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The development of methodology for clinical measurement of 5-lipoxygenase pathway intermediates from human peripheral blood mononuclear cells

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ABSTRACT

Recent studies have shown a correlation between 5-lipoxygenase (5-LO) pathway up-regulation and cardiovascular risk. Despite the existence of several assays for products of the 5-LO pathway, a reliable method for clinical determination of 5-LO activity remains to be established. In the present communication, we report conditions that allow measurement of 5-hydroxyeicosatetraenoic acid (5-HETE) and leukotriene B_4 (LTB_4) in peripheral blood mononuclear cells (PBMCs) isolated from the blood of atherosclerosis patients before and after stimulation by the calcium ionophore, A23187. LTB_4, a potent mediator of inflammation-linked cardiovascular disease, was measured using an existing competitive enzyme immunoassay (EIA) kit after making specific methodological improvements that allowed PBMCs to be used in this format for the first time. LTB_4 was also measured by LC/MS/MS along with 5-HETE, a direct by-product of the action of 5-LO on arachidonic acid and a molecule for which no commercial EIA kit exists. The LC/MS/MS assay was validated over a range of 0.025–25 ng/mL for LTB_4 and 0.1–25 ng/mL for 5-HETE. The EIA method has a validated range covering 0.025–4 ng/mL. When both assays were applied to analyze LTB_4 from stimulated PBMCs isolated from 25 subjects with various degrees of atherosclerosis, a high correlation was obtained ($r = 0.9426$, Pearson's correlation coefficient). A high correlation was also observed between the levels of LTB_4 and 5-HETE measured by LC/MS/MS after ionophore stimulation ($r = 0.9159$). Details are presented for optimized sample collection, processing, storage, and analysis in accordance with the logistical demands of clinical analysis.

1. Introduction

5-Lipoxygenase (5-LO) is the primary enzyme controlling the synthesis of leukotrienes (LTs). LT biosynthesis is initiated by activation of cytosolic phospholipase A_2 ($cPLA_2$), which releases arachidonic acid (AA) from membrane glycerophospholipids (Fig. 1) [1]. The enzyme 5-LO subsequently transforms AA into LTs through two distinct catalytic steps. The first involves hydrogen abstraction from the allylic position at carbon-7 of AA followed by addition of molecular oxygen to form 5-hydroperoxyeicosatetraeinoic acid (5-HPETE). This step is facilitated by the integral nuclear envelope protein 5-LO-activating protein (FLAP) [2], believed to act by orienting AA for presentation to 5-LO [3]. The second step involves hydrogen abstraction from position-10 resulting in formation of the conjugated triene epoxide, LTA_4. LTA_4, is ultimately converted to either LTB_4 or cysteinyl leukotrienes (CysLTs) by the respective action of LTA_4 hydrolase or LTC_4 synthase (Fig. 1) [1]. A detailed account of the biosynthesis and metabolism of leukotrienes can be found in a recent review by Murphy and Gijon [4].

Recent genetic studies have identified variants of the 5-LO gene promoter and the FLAP gene as factors for elevated risk of myocardial infarction and stroke [5–8]. Stimulated neutrophils isolated from individuals carrying these variants produced more LTB_4 than from normal subjects and implied a role for unregulated leukotriene levels in inflammation of the arterial wall. LTB_4, in addition to its evolving role in atherosclerosis, is known to be a potent leukocyte chemoattractant [9–13], and has been implicated in a

Abbreviations: 5-LO, 5-lipoxygenase; 5-HETE, 5-hydroxyeicosatetraenoic acid; 5-HPETE, 5-hydroperoxyeicosatetraeinoic acid; AA, arachidonic acid; $cPLA_2$, cytosolic phospholipase A_2; CPT, cell preparation tubes; CV, coefficient of variation; CysLTs, cysteinyl leukotrienes; DMSO, dimethylsulfoxide; EIA, enzyme immunoassay; FA, formic acid; FLAP, 5-lipoxygenase activating protein; LLOQ, lower limit of quantitation; LTA_4, leukotriene A_4; LTB_4, leukotriene B_4; MTBE, methyl tert-butyl ether; NSB, non-specific binding; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; QC, quality control; RE, relative error; SRM, selected reaction monitoring; TA, total activity; ULOQ, upper limit of quantitation.

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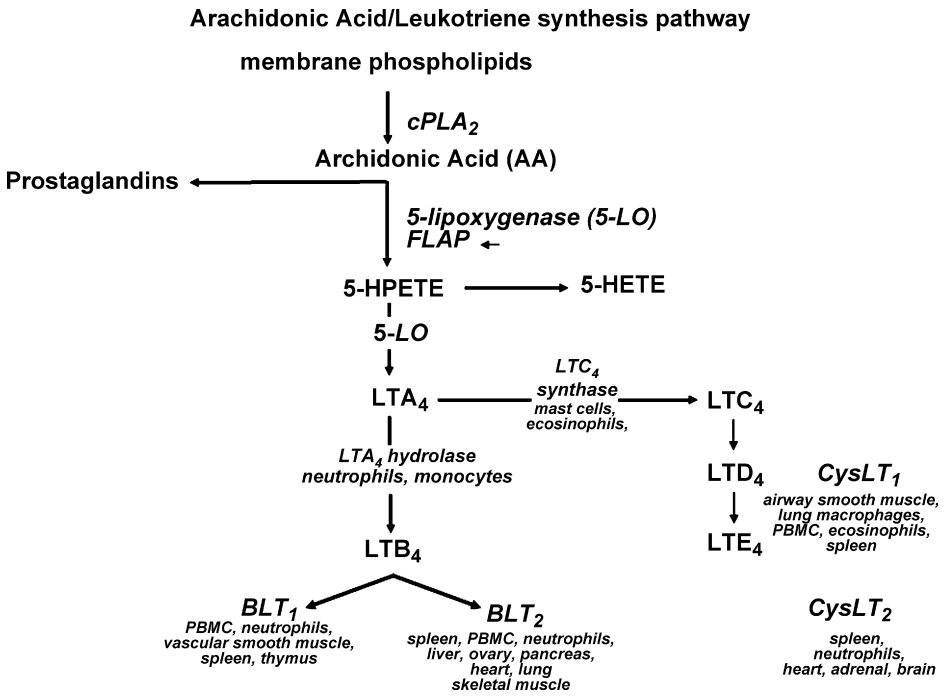

Fig. 1. Schematic diagram of the 5-lipoxygenase pathway showing the biosynthesis of leukotrienes from arachidonic acid.

number of chronic inflammatory diseases which include asthma, allergy and ulcerative colitis [9,13–16].

Several metabolites can be measured as potential indicators of 5-LO pathway activity (Fig. 1). Downstream metabolites, such as LTB_4 and LTC_4, provide important information, but do not give a direct indication of 5-LO activity because of the participation of additional enzymes. The metabolite 5-HPETE offers a more direct readout of 5-LO activity, although its breakdown product, 5-hydroxyeicosatetraenoic acid (5-HETE), is typically measured as a surrogate owing to improved stability. It should be mentioned that a pseudoperoxidase has been identified which can produce 5-HETE from 5-HPETE [17]. In the present investigation LTB_4 and 5-HETE were selected as target analytes to develop methods that will ultimately be used for clinical pharmacodynamic evaluation and to investigate the potential to stratify patients according to myocardial risk.

An enzyme immunoassay (EIA) method for LTB_4 was developed through modifications to a commercial kit as described herein. Unfortunately, because this method relies on a competitive format, concern was expressed over assay selectivity and robustness. As a result of these issues and the potential need to monitor additional metabolites, reversed phase LC/MS/MS using negative ion electrospray ionization (ESI) was also investigated. As demonstrated by previous investigators, LC/MS/MS offers several advantages including selectivity, dynamic range, and has been used to simultaneously profile multiple eicosanoid pathways [18,19].

In this communication, we report methodology for clinical sample collection, processing, storage, and analysis. For the results presented, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using sodium heparin cell preparation tubes (CPTs). PBMCs were investigated based on relative ease of isolation and because multiple leukocytes are represented. The supernatant from washed cells was split and half of the samples were stimulated with calcium ionophore. Aliquots of both sample sets were stored frozen prior to analysis by EIA and LC/MS/MS. Results are presented from the validation of both methods along with a successful cross-validation for LTB_4 demonstrated by analysis of a common sample set. Both methods are currently being used in an expanded investigation involving genotypic profiling to test our ability to stratify atherosclerosis patients based on 5-LO pathway activity.

2. Materials and methods

2.1. Materials for EIA

All human blood samples were both obtained in accordance with the Eli Lilly and Company Biological Donor Program and with informed consent of all study participants. sodium heparin CPTs were purchased from Becton Dickenson, Vacutainer # 362753. LTB_4 EIA kits were purchased from Assay Designs (Leukotriene B_4 enzyme immunoassay kit, Cat. # 901-068). 1X Dulbecco's phosphate-buffered saline (DPBS) was from Gibco, Cat. # 14040. Albumin from bovine serum, minimum 98% was purchased from Sigma, # A3803 and dimethylsulfoxide (DMSO) was from ATCC, # 4-X.

2.2. Materials for LC/MS/MS

The following standard materials were purchased from Cayman Chemical (Ann Arbor, MI) and were obtained as ethanol stock solutions: 5(S)-HETE, 5(S)-HETE-d_8, 8(S)-HETE, 12(S)-HETE, 15(S)-HETE, LTB_4, LTB_4-d_4, 6-trans-LTB_4, 12-epi LTB_4, and 6-trans-12-epi LTB_4. Stock solutions were prepared by dilution in acetonitrile and stored in silanized amber glass vials at −20 °C. HPLC grade water, acetonitrile, and methanol along with methyl tert-butyl ether (MTBE), and formic acid (FA) were purchased from EM Science (Gibbstown, NJ). Monobasic sodium phosphate, sodium hydroxide (1 M), and phosphate-buffered saline (PBS) were all obtained from Sigma Chemical (St. Louis, MO).

2.3. Sample collection and storage of PBMC

Whole blood (5–7 mL) was collected directly into a CPT and centrifuged for 30 min (VanGuard V6500, 3400 rpm, ~1318XG) at room temperature. The tube was inverted several times to mix

the cells and the plasma was transferred to a 15 mL polypropylene tube. After an additional centrifugation (2000 rpm, 4 °C, 5 min), the supernatant was carefully aspirated with a vacuum apparatus, and the PBMCs were washed by vortexing with 500 μL of PBS containing 0.1% BSA, followed by centrifugation as before. This process was repeated twice; after the second wash, 1000 μL PBS + 0.1% BSA were added to the pellet, followed by gentle vortexing to loosen the cells. The sample was then divided into two equal aliquots in clear screw top siliconized glass tubes. One tube received Calcimycin A23187 (Sigma, CAS 52665-69-7) to a final concentration of 1 μM for stimulation of LTB_4. To the other was added an equal volume of solvent as a control. The tubes were capped and incubated for 2 h in a shaking Dubnoff water bath at 37 °C. Following incubation, the cells were pelleted by centrifugation (2000 rpm, 4 °C, 5 min). The supernatants were transferred to 1.5 mL polypropylene microcentrifuge tubes and stored at −20 °C.

2.4. EIA

The EIA from Assay Designs (Ann Arbor, MI) was performed in general accordance with the manufacturer's instructions. Briefly, all reagents were brought to room temperature, and all standards and samples were run in duplicate. To the appropriate wells were added 100 μL aliquots of standard diluent, samples or standards nos. 1–7 (the manufacturer calls for a five-point standard curve). 50 μL aliquots of assay buffer were added to the non-specific binding (NSB) wells. Then, 50 μL aliquots of LTB_4 EIA Conjugate, Cat. # 80-0102 (blue conjugate), were added to each well, except the total activity (TA) and blank wells, followed by 50 μL of LTB_4 EIA Antibody, Cat. # 80-0624 (yellow antibody), which was added to all wells except the blank, TA and NSB wells. The manufacturer's recommendations call for a 2 h incubation at room temperature on a plate shaker at ∼500 rpm. We compared this with an overnight incubation (18–20 h) at room temperature at ∼500 rpm. After incubation, the wells were aspirated and washed 3× with 400 μL of Wash Buffer, Cat. # 80-1287 (Tris-buffered saline plus detergents) per cycle. The plates were then tapped on a lint-free paper towel and 5 μL of blue conjugate were added to the TA wells. Then, 200 μL of pNpp Substrate, Cat. # 80-0076, were added to every well; the plate was sealed (Costar, Cat. # 4361) and incubated for 2 h at 37 °C. After the addition of an aqueous solution of trisodium phosphate, Cat. # 80-0248, (stop solution), the plate was read immediately at 405 nm, with correction between 570 and 590 nm in a Molecular Devices SpectraMax 384 plate reader.

SigmaPlot, version 8.0 was used for fitting of the calibration curves for the LTB_4 EIA. Data from both the EIA and LC/MS/MS were analyzed and plotted using the program FigP (Biosoft, St. Louis, MO). Statistical analysis was performed using the same program.

2.5. Sample preparation for LC/MS/MS analysis

Samples of washed PBMC supernatant (200 μL) were extracted by semi-automated liquid–liquid extraction performed using a Tomtec Quadra 96 liquid handling system. QC and standard samples were prepared in a proxy matrix (PBS + 0.1% BSA) also extracted by the same procedure. Internal standards (500 pg of 5(S)-HETE-d_8 and LTB_4-d_4) were added to each sample in a volume of 150 μL (PBS + 0.1% BSA). A volume of 2 × 200 μL MTBE was used for extraction. The organic phases were combined, dried under nitrogen, and reconstituted in 100 μL water/acetonitrile/formic acid (1:1:0.004, v/v/v) for LC injection. Eight-point standard curves were analyzed for both 5-HETE (range: 0.1–25 ng/mL) and LTB_4 (range: 0.025–25 ng/mL). Validation quality control samples were prepared at concentrations of 0.25, 12.5, and 25 ng/mL for LTB_4, and 0.10, 12.5 and 25 ng/mL for 5-HETE. These samples were used to assess stability (room temperature, long-term storage, and freeze–thaw). Stability experiments were also conducted using a QC sample prepared from unstimulated washed PBMCs at a concentration approximately 10 times higher than the determined baseline level for both LTB_4 and 5-HETE (i.e. spiked with 1.0 ng/mL). This pooled sample was also used to assess bias introduced through the use of a proxy matrix for standard preparation.

2.6. LC/MS/MS quantitation of 5-HETE and LTB_4

Quantitation of LTB_4 and 5-HETE was performed by LC/MS/MS using a Sciex API-4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). Analysis was conducted in negative ion mode using pneumatically assisted ESI. Quadrupoles Q1 and Q3 were both operated under nominal mass resolution. Quantitation occurred by stable isotope dilution using deuterated internal standards. MS analysis occurred in the selected reaction monitoring (SRM) mode using nitrogen as the collision gas and a dwell time of 200 ms per transition. The SRM transitions for LTB_4 and LTB_4-d_4 were m/z 335.2 → 197.1 and 339.2 → 197.1, respectively. The corresponding SRM transitions for 5-HETE and 5-HETE-d_8 were m/z 319.2 → 115.1 and 327.2 → 116.1, respectively. The applied declustering potential was analyte-specific and ranged from 50 to 120 V. For all analytes, a collision energy of 22 eV and a collision gas setting of 5 was used. The Turbo Ion Spray™ gas settings applied were 30 and 60 (gas 1 and gas 2, respectively).

Reversed phase HPLC was conducted using a YMC (2.1 × 150 mm) ODS AQ 3 μm column (Thermo Fisher, Pittsburgh, PA) operated at 60 °C. Gradient elution was performed at a flow rate of 0.3 mL/min using an Agilent 1100 Liquid Chromatograph. Mobile phase A consisted of 0.1% FA in water (v/v). Mobile phase B consisted of 0.1% FA in methanol (v/v). The following gradient was applied (min/%B): 0.1/25, 0.3/60, 10/90, 11.5/100, 12/100, 12.5/25, 14/25. Under these conditions, LTB_4/LTB_4-d_4 and 5-HETE/5-HETE-d_8 were retained for 8.3 and 11.2 min, respectively. Sample injection (10–20 μL) occurred using a CTC Pal HTS autosampler (Leap Technologies, Carrboro, NC). Prior to injection, the samples were kept at 10 °C in a shallow-well polypropylene plate.

Calibration curves were prepared by spiking standards for 5-HETE and LTB_4 into proxy matrix along with fixed concentrations of the stable isotope labeled internal standards, 5-HETE-d_8 and LTB_4-d_4. Calibration curves were constructed by plotting peak area ratio (standard to internal standard) versus spiked concentration and were fit using least squares linear regression with $1/X^2$ weighting.

3. Results

3.1. EIA performance for LTB_4

The competitive EIA from Assay Designs, while validated for measuring LTB_4 levels in tissue culture medium, human saliva or urine, or porcine EDTA plasma, has never been optimized for use with human PBMCs. These cells are known to be a major source of LTB_4 when stimulated with calcium ionophores such as ionomycin or A23187 [16,20–22]. Given the singular importance of this biomarker for patient stratification and measurement of drug efficacy, we sought to make changes in the protocol that would enhance the dynamic range of the assay to adequately capture signals from PBMCs from both normal donors and patients suffering from cardiovascular disease. Fig. 2 shows a standard curve comparison of LTB_4 measurements for both the standard protocol and that which results from an extended incubation of the polyclonal anti-

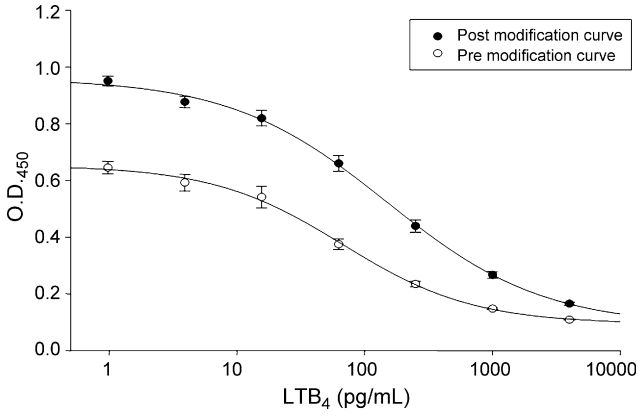

Fig. 2. Comparison of LTB_4 signals by EIA under standard and modified conditions. The standard curves were expanded to include seven points ranging from 0.000976 to 4.00 ng/ml. The post-modification conditions also employed an overnight primary incubation at room temperature (vs. 2 h). The error bars represent the standard error of the mean from four replicates.

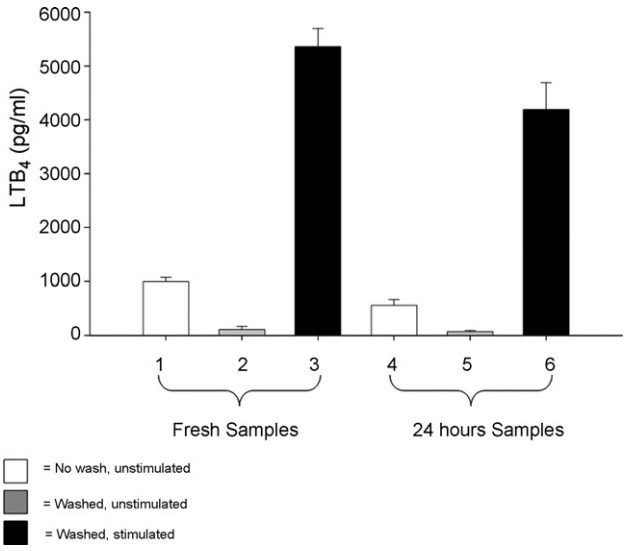

Fig. 3. Graph showing the EIA response for LTB_4 in PBMCs isolated from four donors before and after stimulation with 1.0 μM A23187. Two conditions were compared prior to stimulation: unwashed (white bars) and washed (gray and black bars). The stimulated data (black bars) were all obtained using washed PBMC.

body with the standard/alkaline phosphatase conjugate. We were able to enhance the dynamic range of the assay to an $O.D._{405}$ ranging from ~1.0 to 0.2, which represents about a 30% improvement over the published method. We also extended the standard curve to 4000 pg/mL. Multiple runs gave high reproducibility, as evidenced by the error bars on the graph which indicate the standard error of the mean.

Table 1 provides a summary of the assay parameters for inter- and intra-assay precision, which indicate that the test is performing robustly and reproducibly under the modified conditions.

3.2. LTB_4 signal generation and stability

Calcium influx can play a key biological role in the formation of arachidonic acid from cell membranes via any one of a number of forms of PLA_2 [23,24]. This phenomenon has been mimicked *in vitro* by the use of calcium ionophores, which here takes the form of A23187 [20,25–27]. We found that, in accordance with the literature, stimulating PBMCs, either washed with PBS + 0.1% BSA or left in the plasma matrix, with 1.0 μM A23187, resulted in the generation of LTB_4 over basal levels detected in unstimulated cells; in four out of five samples, the % increase of LTB_4 was much greater in cells that were stimulated after having been washed (Fig. 3). If PBMC cannot be processed immediately for the generation of LTB_4, then they can be stored at 4 °C for at least 24 h in PBS + 0.1% BSA with no apparent loss of response.

PBMCs from four different donors were stimulated with 1.0 μM A23187 and diluted to 1:16 in twofold increments, with good dilutional linearity. We measured freeze–thaw stability of LTB_4 using either the modified EIA or LC/MS/MS and found that beyond two cycles of freeze–thaw (from −20 °C to room temperature), variability begins to increase. Non-enzymatic increase of LTB_4 signal was noted by either modified EIA or LC/MS/MS when extracts were stored at room temperature, whereas a constant signal was seen with extracts stored at 4 °C.

Table 1
EIA validation statistics for LTB_4.

	LTB_4 concentration (pg/mL)			
	125	250	500	1000
Mean (run 1)	153.09	272.35	518.62	941.74
Intra-run precision (%CV)	6.09	10.03	6.69	10.44
Intra-run bias (%RE)	22.47	8.94	3.72	−5.83
n	9	9	9	9
Mean (run 2)	156.99	238.71	552.60	1149.64
Intra-run precision (%CV)	6.05	10.58	1.39	9.18
Intra-run bias (%RE)	25.59	−4.52	10.52	14.96
n	9	9	9	9
Mean (run 3)	154.59	365.15	435.49	1072.20
Intra-run precision (%CV)	5.40	10.37	6.61	12.31
Intra-run bias (%RE)	23.67	46.06	−12.90	7.22
n	9	9	9	9
Overall mean	154.89	292.07	502.23	1054.50
Inter-run precision (%CV)	5.19	21.43	11.34	12.66
Inter-run bias (%RE)	23.91	16.83	0.45	5.45
n	27	27	27	27

Summary of bias and precision data for LTB_4 determined by EIA. Validation QCs ($n = 9$ replicates) were made at four concentrations and assayed using the Assay Designs LTB_4 kit. The bias and precision estimates shown were determined from runs on three separate days.

3.3. LC/MS/MS chromatograms

Reversed phase LC/MS/MS with ESI and negative ion detection has been widely used for eicosanoid profiling including analysis of products of the 5-LO pathway [28–31]. In most of these cases, the referenced assays have been used to profile multiple classes of eicosanoids. The objective of the present method was to establish selective conditions to quantify 5-HETE and LTB_4 under conditions amenable to clinical deployment.

Fig. 4 displays a series of ion mass chromatograms for LTB_4 acquired from LC/MS/MS analysis of a PBMC sample obtained from an atherosclerosis patient. The upper two traces (A and B) display data obtained from washed PBMC plasma before and after stimulation, respectively. The peak at 8.3 min indicated by the asterisk corresponds to LTB_4. This assignment was verified by the corresponding mass chromatogram (bottom trace, C) for the internal standard, LTB_4-d_4. A comparison of the LTB_4 peak intensity from traces A and B indicates an ionophore-induced increase of greater than 30-fold. Injection of known LTB_4 isomers was performed as a test of assay selectivity. This experiment (data not shown) revealed that the peak at 7.8 min (trace B) corresponds to the 6-trans isomer, while the peak at 8.0 min likely contains contribution from both the 12-epi and 12-epi-6-trans isomers.

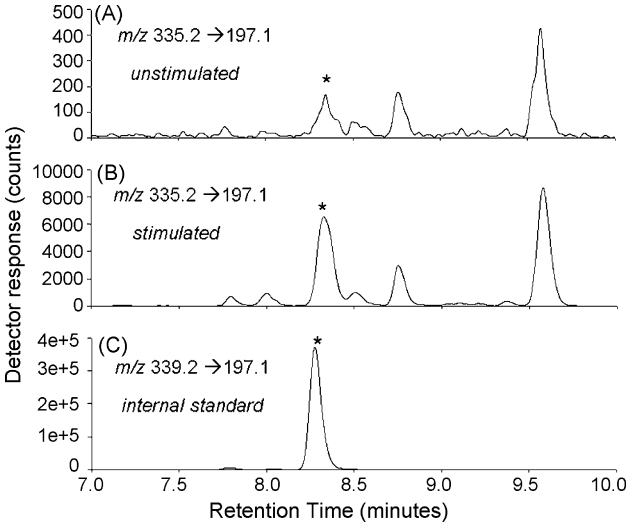

Fig. 4. SRM mass chromatograms from LC/MS/MS analysis of LTB_4 in a PBMCs sample before (panel A) and after (panel B) stimulation with A23187. The peak denoted by the asterisk corresponds to LTB_4 (retention time 8.3 min). The mass chromatogram for the internal standard, LTB_4-d_4, appears in panel C.

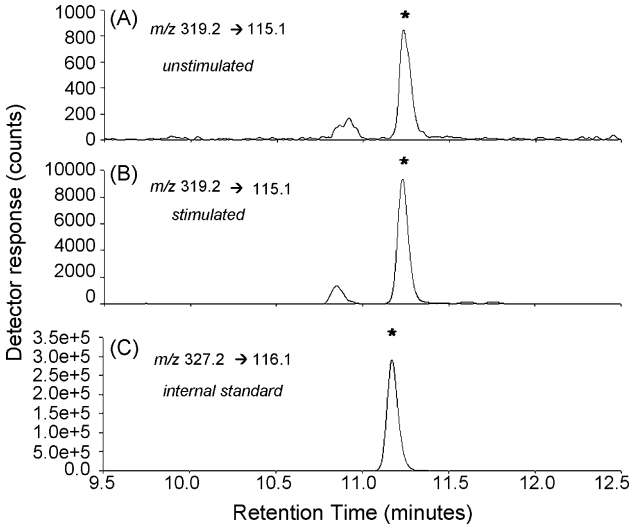

Fig. 5. SRM mass chromatograms from LC/MS/MS analysis of 5-HETE in a PBMC sample before (panel A) and after (panel B) stimulation with A23187. The peak denoted by the asterisk corresponds to 5-HETE (retention time 11.2 min). The mass chromatogram for the internal standard, 5-HETE-d_8, appears in panel C.

Fig. 5 displays an analogous series of ion mass chromatograms for 5-HETE obtained during the same sample analysis. Traces A and B correspond to data obtained before and after stimulation with A23187, respectively. The increase in 5-HETE (denoted by asterisk) was approximately 10-fold. In contrast to the data for LTB_4, the profiles for 5-HETE exhibited fewer peaks, an attribute linked to the SRM transition used which is known to be selective for 5-HETE [27]. Confirmation of the peak assigned as 5-HETE (RT 11.2 min) comes from co-elution with internal standard 5-HETE-d_8 (Fig. 5, trace C). Selectivity was examined by the injection of standards for various HETE isomers (8, 12, and 15-HETE), all of which eluted at earlier retention times (10.4, 10.4, and 10.1, respectively).

3.4. LC/MS/MS validation results

A three-batch validation for LTB_4 and 5-HETE was conducted to assess bias and precision using QC samples prepared in proxy matrix. A total of six replicates were analyzed at the three concentrations (LLOQ, midpoint, and ULOQ). Individual concentrations were interpolated from standard curves prepared in proxy matrix and fit using linear least squares regression with $1/X^2$ weighting.

The results for both LTB_4 and 5-HETE appear in Table 2. The inter-run precision was less than 10% CV at all concentrations for both analytes. The inter-run bias (%RE) for LTB_4 ranged from 2.40% at the midpoint and ULOQ to 14.4% at the LLOQ. For 5-HETE, the inter-run bias (%RE) varied from 1.33% at the ULOQ to 16.7% at the LLOQ.

To test the suitability of the proxy matrix, a single point standard addition experiment was performed. For this test, replicate measurements were made of an actual PBMC sample matrix before and after spiking with 1.0 ng/mL of each analyte. The basal levels of LTB_4 and 5-HETE in the pool were established as 0.0809 and 0.0377 ng/mL, respectively (Table 3). These estimates represent the mean of 12 determinations over 2 runs using a proxy matrix standard curve. The pool was subsequently re-assayed on three separate days ($n = 18$) after spiking each analyte at 1.0 ng/mL. The low observed bias (7.41% for LTB_4 and 5.77% for 5-HETE) and acceptable

Table 2
LC/MS/MS validation statistics for LTB4 and 5-HETE.

	LTB_4 concentration (ng/mL)			5-HETE concentration (ng/mL)		
	0.0250	12.5	25.0	0.100	12.5	25.0
Mean (run 1)	0.0295	12.8	26.0	0.118	13.1	26.3
Intra-run precision (%CV)	6.47	1.04	1.73	9.15	2.08	2.92
Intra-run bias (%RE)	18.0	2.40	4.00	18.0	4.80	5.20
n	6	6	6	6	6	6
Mean (run 2)	0.0305	12.8	25.6	0.122	12.8	25.1
Intra-run precision (%CV)	5.87	3.68	7.58	8.77	18.2	6.85
Intra-run bias (%RE)	22.0	2.40	2.40	22.0	2.40	0.400
n	6	6	6	6	6	6
Mean (run 3)	0.0258	12.8	25.2	0.110	12.7	24.6
Intra-run precision (%CV)	9.11	3.41	9.37	9.45	5.27	9.02
Intra-run bias (%RE)	3.20	2.40	0.800	10.0	1.60	−1.60
n	6	6	6	6	6	6
Overall mean	0.0286	12.8	25.6	0.117	12.9	25.3
Inter-run precision (%CV)	7.15	2.71	6.23	9.12	8.52	6.26
Inter-run bias (%RE)	14.4	2.40	2.40	16.7	2.93	1.33
n	18	18	18	18	18	18

Summary of bias and precision data for LTB_4 and 5-HETE acquired by LC/MS/MS using a proxy matrix (PBS buffer with 0.1% BSA). Validation QCs ($n = 6$ replicates) were analyzed at three different concentrations for each analyte. The data shown were obtained from runs on three separate days.

Table 3
Use of standard addition to demonstrate proxy matrix suitability.

	LTB_4	5-HETE
Endogenous mean ($n = 12$)	0.0809 ng/mL	0.0377 ng/mL
Inter-run precision (%CV)	15.6	26.5
Spiked amount	1.00 ng/mL	1.00 ng/mL
Theoretical concentration	1.08 ng/mL	1.04 ng/mL
Post-spike mean ($n = 18$)	1.16 ng/mL	1.10 ng/mL
Inter-run precision (%CV)	12.3	15.5
Mean bias (%RE)	7.41	5.77

A single point standard addition was used to verify the suitability of PBS buffer containing 0.1% BSA as a suitable proxy matrix for PBMC sample analysis. Basal levels in an un-stimulated PBMC pool were first determined using a proxy matrix standard curve. The relatively low precision (%CV) and bias (%RE) observed after spiking 1.0 ng/mL LTB_4 and 5-HETE support the use of the proxy matrix.

precision supports the use of PBS with 0.1% BSA as a proxy matrix.

Selectivity was demonstrated through the analysis of blank samples as well as the injection of known isomers for each analyte. The absolute carryover for LTB_4 and 5-HETE was 0.05% and 0.2%, respectively. Additional experiments were conducted to verify freeze–thaw, process, and autosampler stability for both analytes (data not shown). As mentioned previously, sample preparation occurred on ice to limit process-related instability.

3.5. Correlation of EIA with LC/MS/MS

PBMCs were processed from 25 individual donors representing a population that had been defined as either atherosclerotic or non-atherosclerotic. There were 10 male and 15 female donors in the group, with a mean age of 61 years. Additional information related to family history along with factors known to correlate with atherosclerosis was obtained, although no attempt was made to stratify this experimental population owing to its finite size and because it exceeded the objectives of the present study.

Fig. 6A displays a correlation plot comparing the LTB_4 results by LC/MS/MS and EIA. All results shown were obtained following stimulation with ionophore. A high degree of correlation was observed ($r = 0.9426$). A second correlation graph in Fig. 6B compares LTB_4 and 5-HETE concentrations determined for the 25 donors using LC/MS/MS. Again, a high correlation was observed ($r = 0.9159$) with LTB_4 concentrations being approximately twice the values found for 5-HETE. Both correlations were determined using Pearson's statistical model.

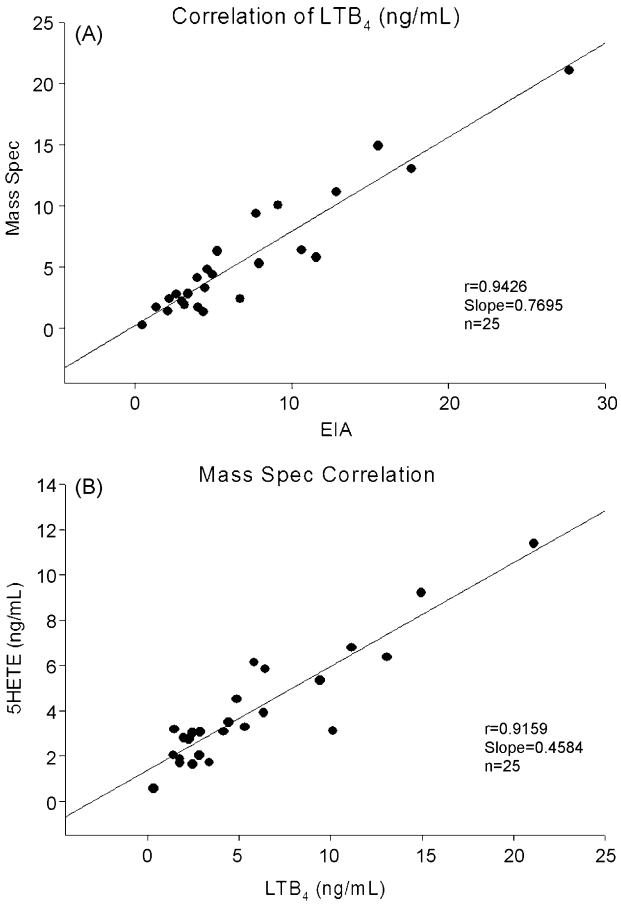

Fig. 6. Correlation plots from the analysis of stimulated PBMCs isolated from the blood of 25 human donors. (A) Displays the correlation obtained when identical samples were analyzed by EIA and LC/MS/MS. (B) Compares the LC/MS/MS results for LTB_4 and 5-HETE obtained for each sample. The correlation coefficients displayed were calculated using Pearson's method.

4. Discussion

As LTB_4 gains recognition as a biomarker associated with cardiovascular risk, it is critical that clinical methods be developed to accurately quantitate this important metabolite. We chose to use PBMCs as a surrogate tissue for measurement because it is easily obtainable, and the release of LTB_4 from PBMCs has been well established in the literature [32]. In this paper, we presented techniques currently being incorporated into a standardized protocol defining proper methods for sample collection, PBMC isolation, stimulation, shipment, and storage. The intent of this effort was to define conditions that allow samples to be collected at the clinic, processed, and shipped to external laboratories for analysis either by EIA or LC/MS/MS.

Two separate methodologies for detecting LTB_4 were simultaneously pursued to provide flexibility and expanded options for clinical analysis. The results contained herein demonstrate that both analytical methods are sufficiently sensitive to measure LTB_4 derived from isolated human PBMCs without ionophore stimulation. Moreover, the validation statistics of each method suggest that either approach can be used in the clinic to support ongoing efforts related to patient stratification and/or pharmacodynamic assessment.

Immunoassays represent the current standard for assessing biochemical markers in the clinic owing to advantages such as throughput and a relative ease of deployment. Unfortunately, it is difficult to produce highly selective assays when using immunoassays for small molecules. This is particularly true for lipids owing to the preponderance of isomeric forms. Further, despite modifications to an existing EIA kit for LTB_4, the use a competitive format (i.e. single antibody) leads to general concern over assay robustness. Such concerns were minimized in the present assay by incorporating wash steps to achieve higher selectivity and reproducibility. As a test of selectivity, we examined the potential for cross-reactivity to 6-trans-12-epi-LTB_4 and 6-trans-LTB_4 via EIA, and found interference to be <5% for either analyte (data not shown). Further confirmation of EIA selectivity was obtained by cross-validation with LC/MS/MS (Fig. 6A). Data generated by either method using samples split following PBMC stimulation exhibited high correlation ($r = 0.9426$). In addition, both methods gave comparable concentrations as indicated by the slope of the correlation plot ($m = 0.7695$).

Although LC/MS/MS lacks the throughput potential of EIA, it has the distinct advantages of selectivity and the ability to profile multiple metabolites within a single assay. As illustrated in Figs. 4 and 5, resolution of regioisomers was accomplished by reversed phase LC in conjunction with additional selectivity in the case of 5-HETE pro-

vided by a unique SRM transition. It is important to acknowledge that the present method does not permit resolution of enantiomers (i.e. R and S), a capability demonstrated by the normal phase chiral LC/MS/MS method of Lee et al. [33]. This technology was not applied to this initial investigation as it was not deemed to be practical for routine clinical deployment.

The LC/MS/MS results for LTB_4 and 5-HETE were quite similar (Fig. 6B) suggesting that a single analyte may be indicative of 5-LO pathway activity. Conversely, we recognize that additional metabolites (e.g. 5-HPETE, 20-hydroxy LTB_4, 20-COOH LTB_4) may need to be measured. Given the well-documented ability of LC/MS/MS to simultaneously profile multiple eicosanoid pathways, we feel that the current assay provides a good starting point to develop an expanded method should the need arise.

5. Conclusions

Given the recent linkage of LTB_4 to cardiovascular disease, the means to accurately measure intermediates of the 5-LO pathway in the clinic is extremely important to the search for meaningful biomarkers to help identify patients who might benefit from interventive therapies. The results presented herein show that robust and reproducible LTB_4 production from washed, stimulated PBMCs can be measured by either EIA or LC/MS/MS and that the results are highly correlated when separate measurements are made on the same sample. Further, the additional measurement of 5-HETE by LC/MS/MS, opens the possibility of mining additional intermediates along the 5-LO pathway to further our understanding of the role of leukotriene markers in cardiovascular risk.

We are currently applying the methods outlined in this communication in conjunction with genotypic profiling to explore the potential of patient stratification according to 5-LO-mediated atherosclerosis risk. Pharmacodynamic assessment is also being explored to assist with clinical translation. Both activities are part of a larger objective, which is to deliver tailored therapy.

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