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Development of a semi-automated LC/MS/MS method for the simultaneous quantitation of 14,15-epoxyeicosatrienoic acid, 14,15-dihydroxyeicosatrienoic acid, leukotoxin and leukotoxin diol in human plasma as biomarkers of soluble epoxide hydrolase activity *in vivo*

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ABSTRACT

Substrates and products of soluble epoxide hydrolase (sEH) such as 14,15-epoxyeicosatrienoic acid (14,15-EET), 14,15-dihydroxyeicosatrienoic acid (14,15-DHET), leukotoxin, and leukotoxin diol are potential biomarkers for assessing sEH activity in clinical trial subjects. To quantify them, we have developed and validated a semi-automated and relatively high-throughput assay in a 96-well plate format using liquid chromatography–mass spectrometry. 14,15-EET, 14,15-DHET, leukotoxin and leukotoxin diol, as well as their deuterium labeled internal standards were extracted from human plasma by liquid–liquid extraction using ethyl acetate. The four analytes were separated from other endogenous lipid isomers using liquid chromatography coupled with tandem mass spectrometry. The method was validated over a concentration range of 0.05–50 ng/mL. The validation results show that the method is precise, accurate and well-suited for analysis of clinical samples. The turn-around rate of the assay is approximately 200 samples per day.

1. Introduction

Soluble epoxide hydrolase (sEH) is an important enzyme in the metabolism of bioactive lipids [1–3]. sEH converts arachidonic-acid-derived epoxides such as 8,9-epoxyeicosatrienoic acid (8,9-EET), 11,12-EET and 14,15-EET to 8,9-dihydroxyeicosatrienoic acid (8,9-DHET), 11,12-DHET and 14,15-DHET, respectively [1–3] (Fig. 1). Moreover, it hydrolizes linoleic-acid-derived epoxide 9,10-epoxyoctadec-12(Z)-enoic acid or leukotoxin (LT) to leukotoxin diol (LTdiol) [4] (Fig. 1).

EETs are produced primarily by epoxygenase CYP enzymes in the vasculature endothelial cells and they are known as modulators of cardiovascular functions [5–7]. EETs act as vasodilators in a number of vascular beds [8]. They also have been shown to function as endothelium-derived hyperpolarizing factors in the coronary circulation [9]. Therefore, conversion of EETs to DHETs by sEH generally is believed to produce less desirable cardiovascular effects [10,11], although some studies suggest DHETs are also potent vasodialators [12]. LT is produced by leukocytes and is shown to be cytotoxic [13]. However, LTdiol, the hydrolysis product of leukotoxin, is found to be more cytotoxic than LT [4,14]. Consequently, inhibition of sEH may confer a cyto-protective effect. Small molecule inhibitors of sEH have been actively pursued as drug candidates for cardiovascular indications. One preclinical study showed that chronic inhibition of sEH lowered blood pressure in angiotensin-induced hypertension in rats [15]. In 2005, another study demonstrated that an oral sEH inhibitor was antihypertensive and reduced renal damage in salt-sensitive hypertension in rats [16].

Often a challenge in clinical drug development is early assessment of engaging the target mechanism. For sEH inhibitors, endogenous substrates and products of sEH-mediated metabolism can be utilized as biomarkers to assess sEH activity. Although these biomarkers are produced predominantly in certain tissues [13,17], changes in their blood/plasma levels are expected to reflect those in tissues. The presence of an active sEH inhibitor should result in an increase of epoxide/diol ratios in blood/plasma.

To test this hypothesis, a robust, sensitive and high-throughput analytical method is needed to simultaneously quantify multiple biomarkers in human plasma. A few analytical assays for analysis

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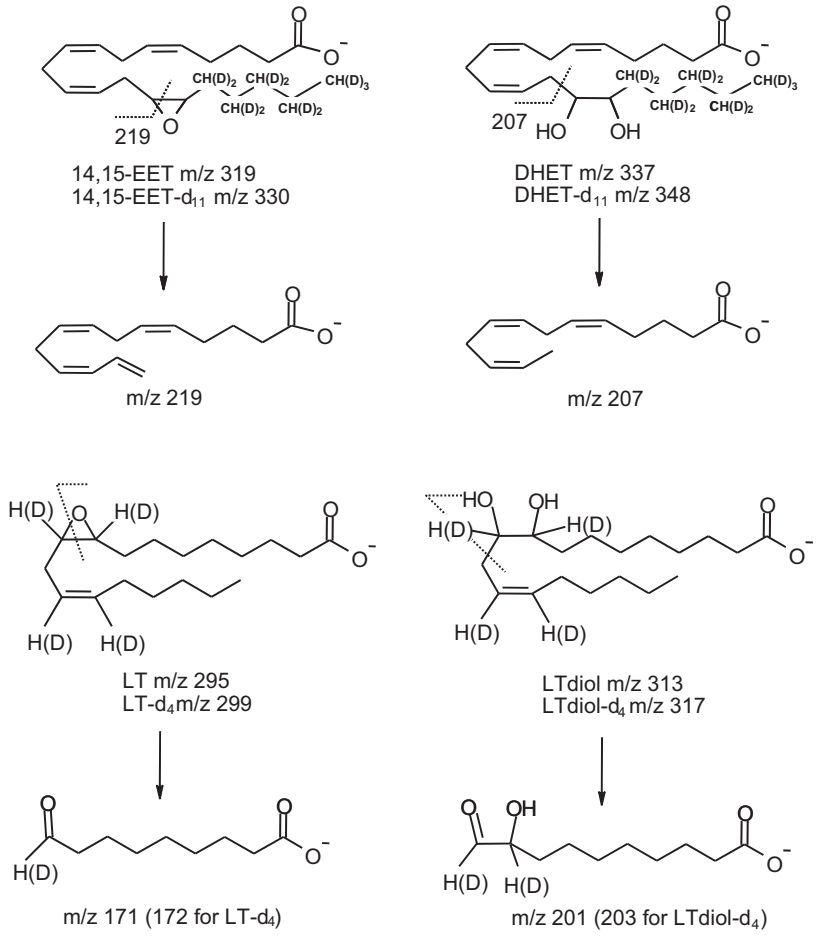

Fig. 1. Structures of parent and daughter ions (proposed) of 14,15-EET(-d_{11}), 14, 15-DHET (-d_{11}), LT (-d_4) and LT diol (-d_4).

of EETs and DHETs have been reported. Miller et al. published an ultra performance liquid chromatography (UPLC)/mass spectrometry (MS) assay for arachidonic acid metabolites including EETs and DHETs in human cerebrospinal fluid (CSF) and rat brain [18]. Newman et al. published a LC/MS method that quantifies LT, LTdiol, EETs and DHETs in human urine with lower limits of quantitation (LLOQs) at around 1 nM (0.3 ng/mL) for LT and EETs and at around 0.3 nM (0.1 ng/mL) for LTdiol and DHETs using 4 mL urine [19]. A few other LC/MS or gas chromatography (GC)/MS assays for EETs and DHETs were also reported [20–26]. Up to date, most reported methods were developed using various tissues/cell cultures or urine. Very few methods have been developed for human or animal plasma. Karara et al. reported a GC/MS method to quantify 8,9-, 11,12- and 14,15-EET in rat and human plasma [26]. Goulitquer reported another GC/MS method for analysis of EETs in the phospholipid fraction of human red blood cells and plasma with a quantification limit of 12.5 ng/mL [24]. More recently, Jianga et al. reported measurement of trans-EET in rat plasma with an LC/MS method [27]. To our knowledge, most of the published plasma methods use long gradients (>30 min) and labor intensive sample preparation processes. Therefore the sample throughput is limited and not suitable for analysis of clinical samples. In addition, given the low level of EET (~0.1 ng/mL) in human plasma [26], it is a challenge to achieve a lower limit of quantitation (LLOQ) below 0.1 ng/mL.

In this study we have developed and validated a semi-automated, relatively high-throughput and sensitive LC/MS assay in 96-well plate format for simultaneous quantitation of 14,15-EET, 14,15-DHET, LT and LTdiol in human plasma. The robustness of this method was demonstrated through a three-day validation over the range of 0.05–50 ng/mL and proved to be sufficient for analysis of these biomarkers from human plasma with a turn-around rate of approximately 200 samples per day.

2. Experimental

2.1. Chemicals and reagents

LT, LTdiol, LT-d_4, LTdiol-d_4, 14,15-EET, 14,15-DHET, 14,15-EET-d_{11}, 14,15-DHET-d_{11} were purchased as 0.1 mg/mL solution in methyl acetate or ethanol from Cayman Chemicals (Ann Arbor, MI). Formic acid, HPLC grade water, ethyl acetate, hexane, isopropanol and acetonitrile, were purchased from Sigma Aldrich (St. Louis, MO).

2.2. Equipment

An Eppendorf 5810R centrifuge with a rotor capacity for four 96-well plates (Brinkmann Instrument, Westbury, NY) was used for sample centrifugation. A TomTec Quadra 3 SPE (Tomtec Inc, Hamden, CT) was used for liquid transfer. Arctic White LLC 96-well round 2-mL plates with silicone and PTFE film seal mats (Arctic White LLC, Bethlehem, PA) were used for the liquid–liquid extraction (LLE). Waters 1 mL 96-well round collection plate (Waters, Milford, MA) and Arctic White Seal 96-well silicone plate covers (Arctic White LLC, Bethlehem, PA) were used for sample reconstitution and injection. A CTC PAL auto sampler (Leap Technologies, Carrboro, NC) was used for sample injection and an Agilent 1100

(Agilent, Palo Alto, CA) was used for HPLC separation. An API-4000 mass spectrometer (Applied Biosystems, Bedford, MA) was used for sample analysis.

2.3. Preparation of standards and quality control (QC) samples

Stock solutions of LT, LTdiol, LT-d_4, 9,10-LTdiol-d_4, 14,15-EET, 14,15-DHET, 14,15-EET-d_{11} and 14,15-DHET-d_{11} at 0.1 mg/mL were stored at −20 °C. The stock solutions of LT, LTdiol, 14,15-EET and 14,15-DHET were combined and diluted with 40/60 acetonitrile/water to make the working solution 1 (WS1) containing 10 μg/mL of each analyte. WS1 was further diluted with 40/60 acetonitrile/water to make working solutions WS2 and WS3 at a concentration 0.5 and 0.05 μg/mL, respectively. Standards and QC samples were prepared in 3/97 acetonitrile/water. WS1 was used to prepare standards at 10, 20 and 50 ng/mL and QC samples at 10, 50 ng/mL. WS2 was used to prepare standards at 0.5, 1, 2, and 5 ng/mL and QC samples at 0.5 ng/mL. WS3 was used to prepare standards at 0.05, 0.1, and 0.2 ng/mL and QC samples at 0.05 and 0.1 ng/mL. QC samples were divided into three aliquots of 5 mL each. Two aliquots were frozen at −80 °C and analyzed in validation Day 2 and Day 3. In the first validation run, freshly prepared QC samples were analyzed against freshly prepared calibration standards. For each subsequent validation run, frozen aliquots of the QC samples were thawed at room temperature and analyzed against freshly prepared calibration standards.

2.4. Sample extraction

A volume of 0.7 mL of blank (3/97 acetonitrile/water), standard, QC, or unknown plasma sample was aliquoted into 2 mL 96-well Arctic White extraction plate for LLE. The sample volume of 0.7 mL was chosen based on the plate capacity to allow sufficient sample mixing for an optimal LLE extraction. An aliquot of 25 μL deuterium labeled internal standard (ISD) mixture (100 ng/mL LT-d_4, 50 ng/mL 9-10-LTdiol-d_4, 200 ng/mL 14,15-EET-d_{11} and 200 ng/mL 14,15-DHET-d_{11}) in 40/60 acetonitrile/water was added to each well and gently vortex-mixed. The amount of ISD added was carefully chosen to achieve sufficient signal/noise ratio and negligible contribution to the unlabeled MRM channels due to the presence of impurities. After sample mixing, 0.7 mL of ethyl acetate was added to each well and the plate was sealed with a silicone and PTFE film seal mat. The plate was then vigorously vortex-mixed for 5 min and then centrifuged at 3100 × *g* for 3 min. The supernatants were transferred into a 96-well collection plate with TomTec Quadra 3 SPE. The LLE extraction was repeated for three times and supernatants from the three extractions were combined and dried down under nitrogen flow at room temperature. The samples were reconstituted in 70 μL 40/60 acetonitrile/water.

2.5. Chromatographic conditions

An Agilent 1100 HPLC system and a Kinetex 2.6 μm C18 100 Å 50 mm × 4.6 mm HPLC column (Phenomenex, Torrance, CA) were used for HPLC separation. The column was kept at ambient temperature (23 °C). Mobile phase A was 0.1% formic acid in water and mobile phase B was acetonitrile. The flow rate was 0.45 mL/min and LC gradient was as follows: 40% B for at 0 min, 45% B at 0.5 min, 60% B at 2.2 min, 80% B at 4 min, 95% B at 4.01 min, 95% B at 5 min, 40% B at 5.01 min and 40% B at 6.3 min. A typical injection volume was 10 μL using partial loop injection mode.

2.6. Mass spectrometric conditions

A triple quadrupole mass spectrometer API-4000 with an electrospray ionization interface (ESI), operating in negative mode was used. The instrument was optimized by infusing a 0.1 μg/mL solution of LT, LTdiol, 14,15-EET and 14,15-DHET in acetonitrile at 10 μL/min with a flow of 0.45 mL/min 50/50 0.1% formic acid in water/acetonitrile from the Agilent 1100 pump into the mass spectrometer. The multiple-reaction-monitoring (MRM) transitions of m/z 295 → 171, m/z 313 → 201, m/z 319 → 219, and m/z 337 → 207 were chosen for LT, LTdiol, 14,15-EET and 14,15-DHET, respectively. The MRM transitions of m/z 299 → 172, m/z 317 → 203, m/z 330 → 219, and m/z 348 → 207 were chosen for LT-d_4, 9-10-LTdiol-d_4, 14,15-EET-d_{11} and 14,15-DHET-d_{11}, respectively. The MRM transitions were selected based on signal to noise ratio and selectivity. The proposed fragmentation pathways are shown in Fig. 1. MRM transitions for the early eluting LTdiol, 14,15-DHET and their ISDs were acquired from 0 to 2.8 min. MRM transitions for the late eluting LT, 14,15-EET and their ISDs were acquired from 2.8 to 4.8 min. The dwell times were 150 and 90 ms for the analytes and the ISDs, respectively. The optimized mass spectrometric conditions were: ion source temperature at 500 °C; ion spray voltage at −5000 V; curtain gas at 20 psi (nitrogen); nebulizing gas at 80 psi (zero air); TIS gas at 60 psi (zero air); collision energy at −23, −29, −16, −24 eV for LT, LTdiol, 14,15-EET and 14,15-DHET, respectively. The instrument parameters for the deuterium labeled ISDs were the same as the unlabeled compounds.

2.7. Data analysis

Data were acquired and processed using Analyst™ (Version 1.4.2, Applied Biosystems/MDS Sciex, Canada). Calibration plots of analyte/ISD peak area ratios and analyte concentrations were constructed and a weighted $1/y^2$ linear regression was applied. Concentrations in QC and unknown samples were determined from the appropriate calibration lines and bias and precision of the method was calculated from measured concentrations of QC samples. Precision was calculated as the standard deviation of measured concentrations from six repeats. Bias was calculated as the difference between the average of measured concentrations and the target QC concentrations.

3. Results and discussion

3.1. Development of the extraction method

To achieve sufficient sensitivity for quantification of all four analytes in human plasma, an efficient and reliable sample extraction method was required. Both solid phase extraction (SPE) and LLE were evaluated during the assay development process. The method for SPE was optimized using Waters Oasis HLB 30 mg SPE plate with 5/95 methanol/water wash and with elution conditions ranging from 50% to 100% acetonitrile in water under both acidic and neutral conditions. LLE was tested using ethylacetate and 98/2 hexane/isopropanol under both acidic and neutral conditions. Since the 14,15-EET and LT were found to be slightly unstable under acidic condition, extraction recovery was only accessed under neutral condition. Due to the presence of endogenous analytes in human plasma, the extraction recovery was evaluated using the deuterium labeled compounds. LLE with ethyl acetate under neutral condition was found to provide the greatest extraction recovery (>70% for all four analytes) and the best sample clean-up (data not shown), and therefore was chosen as the final extraction procedure.

3.2. LC optimization

Since all four analytes were analyzed in one run, optimized LC conditions were required to ensure sufficient separation from

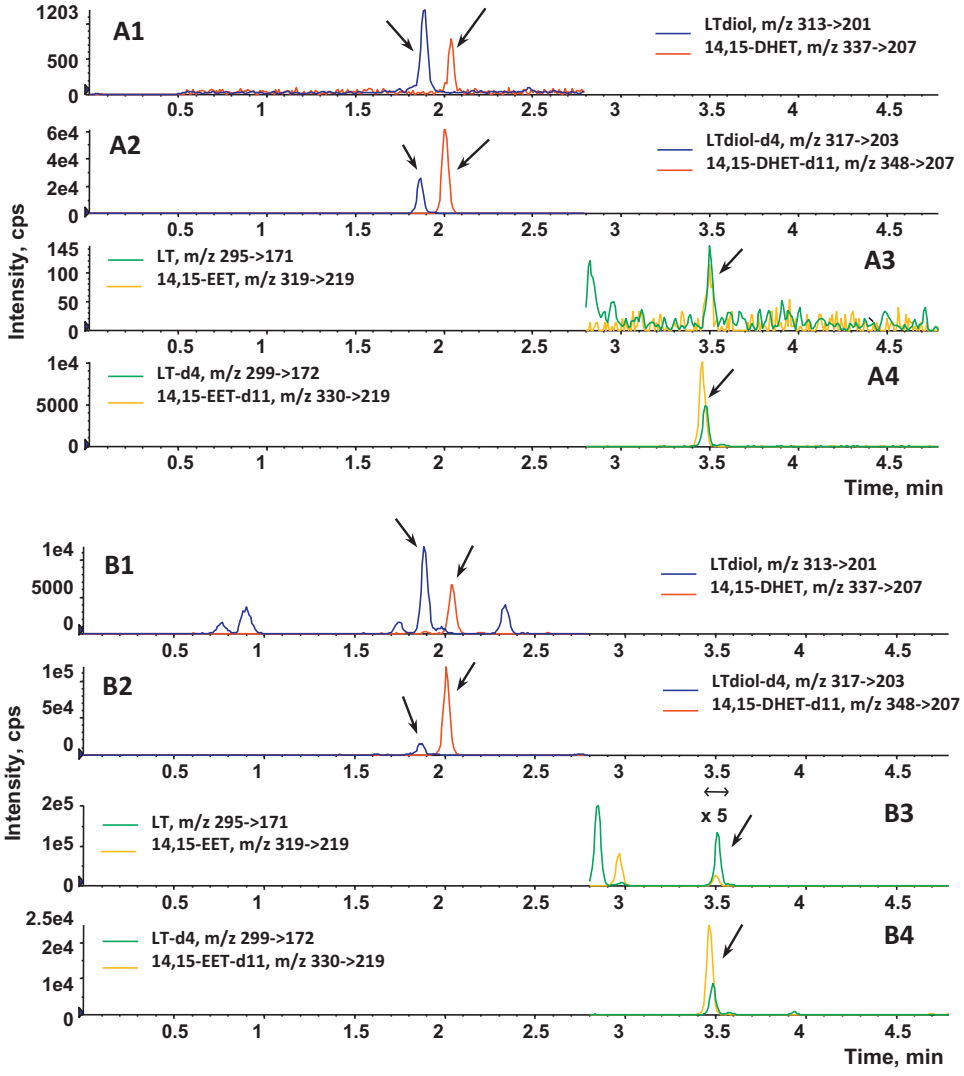

Fig. 2. Chromatograms of all four biomarkers in a LLOQ sample at 0.05 ng/mL (A) and a human plasma sample (B). (1) LTdiol (blue) and 14,15-DHET (red). (2) LTdiol-d_4 (blue) and DHET-d_{11} (red). (3) LT (green) and 14,15-EET (yellow). (4) LT-d_4 (green) and 14,15-EET-d_{11} (yellow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

endogenous interferences. In addition, a relatively short run-time was needed ensure sufficient sample turn-around. Mobile phases were chosen to be 0.1% formic acid in water and acetonitrile. Neutral conditions were also investigated. However the signal-to-noise ratio was lower under neutral conditions compared to acidic conditions. HPLC columns from many commercial sources were evaluated for chromatographic separation. The Phenomenex Kinetex 2.6 μm C18 (100 A 50 mm × 4.6 mm) column was chosen due to high resolution, good peak shape, and short run-time (6 min cycle). LTdiol and 14-15-DHET were baseline separated using this column. Peaks for LT and 14,15-EET could not be baseline resolved; however, all four analytes were separated from endogenous interference peaks (Fig. 2B1 and B3).

3.3. Matrix effect assessment

Since human plasma contains detectable amounts of the investigated analytes, the standard curve was prepared in a solution of 3/97 acetonitrile/water. Because a surrogate matrix was used, the impact of matrix effect was investigated using another set of standard curves prepared in pooled control human plasma (Bioreclamation, Hicksville, NY). As shown in Fig. 3, the standard curves prepared in human plasma were parallel to those prepared in solution. In addition, when the standard curves prepared in human plasma were back extrapolated, their intercepts on axis *x* were identical to the concentrations of the four analytes measured using the standard curve prepared in solution. These results suggested that calibration curves prepared in solution were suitable for analysis of plasma samples.

A thorough matrix effect investigation was also conducted. A mixture of all four analytes and their corresponding ISDs were post-column infused into the mass spectrometer after an injection of 10 μL methanol or control human plasma extract. The signal intensities for the four analytes and the corresponding ISDs were plotted against time as shown in Fig. 4. The traces for all four analytes and ISDs obtained after a blank injection overlapped well with those after a plasma-extract injection in regions where the target analytes eluted. In addition, the analyte/IS ratios (Fig. 4, secondary *y* axis) remained constant and were identical between blank injection and plasma extract injection, indicating that the labeled ISDs corrects for any matrix effect that may occur. The small deviations at ~3.2 and ~3.9 min in the LT analyte/IS ratio trace were due to the presence of a large endogenous interference peak and the endogenous LT peak, respectively.

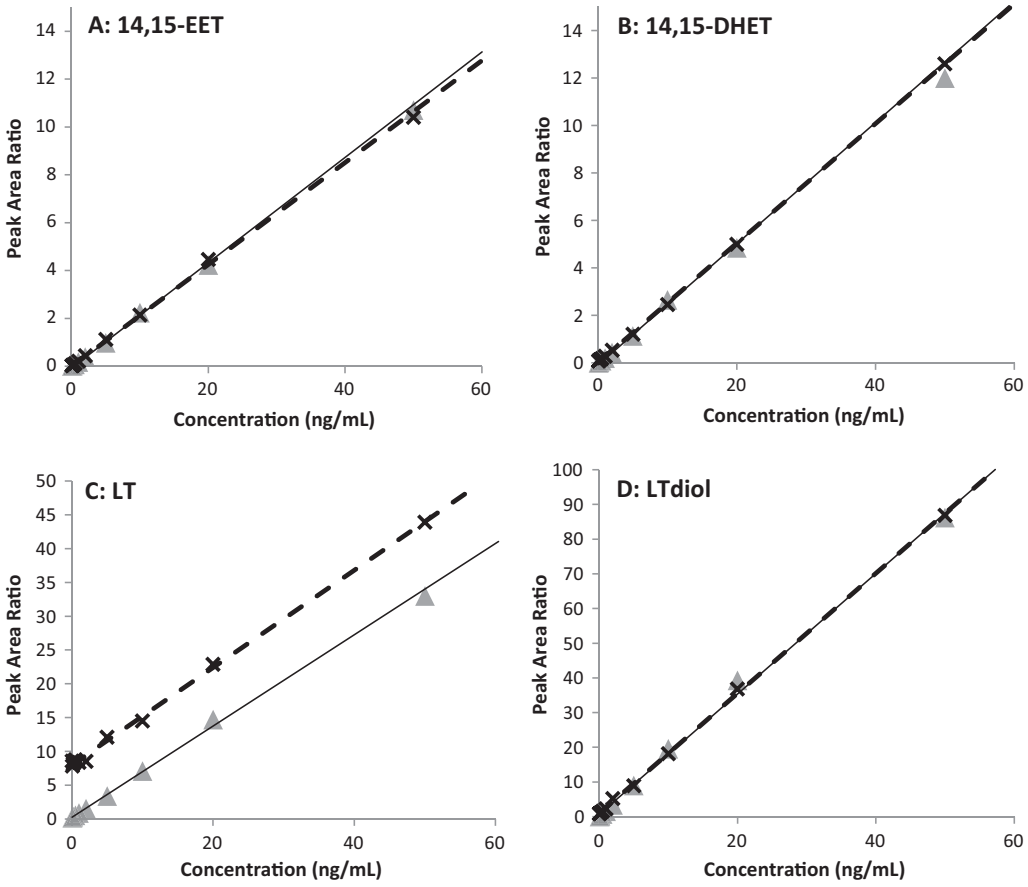

Fig. 3. Standard curves prepared in neat solution vs in human plasma. Neat solution: grey triangle, solid line represents linear regression of data. Human plasma: black cross, dotted line represents linear regression of data.

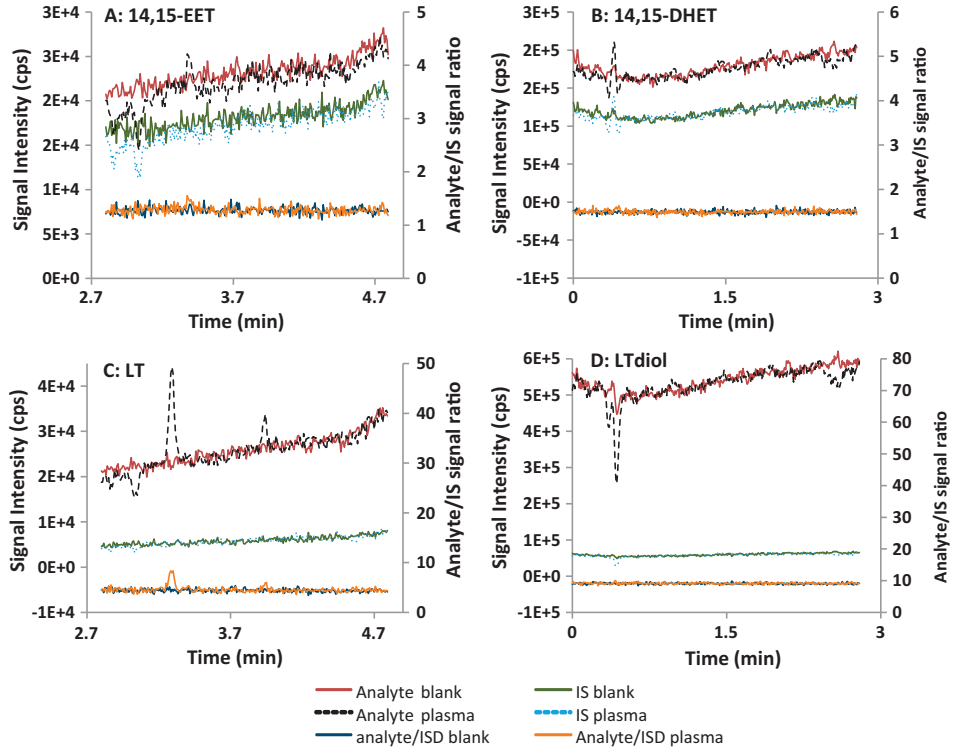

Fig. 4. Assessment of matrix effect by post-column infusion of mixtures of four biomarkers and their corresponding deuterium labeled ISDs after injection of a plasma extract sample or a solvent blank. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.4. Selectivity, linearity and performance

Since human plasma samples contain detectable levels of the four analytes, it is a challenge to demonstrate that the method is selective. The selectivity of the method was demonstrated by analysis and evaluation of an incurred human plasma sample using a much shallower gradient. The use of the shallow gradient resulted in a significantly increase in peak retention and resolution. The peak resolutions between LTdiol and DHET as well as between LT and the preceding endogenous peak (~2.8 min in Fig. 2) were more than doubled for the shallow gradient as compared to the regular gradient (data not shown). In the mean time, the peak area ratios (Analyte/ISD) from the shallow gradient were identical to those obtained from the regular LC gradient (data not shown), indicating that the selectivity of the method is satisfactory. The linearity of the method was evaluated by analyzing 10 calibration standards in duplicate over a range of 0.05–50 ng/mL. The R^2 value obtained from a $1/y^2$ weighted regression was better than 0.995 for all four analytes. At all QC concentrations examined, the bias and precision (%CV) values were less than 15% across the three-day validation for all target analytes (Table 1).

3.5. Stability during freeze–thaw cycles

The freeze–thaw stability of all four analytes were assessed by subjecting QC samples at 0.1, 0.5 and 10 ng/mL concentrations to three freeze–thaw cycles from −80 °C to room temperature and then comparing the mean concentrations against those of the freshly prepared samples in replicates of three. In this experiment the percent biases were less than 10% and %CVs were less than 6% for all four analytes at all three concentrations. In addition, the freeze–thaw stability of all four analytes in pooled blank human plasma was also assessed in replicate of three. The %CV from this experiment was less than 9% for all four analytes. Therefore, the four analytes are stable for at least three freeze–thaw cycles.

3.6. Stability in processed samples

The stability of analytes in extracted human plasma was assessed by re-injecting validation samples after storage at 23 °C for 20 h. The accuracy and precision of these samples were found to be acceptable (less than 15%) and no change in sensitivity was observed, indicating that processed samples are stable when stored in the autosampler at 23 °C for at least 20 h.

3.7. Quantitation of the four analytes in plasma samples from healthy subjects

The four analytes were quantified from human plasma samples obtained from 12 healthy subjects (7 males and 5 females). The plasma samples were obtained from Bioreclamation Inc (Westbury, NY, USA). Human plasma was collected following a standard operating procedure at Bioreclamation that ensured ethical approval, donor consent and sample integrity and the samples were used in accordance with current GSK policies (POL-GSL-410 – Standards for Collecting, Obtaining and Using Human Biological Samples in Research). Mean concentration and standard error (SE) were calculated for each analyte. The levels of LT were 1.09 ± 1.10 ng/mL (mean ± SE) with a range from 0.393 to 4.36 ng/mL. The levels of LTdiol were 4.05 ± 5.41 ng/mL (mean ± SE) with a range from 1.15 to 20.6 ng/mL. The levels of EET were 0.101 ± 0.028 ng/mL (mean ± SE) with a range from 0.055 to 0.143 ng/mL. The levels of DHET were 0.206 ± 0.044 ng/mL (mean ± SE) with a range from 0.147 to 0.292 ng/mL.

Table 1
Mean, precision and bias for QC samples.

Conc. (ng/mL)	14,15-EET					14,15-DHET					9,10-LT					9,10-LTdiol				
	0.05	0.1	0.5	10	50	0.05	0.1	0.5	10	50	0.05	0.1	0.5	10	50	0.05	0.1	0.5	10	50
Run 1 (n = 6)																				
Mean	0.0493	0.111	0.561	11.4	51.3	0.0489	0.103	0.527	10.5	48.1	0.0491	0.107	0.546	11.2	52.0	0.0453	0.101	0.519	10.3	48.9
%CV	14.7	4.45	2.70	1.82	2.71	2.88	4.10	1.32	0.500	3.45	13.9	7.22	5.07	3.37	3.10	4.67	4.71	1.87	1.38	5.27
%Bias	−1.48	10.6	12.2	13.8	2.53	−2.22	2.74	5.39	4.77	−3.82	−1.73	7.46	9.12	4.09	4.09	−9.36	0.79	3.86	2.77	−2.37
Run 2 (n = 6)																				
Mean	0.0518	0.107	0.502	9.76	48.7	0.0518	0.111	0.507	9.74	49.6	0.0499	0.104	0.483	9.70	48.5	0.0521	0.112	0.511	9.83	51.2
%CV	10.2	6.90	3.15	6.96	4.67	7.50	3.44	6.13	4.30	3.34	14.7	11.6	3.15	5.01	7.95	6.99	2.52	2.52	3.83	4.41
%Bias	3.56	6.93	0.370	−2.40	−2.58	3.66	10.7	1.49	−2.63	−0.74	−0.250	3.66	−3.37	−3.02	−3.08	4.27	11.7	2.11	−1.73	2.45
Run 3 (n = 6)																				
Mean	0.0556	0.106	0.485	9.76	49.3	0.0563	0.108	0.491	9.99	54.99	0.0512	0.0980	0.468	9.64	51.2	0.0570	0.110	0.494	9.94	54.5
%CV	5.80	11.5	5.28	3.39	5.39	4.65	4.12	4.94	3.83	4.86	5.70	8.43	4.03	2.85	6.26	14.4	14.7	2.41	2.69	5.66
%Bias	11.1	6.45	−2.97	−2.36	−1.48	12.5	8.25	−1.78	−0.07	9.99	2.36	−1.54	−6.47	−3.56	2.33	14.1	10.3	−1.21	−0.64	8.93
Total																				
Mean	0.0522	0.108	0.516	10.3	49.8	0.0528	0.107	0.509	10.1	50.9	0.0501	0.103	0.499	10.2	50.5	0.0515	0.108	0.508	10.0	51.5
%CV	11.1	7.54	7.41	8.62	4.66	8.39	4.89	5.21	4.46	7.07	11.8	9.40	8.03	8.18	6.46	13.7	9.81	3.03	3.29	6.67
%Bias	4.40	7.54	3.20	2.97	−0.490	5.61	7.31	1.71	0.756	1.81	0.167	3.15	−0.220	1.85	1.09	3.01	7.51	1.63	0.128	3.04

4. Conclusions

A semi-automated 96-well format LLE extraction-LC/MS method was developed and validated for quantification of four biomarkers LT, LTdiol, 14,15-EET and 14,15-DHET over a range of 0.05–50 ng/mL. The method is accurate and selective and provides a relatively high throughput of approximately 200 samplers per day. The method also was partially validated in other matrixes such as plasma (rat, dog and monkey), blood (rat and human), and urine (human, dog and monkey) (data not shown). The high sensitivity and high throughput makes this method suitable for supporting future human clinical studies. The results from these studies will help provide early assessments on mechanism engagement of sEH inhibitors in human clinical trials.

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