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A highly sensitive and selective method for the determination of leukotriene B_4 (LTB_4) in ex vivo stimulated human plasma by ultra fast liquid chromatography–tandem mass spectrometry

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ABSTRACT

Leukotriene B_4 (LTB_4) is an important inflammatory component in a number of diseases and has been used as a pharmacodynamic (PD) biomarker. In this report, a highly sensitive and selective ultra fast liquid chromatography–tandem mass spectrometry (UFLC-MS/MS) method for the determination of LTB_4 in plasma from ex vivo stimulated human blood, using leukotriene B_4-d_4 (LTB_4-d_4, contains four deuterium atoms at the 6, 7, 14, and 15 positions) as the internal standard (IS), was developed and validated. The chromatographic separation of LTB_4 from its three isomers and an unknown interference peak from human plasma was crucial to achieve accurate determination of 0.2 ng/mL (LLOQ) of LTB_4. LTB_4 and the IS were extracted with methyl tertiary butyl ether (MTBE) from 200 μL human plasma. Reversed-phase HPLC separation was carried out with a Phenomenex Synergi Hydro-RP column (100 mm × 3 mm, 2.5 μm). MS/MS detection was set at mass transitions of 335.0 → 194.9 *m/z* for LTB_4 and 339.0 → 196.9 *m/z* for LTB_4-d_4 in Turbo Ionization Spray (TIS) negative mode. The dynamic range of the method is 0.2–200 ng/mL. LTB_4 was found to be stable in human plasma for at least three freeze (−20 °C)/thaw cycles, and on the benchtop (room temperature) for at least 6 h. The stock solution storage stability study demonstrated that the LTB_4 stock solution, in 50:50 acetonitrile:water, was stable at 4 °C for at least 198 days. The processed samples were found to be stable for at least 72 h at room temperature. The long-term sample storage stability test demonstrated that LTB_4 human plasma samples were stable at a storage temperature of −20 °C for at least 198 days. In addition, intraday and interday accuracy and precision, sensitivity, linearity, and recovery were evaluated. An additional partial validation was conducted to decrease the plasma sample volume from 200 to 100 μL. All the data reported in this study fulfilled the requirements and recommendations in the FDA guidance for bioanalytical method validation. Comparison of the validated UFLC-MS/MS method with an ELISA method using ex vivo stimulated samples indicated that although results from the two assays correlated relatively well, the UFLC-MS/MS method has been shown to be superior in selectivity and dynamic range to an ELISA method in our study. The validated UFLC-MS/MS method was successfully used to analyze samples generated from two clinical studies. The excellent assay performance and incurred sample reproducibility (ISR) results obtained from the study sample analysis demonstrated the assay is robust and reliable.

1. Introduction

Leukotriene B_4 [LTB_4; (5S,12R)-5,12-dihydroxy-(Z,E,E,Z)-6,8,10,14-eicosatetraenoic acid] is a potent chemotactic agent generated enzymatically in leukocytes from arachidonic acid via the 5-lipoxygenase pathway. Previous studies demonstrated that LTB_4 is generated in several cell types including polymorphonuclear leukocytes (PMNs), macrophages, and monocytes [1–6]. As a potent chemotactic agent, LTB_4 is regarded as an important mediator in several pathological processes such as inflammatory and allergic responses. To understand the role of LTB_4 in these pathological processes, an accurate and reliable analytical method to determine the concentration of LTB_4 in biological fluids is

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required. However, due to the very low concentration of endogenous LTB_4 and the potential interference from other endogenous compounds in the biological fluids, it is challenging to develop a sufficiently sensitive and selective method.

Various techniques have been reported for determination of LTB_4 in biological fluids, including radioimmunoassay (RIA) [7–9], gas chromatography-MS [10–13], enzyme immunoassays [14], high performance liquid chromatography (HPLC) [15–20], and high performance liquid chromatography–mass spectrometry (HPLC-MS) [21]. Among these techniques, RIA, enzyme immunoassay, HPLC-UV/HPLC-PDA, and HPLC-MS may lack sufficient specificity and/or sensitivity for LTB_4, considering the complexity of biological samples and endogenous level of LTB_4 at ng/mL or even pg/mL. Gas chromatography/Mass spectrometry is quite specific and sensitive for LTB_4 analysis, however, tedious and time-consuming sample preparation including derivatization and even HPLC purification is required prior to GC/MS analysis. The complicated derivatization and purification procedures are not only inefficient, but also result in low recovery. Some studies have used GC–tandem mass spectrometry to further improve the sensitivity and selectivity [11,22–24].

HPLC-MS/MS techniques have been widely and extensively used for bioanalysis due to their unique advantages of sensitivity, selectivity, robustness, and linearity. However, using this approach for quantification of LTB_4 in biological fluids is rare. Recently, Chappell et al., [25] developed and validated an ultra high pressure liquid chromatography–tandem mass spectrometry (UHPLC, also called UFLC or UPLC) method for measurement of cysteinyl leukotrienes and LTB_4 in human sputum. The low limit of quantification (LLOQ) for LTB_4 was 39.0 pg/mL and LTB_4 was well separated from its two isomers 6-trans-LTB_4 and 6-trans-12-epi-LTB_4. However, the separation of LTB_4 from its third isomer 12-epi-LTB_4 was not mentioned in their study, and is worthy to test and ensure the method selectivity. None of the LC-MS/MS assays reported in the literature for the determination of LTB_4 have achieved baseline separation of LTB_4 and its three isomers in plasma from ex vivo stimulated human blood, with lower limit of quantitation (LLOQ) at 0.2 ng/mL.

In this report, we describe a highly sensitive, selective, and robust UFLC–MS/MS method for determination of LTB_4 in plasma from ex vivo stimulated human blood. Good chromatographic separation of LTB_4 from its three isomers and an endogenous interference was attained, which was crucial for accurate determination of LTB_4 as low as 0.2 ng/mL using 0.1 or 0.2 mL sample volume. Although we were able to achieve an LLOQ of 20 pg/mL by using 0.5 mL of sample volume, the 0.2 ng/mL LLOQ is sensitive enough for the analysis of ex vivo stimulated human plasma samples based on results obtained from previous studies using ELISA method. Therefore, the 0.2 ng/mL LLOQ assay was validated and used to support clinical studies. Over 2000 human plasma samples have been successfully analyzed with this validated method to support two clinical studies. The data obtained from the validated UFLC–MS/MS method will be discussed and compared with the data obtained from ELISA method using some ex vivo stimulated human plasma samples.

2. Experimental

2.1. Chemicals and reagents

Calcium ionophore A23187 was purchased from Sigma–Aldrich Chemical Corporation (St. Louis, MO, USA). LTB_4 ELISA kit was from Enzo Life Sciences Inc. (Farmingdale, NY, USA). LTB_4 (purity ≥97%) and internal standard LTB_4-d_4 (chemical purity ≥97%, isotopic purity >99%) was purchased from Cayman Chemicals (Ann Arbor, MI, USA). HPLC-grade methanol (MeOH) and acetonitrile (ACN) were obtained from Mallinckrodt (Hazelwood, MO, USA) and EMD (Gibbstown, NJ, USA), respectively. GR-grade methyl tert-butyl ether (MTBE) was obtained from Fisher Scientific (Pittsburgh, PA, USA). Formic acid and ammonium acetate were purchased from Sigma–Aldrich Chemical Corporation (St. Louis, MO, USA). Acetic acid was purchased from J. T. Baker. Deionized water (18.2 MΩ cm) obtained from a US Filter System (Warrendale, PA, USA) was used to prepare HPLC mobile phase.

Blank human plasma matrix was prepared through the following process: Human blood (sodium heparin as the anticoagulant) was collected from healthy donors through normal blood donor service provided by Scripps Research Institute (La Jolla, CA, USA). Blood was diluted 1:1 with RPMI-1640 (HyClone Laboratories, Inc., Logan, UT, USA), then centrifuged at 833 × *g* for 10 min. Plasma portion was collected and analyzed for basal level of LTB_4. After screening for LTB_4 levels in different blood donors, two donors were found with LTB_4 levels less than 20% of the assay LLOQ (0.2 ng/mL). The pooled human plasma prepared from these two donors was used to prepare the double blanks, blanks, calibration standards, and QC samples. The blank plasma was stored at −20 °C.

2.2. Preparation of standard stock solutions, and working standard for ELISA assay

Blank plasma was diluted 80-fold with assay buffer provided in LTB_4 ELISA kit. The working standard solution of LTB_4 was diluted in blank plasma from stock (12,000 pg/mL) to 3,000, 750, 188, 46.9 and 11.7 pg/mL respectively. Diluted standards were used within 60 min of preparation.

2.3. Preparation of standard stock solutions, working standard solutions and quality control (QC) samples for UFLC–MS/MS assay

The standard stock solution of LTB_4 was approximately 100 μg/mL in ethanol. Subsequently diluted standard stock 1 and 2 were 10 and 0.1 μg/mL in 1:1 ACN/H_2O., respectively. From these stock solutions, eight standard spiking solutions (0.8, 1.6, 4, 40, 200, 480, 640, and 800 ng/mL) were prepared in 1:1 ACN/H_2O. Separate preparation of QC stock solutions was carried out and used for QC sample preparation. Four levels of QC samples (LLOQ 0.2 ng/mL, Low-QC 0.6 ng/mL, Mid-QC 30 ng/mL, and High-QC 150 ng/mL) were prepared from blank human plasma. The QC samples were then aliquoted and stored at -20 °C until use. Internal standard (IS) stock solution was approximately 100 μg/mL of LTB_4-d_4 in ACN. The IS spiking solution containing 200 ng/mL of LTB_4-d_4 was diluted from IS stock with 1:1 ACN/H_2O. All prepared stock solutions and spiking solutions were stored at 4 °C.

2.4. Sample preparation for ELISA assay

Calcium ionophore working solution was freshly made by diluting ionophore stock (1.67 mg/mL in DMSO) 10-fold in deionized water. Heparinized human blood was diluted 1:1 with RPMI-1640, then stimulated with calcium ionophore at final concentration of 7.2 μg/mL for 30 min at 37 °C. Blood was centrifuged at 833 × *g* for 10 min, and plasma portion was collected. For ELISA, plasma was further diluted 80-fold in assay buffer provided in the ELISA kit, then directly assayed for LTB_4 by following the manufacturer's instructions.

2.5. Sample preparations for UFLC–MS/MS assay

The working standards were prepared freshly in blank plasma on each day prior to use. Blank human plasma (200 microliters (μL) was added into a test tube and spiked with 50 μL of corresponding standard spiking solution. At the same time, 200 μL of

QC sample was added into a test tube and 50 μL of 1:1 ACN/H_2O was added. To each sample except for the double blank, 50 μL of IS spike solution (200 ng/mL of LTB_4-d_4) was added and sample was vortexed thoroughly. 100 μL of 0.1% formic acid solution was added and LTB_4 was extracted with 2 mL of MTBE for 5 min by vortexing. The resulting sample was centrifuged at 3000 rpm for 5 min and the organic layer was transferred to a clean test tube. After drying down the organic phase under nitrogen stream in a water bath at 45 °C for approximately 10 min, 100 μL of reconstitution solution (60:32:8 H_2O/ACN/MeOH) was added into each tube. The sample was vortexed and transferred into HPLC sample vial and 10 μL of the extracted sample was injected.

2.6. UFLC/MS/MS system

A Shimadzu UFLC system (Shimadzu Scientific Instruments, Columbia, MD, USA) was used for LTB_4 separation. It contained LC-20AD pumps, a model LC-20AC autosampler, and a model DGU-$20A_3$ Degasser. A 100 mm × 3 mm, 2.5 μm Synergi Hydro-RP C18 column (Phenomenex Inc., Torrance, CA, USA) was used. An isocratic and a subsequent washing program were carried out using an aqueous mobile phase A of 5 mM ammonium acetate in water and an organic mobile phase B of 80:20 ACN/MeOH. Flow rate was kept at 0.65 mL/min and injection volume was 10 μL. The mobile phase program was as follows: hold at 40% B for 5.0 min, then increase to 100% B in 0.1 min, hold at 100% B for 1.4 min, finally back to initial condition and equilibrate for 1.4 min. The retention time of both LTB_4 and LTB_4-d_4 was about 3.8 min.

An Applied Biosystems 4000 QTRAP mass spectrometer (AB Sciex, Foster City, CA, USA) with an electrospray ionization (ESI) source was used in negative ion mode. LTB_4 and LTB_4-d_4 were monitored at m/z 335.0 → 194.9 and 339.0 → 196.9 transition in MRM mode, respectively. Instrument conditions were as follows: curtain gas 20 psi, nebulizer gas 40 psi, turbo gas 60 psi, temperature 650 °C, collision gas medium, voltage −4000 V.

Data were acquired and processed using Analyst Version 1.4.2, and the concentrations were calculated using linear regression with $1/x^2$ weighting with Watson LIMS version 7.3 (Thermo Fisher Scientific, Inc., Philadelphia, PA).

2.7. Sample preparation and instrument conditions for method partial validation of the UFLC–MS/MS assay

Due to sample constraints, a method partial validation reducing the sample volume from 200 to 100 μL was carried out after full method validation. In brief, 100 μL of blank human plasma was added into a test tube and spiked with 25 μL of corresponding standard spiking solution, 25 μL of IS spike solution (200 ng/mL of LTB_4-d_4), and 50 μL of 0.1% formic acid solution. After the sample was vortexed thoroughly, LTB_4 was extracted with 2 mL of MTBE for 5 min by vortexing. The resulting sample was centrifuged at 3000 rpm for 5 min and the organic layer was transferred to a clean test tube. After drying down the organic phase under nitrogen stream in a water bath at 45 °C for approximately 10 min, 100 μL of reconstitution solution (60:32:8 H_2O/ACN/MeOH) was added into each tube. The sample was vortexed and transferred into HPLC sample vial and 20 μL of the extracted sample is injected.

Instrument conditions for the adapted method were the same except that the gradient program was slightly adjusted, maintaining similar separation and retention times.

3. Results and discussion

3.1. Ex vivo stimulated human plasma

The major reason for using plasma from ex vivo stimulated blood for the measurement of LTB_4 is that human plasma has

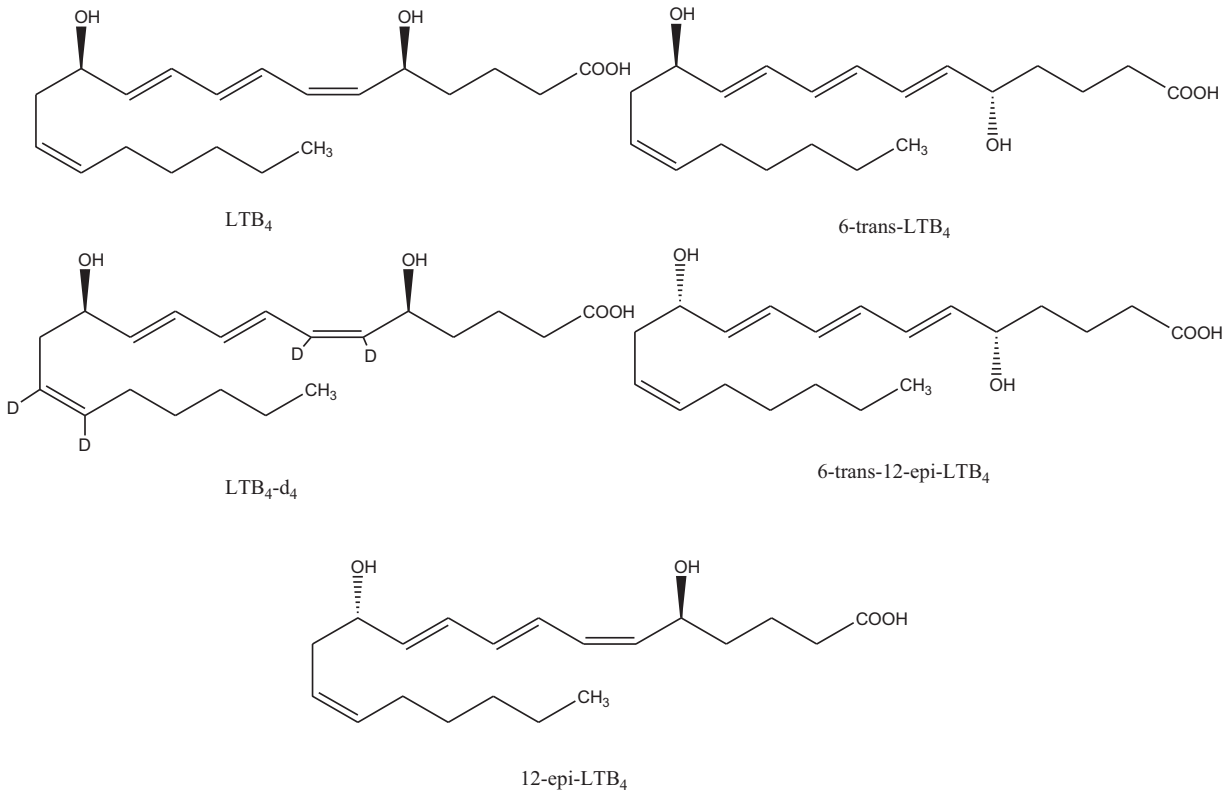

Fig. 1. Structures of LTB_4, LTB_4-d_4, three LTB_4 isomers 6-trans-LTB_4, 6-trans-12-epi-LTB_4, and 12-epi-LTB_4.

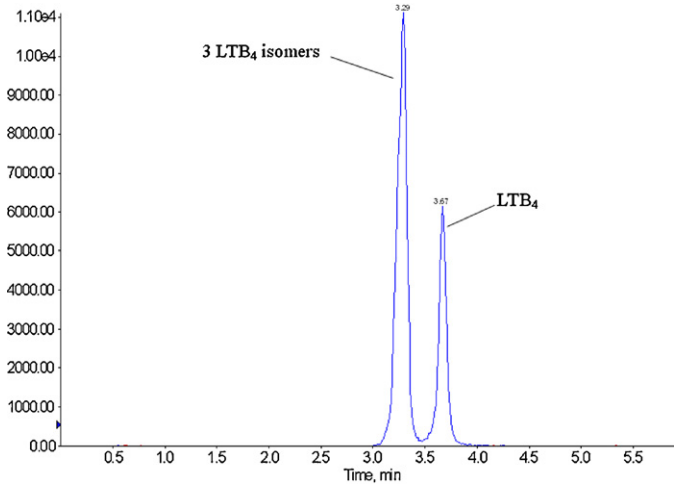

Fig. 2. Chromatogram of LTB_4 and its 3 isomers (6-trans-LTB_4, 6-trans-12-epi-LTB_4, 12-epi-LTB_4) in neat solution (mixture of above 4 individual compound solution at 50 ng/mL in 1:1 ACN/H_2O with equal volume).

very low endogenous LTB_4 levels which are difficult to measure accurately with currently available ELISA or UFLC–MS/MS methods. Furthermore, high inter-individual variability of endogenous LTB_4 levels in healthy subjects and disease populations are common. To obtain a matrix that best mimics the study samples, more than 10 healthy subjects were screened for endogenous LTB_4 levels in human plasma. Two of the subjects showed LTB_4 levels less than 20% of the UFLC–MS/MS assay LLOQ (0.2 ng/mL). The pooled plasma from these two subjects was used for the preparation of double blanks, blanks, calibration standards, and QC samples for the UFLC–MS/MS method validation and sample analysis.

3.2. UFLC–MS/MS assay

3.2.1. Optimization of UFLC–MS/MS assay conditions

The MS/MS conditions were optimized in manual tuning mode by infusing 0.25 μg/mL of LTB_4 standard solution with continuous mobile phase. Negative mode at MRM transition 335.0 → 194.9 demonstrated the highest sensitivity for LTB_4 using 5 mM ammonium acetate in H_2O as mobile phase. Other common buffers, such as formic acid, acetic acid, and ammonium formate were also used for comparison in terms of sensitivity and HPLC separation.

Fig. 1 depicts the structure of LTB_4, LTB_4-d_4 (internal standard), and three LTB_4 isomers 6-trans-, 6-trans-12-epi-, and 12-epi-LTB_4. To achieve baseline separation of LTB_4 and its 3 isomers and potential interference from plasma, a UFLC system, a 100 mm × 3 mm Synergi Hydro-RP C_{18} column with 2.5 μm particle size, and ammonium acetate mobile phase were used. Fig. 2 is a typical chromatogram of LTB_4 and its 3 isomers demonstrating baseline separation of LTB_4 from its 3 isomers which co-eluted at about 3.3 min. Representative chromatograms of blank plasma (with internal standard) and an LLOQ sample at 0.2 ng/mL in human plasma are shown in Figs. 3 and 4, respectively. This separation was achieved in 8 min and all LTB_4 isomer peaks were well resolved. It should be mentioned that considerable effort was spent on screening of columns and HPLC conditions to separate LTB_4 from its 3 isomers and other potential interferences. Fig. 5 demonstrates the typical chromatograms of LTB_4 in human plasma using 3 columns: Phenomenex Gemini 30 mm × 2 mm, Phenomenex Luna C18-HST 50 mm × 2 mm, and Phenomenex Synergi Fusion RP 100 mm × 3 mm. It is also worth to mention that the major reason of using UFLC system is to help achieving the baseline separation, with improved peak shape and sensitivity. At the initial stage of the assay development, a regular HPLC system was used and an unknown peak was found to interfere with the quantitation of LTB_4. This issue was resolved by switching to UFLC.

Table 1
Method validation results of LLOQ samples.

	Sample volume: 200 μL	Sample volume: 100 μL
	LTB_4 concentration, 0.2 ng/mL	LTB_4 concentration, 0.2 ng/mL
Mean (n = 6)	0.216	0.190
%CV	7.84	8.60
%Nominal	108	95.0

3.2.2. Selectivity and sensitivity

Selectivity is defined as the ability of a chromatographic method to measure a response from the analyte without interference from the biological matrix. Figs. 2–4 clearly demonstrated that LTB_4 was well separated from the other three isomers and potential interference from blank plasma. It should be noted that commercially available human plasma can contain high levels of endogenous LTB_4.

The lower limit of quantitation (LLOQ) was defined as the concentration at which the signal/noise was ≥5. The validation was conducted with a target LLOQ of 0.2 ng/mL for LTB_4 in human plasma. To evaluate the sensitivity, six samples prepared at the LLOQ level were analyzed and the concentrations were calculated with the calibration curve. The data, shown in Table 1, demonstrated that the method met the acceptance criteria for sensitivity (accuracy within 100 ± 20% and %CV no more than 20%). Therefore, the method was sensitive enough to determine LTB_4 in human plasma at a concentration of 0.2 ng/mL when using either a 200 μL or a 100 μL sample volume.

3.2.3. Back-calculated concentrations of calibration standards

The back-calculated concentrations of calibration standards using 200 or 100 μL sample volume did not differ by more than 15% from the nominal concentrations (20% at the LLOQ) and the %CV for each concentration level was not more than 15% (20% at the LLOQ).

3.2.4. Linearity

The linearity of the method was evaluated at a linear range of 0.2–200 ng/mL for LTB_4. Linear regression (with a weighting factor of $1/x^2$) was used to produce the best fit for the concentration-detector response relationship for LTB_4 in human plasma. All calibration curves had a coefficient of determination (r^2) ≥ 0.9975.

3.2.5. Intraday and interday accuracy and precision

The intraday and interday accuracy and precision of this method were investigated at three different QC concentrations of LTB_4 (0.6, 30, and 150 ng/mL). The statistical results of the QC samples using 200 or 100 μL sample volume are shown in Tables 2a and 2b, respectively.

The results demonstrated that the intraday and interday precision and accuracy of the method met the acceptance criteria (accuracy within 100 ± 15% and %CV no more than 15%) when using either a 200 or a 100 μL sample volume.

3.2.6. Recovery

The recovery of the sample preparation was evaluated by comparing the mean peak area of LTB_4 in QC samples with the mean peak area of directly post-spiked LTB_4 samples (at the same concentrations) in extracted plasma. Recovery was determined for LTB_4 at three QC concentration levels. For each concentration, three measurements were performed. The results indicated that the recovery of LTB_4 was in the range of 99–106%. Recovery of LTB_4-d_4 (IS) was

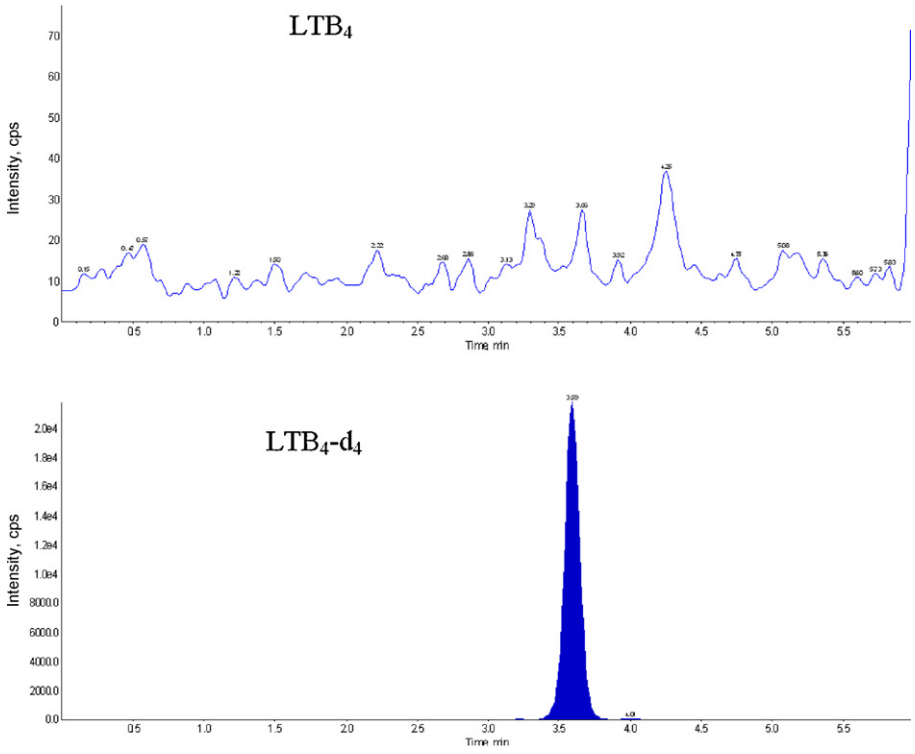

Fig. 3. Typical chromatogram of blank human plasma (with internal standard).

determined at a concentration of 50 ng/mL, which is the concentration used in the method. Nine measurements were performed for LTB_4-d_4 (IS). The results indicated that the average recovery for LTB_4-d_4 (IS) was 101%.

3.2.7. Dilution integrity

During the analysis, it may be necessary to dilute the plasma samples if the analyte concentrations are above the upper limit of quantitation (ULOQ). In order to verify that the dilution of a high

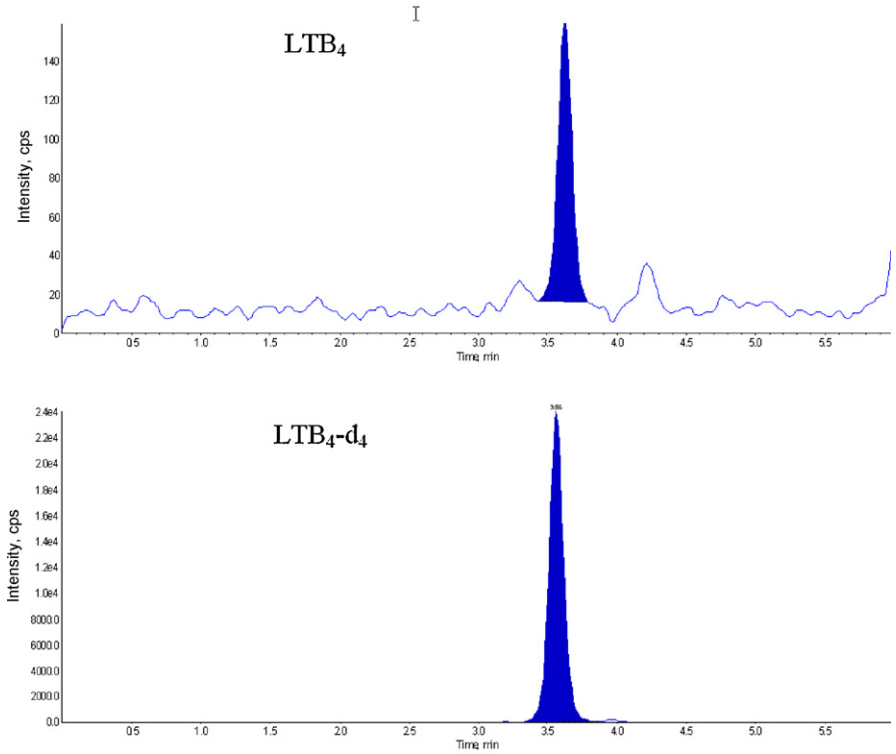

Fig. 4. Typical chromatogram of LLOQ sample at 0.2 ng/mL.

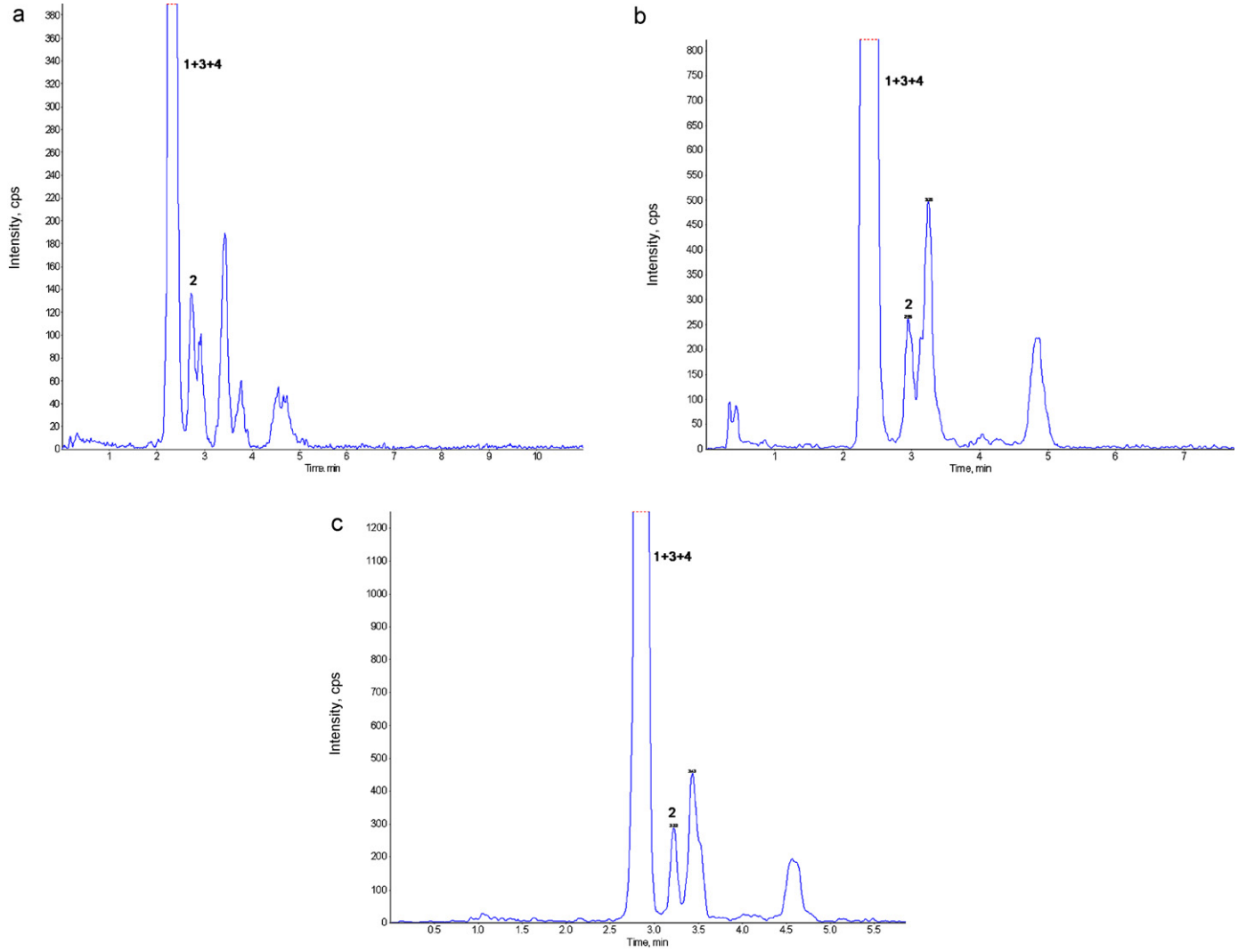

Fig. 5. Typical chromatogram of a real sample for LTB_4 in human plasma on 3 regular columns. (a) Phenomenex Gemini 30 mm × 2 mm; (b) Phenomenex Luna C18-HST 50 mm × 2 mm; (c) Phenomenex Synergi Fusion RP 100 mm × 3 mm. (1: 6-trans-12-epi-LTB_4; 2: LTB_4; 3: 6-trans-LTB_4; 4: 12-epi-LTB_4) (instrument conditions not shown).

concentration sample would yield an analytical response that is within the dynamic range and acceptable tolerance limits of the assay, blank plasma was fortified with LTB_4 at a concentration 10 times above the ULOQ of the method (2000 ng/mL). Six replicates were prepared by diluting the samples 20-fold with blank plasma and assayed against a freshly prepared LTB_4 calibration curve. The dilution integrity evaluation was considered acceptable if the mean of the obtained concentration was within 100 ± 15% of the nominal concentration and the %CV was no more than 15%. The results obtained were acceptable by the above criteria.

Table 2a
Intraday and Interday Accuracy and Precision for LTB_4 using 200 μL sample volume.

Day ID		LTB_4 Concentration (ng/mL)		
		0.6	30	150
Intraday 1	Mean (n = 6)	0.608	30.7	158
	%CV	4.50	1.42	1.25
	%Nominal	101	102	105
Intraday 2	Mean (n = 6)	0.585	30.1	156
	%CV	3.78	1.16	1.66
	%Nominal	97.5	100	104
Intraday 3	Mean (n = 6)	0.572	29.7	154
	%CV	3.12	0.928	1.53
	%Nominal	95.3	99.1	103
Interday results	Mean	0.588	30.1	156
	%CV	4.49	1.76	1.77
	%Nominal	98.0	100	104

3.2.8. *Stability of LTB_4*

To evaluate the benchtop stability, freeze/thaw stability and long-term stability of LTB_4 in human plasma, QC samples at three concentration levels (0.6, 30, and 150 ng/mL) with six replicates at each concentration level were subjected to corresponding period of time and then analyzed against a freshly prepared calibration curve. LTB_4 was considered stable if the mean of the obtained concentrations at each level was within 100 ± 15% of the nominal concentrations.

Table 2b
Intraday Accuracy and Precision for LTB_4 using 100 μL sample volume.

	LTB_4 Concentration (ng/mL)		
	0.6	30	150
Mean (n = 6)	0.587	29.6	149
%CV	4.44	1.12	0.784
%Nominal	97.8	98.7	99.3

Table 3
Stability Data of LTB_4.

Stability	Stable for at least
Benchtop stability in human plasma	6 h at room temperature [a]
Freeze/thaw in human plasma	3 cycles at −20 °C/room temperature[a]
Longterm stability in human plasma	198 days at −20 °C[a]
Processed sample stability	72 h at room temperature[a]
Benchtop stability of stock solution	20 h at room temperature[b]
Longterm stability of stock solution	198 days at 4 °C[b]

[a] Considered stable if the mean of the obtained concentrations at each level was within 100 ± 15% of the nominal concentrations.
[b] Considered stable if the percent difference of the mean peak area ratio of samples compared to those of the fresh prepared samples were no more than ±10%.

To assess the processed sample stability of LTB_4, processed QC samples at three concentration levels, which were kept at room temperature for 72 h, were re-injected after the initial injection and calculated against a freshly prepared calibration curve.

The stock solution stability on the benchtop and in storage conditions (4 °C) was tested by comparing with freshly prepared LTB_4 stock solution. The percentage difference was 3.3% after 13 h on the benchtop and 5.9% after 244 days at 4 °C storage conditions.

All stability data of LTB_4 are summarized in Table 3. No instability was observed under all the tested conditions.

3.2.9. Sample analysis and ISR evaluation

This assay was successfully used in single ascending dose (SAD) and multiple ascending dose (MAD) Phase 1 clinical studies to establish PK/PD relationships and select doses for phase 2 studies (a separate manuscript in preparation to discuss PK/PD relationship). Assay results were made available on an interim basis throughout the course of both studies to guide dose selections and assessment of target occupancy. Over 2000 samples were analyzed. Fig. 6 shows a typical chromatogram of a study sample with the measured concentration of 65 ng/mL.

To evaluate the Incurred Sample Reassay (ISR) of this method, 108 study samples were selected and analyzed. The criteria specify that the % difference, as calculated as (repeat value − original value)/(mean of original value and repeat value) × 100, of at least 2/3 of all the analyzed ISR samples should be within ±20%. The results demonstrated that 103 of the 108 samples were within ±20% and the other 5 samples were within ±30%, which met the acceptance criteria.

The successful sample analysis and ISR test proved this method is sufficiently sensitive, selective, and accurate for determination of LTB_4 in human plasma.

3.3. ELISA assay

The LTB4 ELISA kit is a competitive immunoassay for the quantitative determination of LTB4 in biological fluids. The kit uses a polyclonal antibody to LTB4 to bind, in a competitive manner, the LTB4 in the sample or an alkaline phosphatase molecule which has LTB4 covalently attached to it. After the incubation, excess conjugate and unbound sample is removed and the substrate is added to determine the enzyme activity. The optical density (OD) is measured on a microplate reader at 405 nm. The OD is inversely proportional to the concentration of LTB4 in either standards or samples. The standard curve is plotted as percentage of the maximum binding (without competition). The typical standard curve is shown in Fig. 7a. The concentration of LTB4 in plasma was extrapolated from the standard curve, and representative data was shown in Fig. 7b.

3.4. Comparison of the UFLC–MS/MS method with the ELISA method

We analyzed 48 samples by UFLC–MS/MS and ELISA in parallel, and the results are shown in Fig. 8. On average, LTB_4 concentrations measured by ELISA appeared to be lower than UFLC–MS/MS by approximately 15%. Although the two methods correlated relatively well with $R^2 = 0.92$, the UFLC–MS/MS method has clear advantages over ELISA. First, based on the information provided by

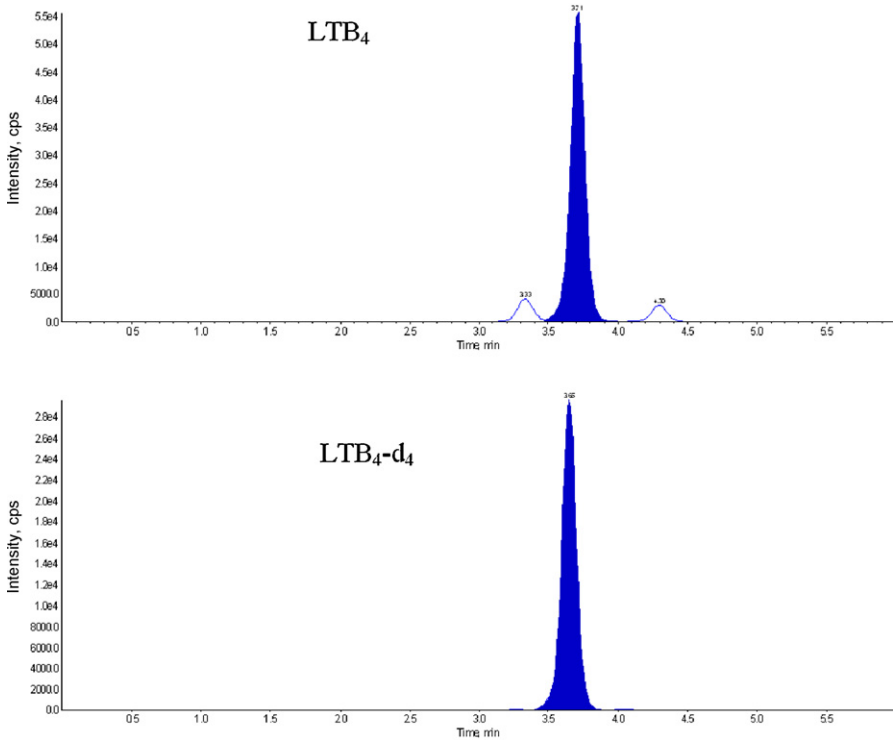

Fig. 6. Typical chromatogram of a study sample.

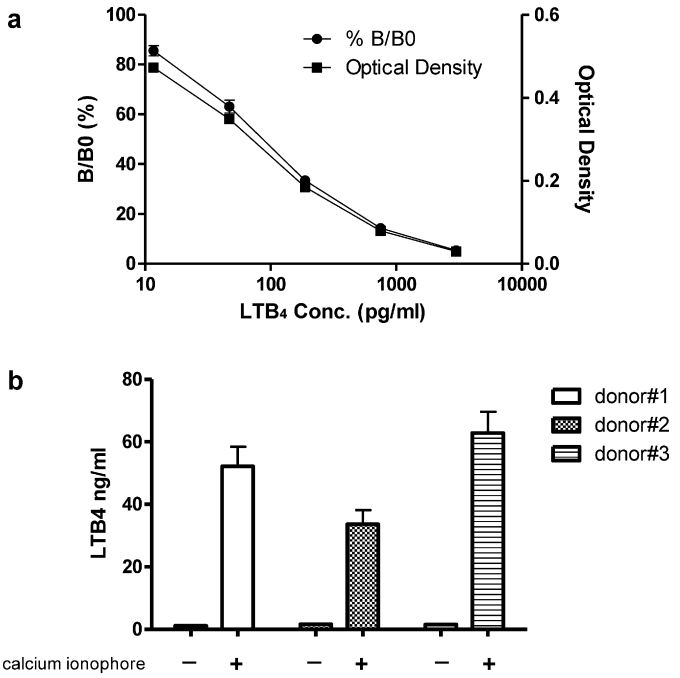

Fig. 7. Typical LTB_4 standard curve (a) and LTB_4 level in human plasma from healthy subject (b) by ELISA.

the manufacturer, the anti-LTB_4 antibody for ELISA demonstrates cross-reactivities to 3 other LTB_4 isomers: 5.5% for 6-trans-12-epi-LTB_4, 4.9% for 6-trans-LTB_4, and 0.94% for 12-epi-LTB_4. Thus, the LTB_4 ELISA method will detect other isomers and be unable to differentiate them from LTB_4. Therefore, PD effects may potentially be underestimated using the ELISA method if the compound under investigation does not affect the pathway leading to the production of these isomers. Second, ELISA is known to be highly matrix dependent, which results in overestimation or underestimation of the actual concentration. Matrix effects in plasma may have been the cause of underestimation of LTB_4 concentration by ELISA in our study. Third, ELISA generally has a narrow dynamic range with limited upper level of quantitation. For example, the ELISA used in this study has a dynamic range up to 3 ng/mL, while the UFLC–MS/MS method we developed has a dynamic range up to 200 ng/mL. In our clinical study, the level of LTB_4 in plasma after ex vivo stimulation was quite variable between individuals, and many samples were above the upper limit of quantitation for ELISA. The individual variation makes it challenging to establish a standard dilution for all samples to fall within the quantifiable range of the ELISA, while the UFLC–MS/MS method is able to overcome this issue.

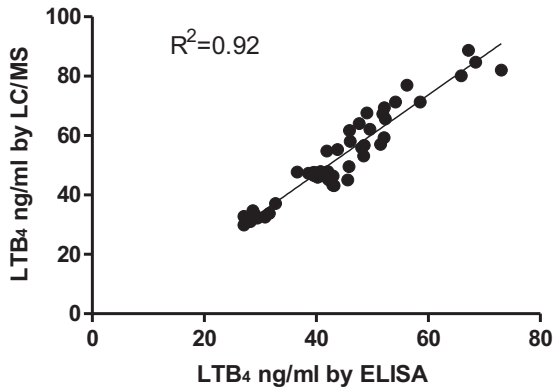

Fig. 8. Correlation of LC-MS/MS vs. ELISA on LTB_4 measurement.

4. Conclusion

A novel UFLC–MS/MS method was developed and validated for the determination of LTB_4 in plasma from ex vivo stimulated human blood. The method is sensitive, selective and robust. The validated method has been successfully used to support two clinical studies in dose selections and general assessment of target occupancy. The validated UFLC–MS-MS assay has been shown to be superior in selectivity and dynamic range to an ELISA assay in our study. The validated LC-MS/MS method for the LTB_4 biomarker will have utility for additional clinical study applications.

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