of the EET-EAs and DHET-EAs were below the instrumental limits of detection, the method can be applied to further work using enzymatic inhibitors and/or transgenic mice to raise levels of EET-EAs and DHET-EAs and to understand EET-EA and DHET-EA endogenous distribution and biological function in endocannabinoid signaling.

2 Materials

All solvents used should be Optima grade or 18 M Ω ultrapure water, unless otherwise indicated.

2.1 Standards for Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

1. 5,6-; 8,9-; 11,12-; and 14,15-EET-EA and standard solutions (Cayman Chemical, Ann Arbor, MI, USA): Make up as 5 μ M solution in methanol and store at -20°C under nitrogen gas until use (*see* Note 1).
2. AEA standard solution (Cayman Chemical, Ann Arbor, MI, USA): Make up as 5 μ M solution in methanol and store at -20°C under nitrogen gas until use.
3. 5,6-; 8,9-; 11,12-; and 14,15-DHET-EA standards were prepared by enzymatic hydrolysis from their respective EET-EAs. In 10 – 75 mm glass tube, to 100 μ L of purified recombinant mouse sEH [13] ($[\text{MsEH}]_{\text{final}} = 0.6 \mu\text{M}$, 0.04 mg/mL) in sodium phosphate buffer (0.1 M pH 7.4) containing 0.1 mg/mL of BSA, 1 μ L of

¹Store lipid standards under nitrogen at -20°C to minimize oxidation.

solutions of individual EET-EAs in DMSO was added ($[S]_{\text{final}} = 0\text{--}2.5\ \mu\text{M}$). The tubes were sealed and incubated at $37\ ^\circ\text{C}$ for 2 h. The reaction was then stopped by adding $100\ \mu\text{L}$ of chilled methanol containing $0.5\ \mu\text{M}$ of AEA as internal standard. The mixture was then centrifuged ($3000 \times g \times 5\ \text{min}$) to remove any precipitated salt. The supernatant was transferred to vials, which were kept at $-20\ ^\circ\text{C}$ until LC-MS/MS analysis.

2.2 Tissue Collection

1. Liver, spleen, brain, and kidney tissues from saline-perfused Swiss Webster mice were collected just after sacrifice of the animals and immediately stored at $-80\ ^\circ\text{C}$ until analysis. All procedures and animal care adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications 8th Edition 2011) and were performed in accordance with the protocols approved by the International Animal Use and Care Committee (IACUC) of the University of California, Davis.
2. The whole liver, spleen, brain, and kidney were used from mice ($n = 3$). Frozen tissues were cut into small pieces with a clean razor blade. Tissues were weighted ($100\text{--}260\ \text{mg}$) and allocated to $2\ \text{mL}$ polypropylene microtubes. Tissues were suspended in $400\ \mu\text{L}$ ice-cold methanol containing 0.1% concentrated acetic acid.
3. Antioxidant solution was prepared by combining $0.6\ \text{mg/mL}$ butylated hydroxytoluene (BHT), $0.6\ \text{mg/mL}$ triphenylphosphine (TPP), and $0.6\ \text{mg/mL}$ ethylenediaminetetraacetic acid (EDTA) in DMSO to give a final concentration of $0.2\ \text{mg/mL}$ in methanol. A $10\ \mu\text{L}$ aliquot was added to each tissue sample to prevent autooxidation.

2.3 Solid-Phase Extraction (SPE)

1. Oasis HLB $3\ \text{mL}$ $60\ \text{mg}$ cartridges (Waters Corporation, Milford, MA, USA).
2. Water/methanol (8:2) with 0.1% acetic acid: Fill a $1\ \text{L}$ graduated cylinder with $800\ \text{mL}$ pure water, $200\ \text{mL}$ methanol, and $1\ \text{mL}$ concentrated acetic acid and store in a $1\ \text{L}$ reagent bottle.
3. 30% glycerol solution: Measure $6\ \text{mL}$ glycerol and $14\ \text{mL}$ methanol in a $25\ \text{mL}$ graduated cylinder and store in a $20\ \text{mL}$ glass screw-cap vial.
4. Methanol: Store methanol in a $500\ \text{mL}$ reagent bottle.
5. Ethyl acetate: Store ethyl acetate in a $500\ \text{mL}$ reagent bottle.
6. SPE vacuum manifold processing station.

2.4 Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

1. LC-MS/MS: We used a Waters Acquity Ultra Performance Liquid Chromatograph coupled to a Waters Xevo TQ-S Mass Spectrometer (Waters Corporation, Milford, MA, USA).

2. Liquid chromatography column: Kinetex 1.7 μm XB-C18 (100 $\text{\AA}$, 150 $\times$ 2.1 mm) with a SecurityGuard ULTRA cartridge for C18 UPLC, sub-2 μm with 2.1 mm internal diameters (Phenomenex, Torrance, CA, USA).
3. Water with 0.1% acetic acid and 1 mM ammonium acetate: Fill a 1 L graduated cylinder with 999 mL LC-MS grade water, 1 mL acetic acid, and 77 mg ammonium acetate and store in a 1 L LC glass solvent bottle.
4. Methanol with 0.1% acetic acid and 1 mM ammonium acetate: Fill a 1 L graduated cylinder with 999 mL LC-MS grade methanol, 1 mL acetic acid, and 77 mg ammonium acetate and store in a 1 L LC glass solvent bottle.
5. Glass screw top vials: 2 mL, 32 $\times$ 12 mm, and 9 mm Amber Glass Screw Thread Vials are used.
6. Glass inserts for screw top vials: Use a 0.150 mL glass insert with attached plastic spring purchased.
7. Caps for screw top vials: Use 9 mm thread cap with bonded plastic PTFE/silicone septa with pre-slit.

3 Methods

3.1 Processing of Enzyme Kinetic Samples

1. Kinetic parameters for the 5,6-EET-EA; 8,9-EET-EA; 11,12-EET-EA; and 14,15-EET-EA were determined under steady-state conditions using partially purified recombinant human sEH (95% pure) and human mEH (80% pure) [14, 15]. Just before usage, the human sEH was diluted in chilled sodium phosphate buffer (0.1 M pH 7.4) containing 0.1 mg/mL BSA to a final concentration of 4 nM (0.25 $\mu\text{g}/\text{mL}$). Whereas, the human mEH was diluted in chilled Tris/HCl buffer (0.1 M, pH 9.0) containing 0.1 mg/mL of BSA to a final concentration of 200 nM (10 $\mu\text{g}/\text{mL}$). Enzyme solutions were kept on ice until usage.
2. A series of substrate (individual EET-EA) stock solutions were prepared in DMSO. In general, 50–100 μL of eight substrate concentrations ranging from 0 to 5 mM were prepared.
3. In 10 $\times$ 75 mm glass tubes, to 100 μL of enzyme solution or buffer for control samples, 1 μL of solutions of individual EET-EAs in DMSO was added ($[\text{S}]_{\text{final}} = 0\text{--}50\text{ }\mu\text{M}$). The mixture was sealed and incubated at 37 $^{\circ}\text{C}$ for 15–120 min depending on substrate and enzyme. Incubation time was optimized to ensure the enzymatic reaction is in steady-state conditions, with less than 5% of the initial substrate metabolized during the whole course of the hydrolysis. The reaction was then stopped by adding 100 μL of chilled methanol containing 0.5 μM of AEA as internal standard.
4. The mixture was then centrifuged (3000 $\times g$ $\times$ 5 min) to remove any precipitated salt. The supernatant was transferred to a vial, which was kept at $-20\text{ }^{\circ}\text{C}$ until LC-MS/MS analysis.

5. The quantity of product (DHET-EA) formed was determined by LC-MS/MS analysis (Subheading 3.3). The kinetic constants (K_M and k_{cat}) were calculated by nonlinear fitting of the Michaelis-Menten equation ($v = (k_{cat} \cdot [E] \cdot [S]) / (K_M + [S])$) using the enzyme kinetic module of SigmaPlot 11.0. Reactions were done in hexaplicate ($n = 6$).

3.2 Tissue Extraction of EET-EAs and DHET-EAs

1. Between 100 and 260 mg of tissues are weighted in 2 mL plastic vials. Then, 10 μ L of the BHT, TPP, and EDTA antioxidant solution was added as well as 400 μ L ice-cold methanol containing 0.1% acetic acid. To correct for possible analyte loss throughout the extraction, 5 μ L of 1 μ M AEA was added as an internal standard (*see Note 2*) just prior to tissue homogenization.
2. Tissue samples are homogenized by placing two steel beads into each vial and shaken using a bead beater operating at 30 Hz for 10 min (*see Note 3*). The resulting homogenate solution was centrifuged at $13,200 \times g \times 10$ min.
3. The supernatant is collected into 2 mL polypropylene vials, and the remaining tissue pellet is washed with 100 μ L ice-cold methanol containing 0.1% acetic acid by vortexing for 1 min to break up and resuspend the pellet (*see Note 4*). Samples are centrifuged again at $13,200 \times g \times 10$ min. This supernatant was combined with the first to give a total volume approximating 0.5 mL. Samples are diluted with 1.5 mL ice-cold water and stored on ice before loading onto the SPE cartridges (*see Note 5*).
4. Oasis HLB cartridges (3 mL, 60 mg) are mounted on a vacuum manifold processing station under vacuum (~ 5 bar). Cartridges are preconditioned to remove impurities with 3 mL ethyl acetate and 6 mL methanol [16].
5. Cartridges are equilibrated by loading 6 mL of water/methanol (8:2) with 0.1% acetic acid under atmospheric pressure (*see Note 6*).
6. Samples are loaded onto the cartridge and left to filter at atmospheric pressure through the cartridge. The approximate flow rate is approximately 1 drop/3 s, monitored by watching the drip rate at the bottom of the cartridge. If the rate is less than this, light vacuum can be applied (*see Note 7*).

²Deuterated standards are not commercially available for EET-EAs and DHET-EAs; therefore, we used AEA (Cayman Chemical, Ann Arbor, MI, USA) as an internal standard for quantifying EET-EAs and DHET-EAs, due to its similar size and structure. We used non-labeled AEA since endogenous levels of AEA were not detected in any tissue. Alternatively, the d4-AEA (Cayman Chemical, Ann Arbor, MI, USA) can be used as an internal standard for extracting EET-EAs and DHET-EAs, especially if there are levels of endogenous AEA in the tissue samples.

³Ensure vials are tightly capped; otherwise, the sample mixture will be lost during homogenization.

⁴Extracting the pellet twice with methanol increases the analyte recovery from the tissue matrix.

⁵Dilute samples with water before loading onto the SPE columns. This will make the sample more polar and facilitate nonpolar analyte adsorption onto the cartridge stationary phase.

⁶Upon equilibration, it is important that the solvent does not go below the column frit at the top of the stationary phase. Doing so will leave the top of the stationary phase dryer than the rest, changing its physical composition and thus the sorption behavior of the analytes, leading to variations in analyte recovery.

⁷Generally, extracting the sample at a flow rate of 1 drop/3 s is acceptable. However, samples may be eluted through the column at slower rates depending on the matrix and cartridge composition. If this is the case, light vacuum can be applied to maintain the desired flow rate.

7. Polar substances from the tissue extracts are eluted through the column using 6 mL of water/methanol (8:2) with 0.1% acetic acid.
8. Apply approximately 10 bar vacuum to the manifold to dry the cartridges (20 min).
9. While cartridges are drying, prepare 2 mL polypropylene Eppendorf vials for the sample eluent, by adding 6 μ L 30% glycerol solution in methanol. Label each vial with their respective sample name and place in the vacuum manifold, just below each sample cartridge.
10. EET-EAs, DHET-EAs, and AEA are eluted with 0.5 mL methanol followed by 1.5 mL ethyl acetate into the 2 mL Eppendorf vials at atmospheric pressure. Apply a 10 bar vacuum to elute the remaining solvent from the cartridge.
11. Samples are evaporated under vacuum using a ScanVac Speed-Vac (Neutec Group Inc., Farmingdale, NY) for approximately 2 h (*see* Note 8).
12. Samples are reconstituted in 50 μ L methanol in a 2 mL polypropylene Eppendorf tube, filtered using Durapore PVDF 0.1 μ m filter units (Millipore, Cork, Ireland), and centrifuged at $13,200 \times g \times 10$ min (*see* Note 9).
13. Filtrates are transferred to 0.15 mL glass inserts in a 2 mL screw top vial and cap. Samples are stored at -20°C until LC-MS/MS analysis.
14. Analyte recovery was validated by performing a spike-and-recovery test, the values of which are given in Table 2 (*see* Note 10).

3.3 LC-MS/MS Analysis

1. Samples are analyzed on a Waters Acquity Ultra Performance Liquid Chromatograph coupled to a Waters Xevo TQ-S Mass Spectrometer in positive electrospray ionization mode. Samples are injected (5 μ L) and separated using a Phenomenex Kinetex column (150×2.1 mm; 1.7 μ m) at 40°C using the following mobile phase gradient, consisting of LC-MS grade water (A) and optima LC-MS grade methanol (B) each containing 0.1% acid and 0.1 mM ammonium acetate: initial conditions of 70:30 A/B for 2.9 min ($t = 2.9$ min), changing to 45:55 at 3 min ($t = 3$ min) and decreasing to 35:65 over 5.5 min ($t = 8.5$ min), decreasing to 28:72 over 4 min ($t = 12.5$ min), decreasing to 18:82 over 2.5 min ($t = 15$ min), decreasing to 5:95 over 1.5 min ($t = 16.5$ min), holding at 5:95 for 1.5 min ($t = 18$ min), reverting to initial conditions of 70:30 in 0.1 min (t

⁸Ensure all solvents are removed prior to reconstitution.

⁹Filtering removes any particulates from the samples to maintain LC column integrity.

¹⁰Analyte recovery and matrix-matched calibration: To determine the extraction efficiency of the method described above, we performed a spike-and-recovery test. The EET-EA and DHET-EA stock solutions were diluted and combined to form a solution having a final concentration of 500 nM. The liver (approximately 600 mg) was cut and homogenized in methanol. 100 μ L of the homogenate was spiked with 2.5 μ L of the 500 nM EET-EA and DHET-EA solution in triplicate. 5 μ L of the 500 nM AEA internal standard was also added to the spiked tissue samples. The rest of the unspiked liver homogenate was divided and extracted to create a matrix-matched calibration curve. All tissue samples (spiked and unspiked) were extracted and analyzed using the SPE-LC-MS/MS-method described above. Samples were reconstituted in 50 μ L methanol. Matrix-matched standards were made by adding 0–6 μ L of the 500 nM EET-EA and DHET-EA stock solution to generate a concentration range from 0 to 60 nM, as well as 5 μ L of the 500 nM AEA. The final volume for each standard was brought to 50 μ L with methanol. A non-matrix-matched calibration curve was produced in methanol (rather than reconstituted matrix) using the same concentrations (0–60 nM with 50 nM AEA).

= 18.1 min), and re-equilibrating for 2.9 min ($t = 21$ min). Mass spectral analysis was accomplished using a capillary voltage of 3 kV, a desolvation temperature of 200 °C, a desolvation gas (nitrogen 99.99%) flow of 800 L/h, a cone gas flow of 150 L/h, nebulizer pressure of 6 bar, and collision gas flow of 0.15 mL/min. LC-MS/MS parameters MRM transitions for each analyte are shown in Table 2, with analyte dwell times of 25 ms. A representative spectrum of the separated EET-EAs and DHET-EAs is shown in Fig. 2.

2. EET-EAs and DHET-EAs are detected by retention time and mass transitions, values of which are given in Table 2.
3. Analyte concentration was calculated using a matrix-matched and non-matrix-matched standard curve with known concentrations (2.5–60 nM) (see Notes 10 and 11). The area of all analytes was achieved through peak integration and dividing its value by the area of the AEA internal standard.

Acknowledgments

This work received support in part from the National Institute of Environmental Health Sciences R01 ES002710 and NIEHS Superfund Program P42 ES004699. A. Rand acknowledges support from the OSCB training grant NIH/NIEHS T32 CA108459 and from the 2016 AACR Judah Folkman Fellowship for Angiogenesis Research, Grant Number 16-40-18-RAND.

References

1. Mechoulam R, Parker LA. The endocannabinoid system and the brain. *Annu Rev Psychol.* 2013; 64:21–47. [PubMed: 22804774]
2. Snider NT, Walker VJ, Hollenberg PF. Oxidation of the endogenous cannabinoid arachidonoyl ethanolamide by the cytochrome P450 monooxygenases: physiological and pharmacological implications. *Pharmacol Rev.* 2010; 62:136–154. [PubMed: 20133390]
3. Sridar C, Snider NT, Hollenberg PF. Anandamide oxidation by wild-type and polymorphically expressed CYP2B6 and CYP2D6. *Drug Metab Dispos.* 2011; 39:782–788. [PubMed: 21289075]
4. McDougle DR, Kambalyal A, Meling DD, Das A. Endocannabinoids anandamide and 2-arachidonoylglycerol are substrates for human CYP2J2 epoxide hydrolase. *J Pharmacol Exp Ther.* 2014; 351:616–627. [PubMed: 25277139]
5. Bornheim LM, Kim KY, Chen B, Correia MA. Microsomal cytochrome P450-mediated liver and brain anandamide metabolism. *Biochem Pharmacol.* 1995; 50:677–686. [PubMed: 7669071]
6. Snider NT, Kornilov AM, Kent UM, Hollenberg PF. Anandamide metabolism by human liver and kidney microsomal cytochrome p450 enzymes to form hydroxyeicosatetraenoic and epoxyeicosatrienoic acid ethanolamides. *J Pharmacol Exp Ther.* 2007; 321:590–597. [PubMed: 17272674]
7. Zelasko S, Arnold WR, Das A. Endocannabinoid metabolism by cytochrome P450 monooxygenases. *Prostaglandins Other Lipid Mediat.* 2015; 116:112–123. [PubMed: 25461979]
8. Imig JD, Hammock BD. Soluble epoxide hydrolase as a therapeutic target for cardiovascular diseases. *Nat Rev Drug Discov.* 2009; 8:794–805. [PubMed: 19794443]
9. Morisseau C, Hammock BD. Impact of soluble epoxide hydrolase and epoxyeicosanoids on human health. *Annu Rev Pharmacol Toxicol.* 2013; 53:37–58. <https://doi.org/10.1146/annurev-pharmtox-011112-140244>. [PubMed: 23020295]

¹¹Matrix-matched calibration curves are used to correct for matrix effects within the samples. In our case, there was no significant matrix suppression or enhancement when compared to the non-matrix-matched calibration curve; therefore, either curve can be used for quantification.

10. Snider NT, Nast JA, Tesmer LA, Hollenberg PF. A cytochrome P450-derived epoxygenated metabolite of anandamide is a potent cannabinoid receptor 2-selective agonist. *Mol Pharmacol*. 2009; 75:965–972. [PubMed: 19171674]
11. Bellien J, Joannides R. Epoxyeicosatrienoic acid pathway in human health and diseases. *J Cardiovasc Pharmacol*. 2013; 61:188–196. [PubMed: 23011468]
12. Wagner K, Inceoglu B, Hammock BD. Soluble epoxide hydrolase inhibition, epoxygenated fatty acids and nociception. *Prostaglandins Other Lipid Mediat*. 2011; 96:76–83. [PubMed: 21854866]
13. Argiriadi MA, Morisseau C, Hammock BD, Christianson DW. Detoxification of environmental mutagens and carcinogens: structure, mechanism, and evolution of liver epoxide hydrolase. *Proc Natl Acad Sci*. 1999; 96:10637–10642. [PubMed: 10485878]
14. Morisseau C, Bernay M, Escaich A, et al. Development of fluorescent substrates for microsomal epoxide hydrolase and application to inhibition studies. *Anal Biochem*. 2011; 414:154–162. [PubMed: 21371418]
15. Morisseau C, Weckslar AT, Deng C, et al. Effect of soluble epoxide hydrolase polymorphism on substrate and inhibitor selectivity and dimer formation. *J Lipid Res*. 2014; 55:1131–1138. [PubMed: 24771868]
16. Yang J, Schmelzer K, Georgi K, Hammock BD. Quantitative profiling method for oxylipin metabolome by liquid chromatography electrospray ionization tandem mass spectrometry. *Anal Chem*. 2009; 81:80–85. [PubMed: 19061410]

Fig. 1.

Determination of the kinetic constants for 5,6-EET-EA; 8,9-EET-EA; 11,12-EET-EA; and 14,15-EET-EA, with the human sEH ($[HsEH]_{final} = 4 \text{ nM}$) in NaPO_4 buffer (0.1 M pH 7.4 containing 0.1 mg/mL of BSA) at 37 °C. Values were obtained from sextuplicate analysis (average $\pm$ standard deviation)

Fig. 2.
LC-MS/MS total ion chromatograph of a 60 nM standard mixture containing all the regioisomers of DHET-EAs and EET-EAs

Kinetic constants of recombinant purified human sEH and mEH for the hydrolysis of all the regioisomers of 14,15-EET-EAs

Table 1

Enzyme	Substrate	K_M (μM)	r^2	k_{cat} (s^{-1})	k_{cat}/K_M ($s^{-1} \mu M^{-1}$)
Human sEH	14,15-EET-EA	16 ± 1	0.99	3.32 ± 0.02	0.20 ± 0.01
	11,12-EET-EA	19 ± 6	0.92	6.0 ± 0.8	0.31 ± 0.07
	8,9-EET-EA	28 ± 3	0.99	1.9 ± 0.1	0.068 ± 0.009
	5,6-EET-EA	22 ± 4	0.96	0.22 ± 0.02	0.010 ± 0.002
Human mEH	14,15-EET-EA	3.8 ± 0.4	0.99	0.61 ± 0.02	0.16 ± 0.02
	11,12-EET-EA	1.2 ± 0.3	0.99	0.17 ± 0.01	0.15 ± 0.04
	8,9-EET-EA	6.2 ± 0.6	0.99	0.10 ± 0.01	0.016 ± 0.002
	5,6-EET-EA	10.2 ± 1.4	0.99	0.11 ± 0.01	0.010 ± 0.002

Constants (K_M and k_{cat}) were calculated by nonlinear fitting of the Michaelis-Menten equation using the enzyme kinetic module of SigmaPlot version 11. Results are value $\pm$ error

LC-MS/MS parameters, instrumental limits of detection and quantification, and analyte recovery from mouse liver tissue

Table 2

Analyte	Retention time (min)	MRM transition	Cone voltage (V)	Collision energy (V)	Limit of detection (nM)	Limit of quantification (nM)	Analyte recovery (%)
14,15-DHET-EA	12.56	383.0 > 285.0 (165.0)	16	12 (32)	3.4	11	163 ± 35
11,12-DHET-EA	13.22	383.0 > 285.0 (165.0)	16	12 (32)	1.7	5.7	117 ± 15
8,9-DHET-EA	14.13	383.0 > 285.0 (165.0)	16	12 (32)	2.9	9.8	88 ± 8
5,6-DHET-EA	15.13	383.0 > 285.0 (165.0)	16	12 (32)	5.4	18	167 ± 18
14,15-EET-EA	15.77	346.3 > 62.2 (285.2)	56	16 (16)	0.11	0.38	93 ± 11
11,12-EET-EA	16.17	346.3 > 62.2 (285.2)	56	16 (16)	1.6	5.4	82 ± 11
8,9-EET-EA	16.40	346.3 > 62.2 (285.2)	56	16 (16)	0.42	1.4	77 ± 14
5,6-EET-EA	16.74	346.3 > 62.2 (285.2)	56	16 (16)	0.79	2.6	11 ± 3
AEA	17.79	348.1 > 62.2	15	25			

Analytes measured were all the regioisomers of DHET-EAs and EET-EAs, extracted from tissues using AEA as an internal standard. Mass spectra were collected in multiple reaction monitoring (MRM) mode, with two analyte transitions used for quantification and qualification (shown in brackets). Limits of detection and quantification were obtained from linear regression by calculating the concentration at which the signal is three and ten times greater than the noise, respectively. Recoveries were calculated after spiking a known concentration (25 nM) of analytes into mouse liver tissue and extracted using solid-phase extraction (SPE) prior to LC-MS/MS analysis. Values were obtained from triplicates and are given as average ± standard error