



## Quantitation of leukotriene B<sub>4</sub> in human sputum as a biomarker using UPLC–MS/MS



Wenying Jian<sup>a,\*</sup>, Richard W. Edom<sup>a</sup>, Xiaohua Xue<sup>b</sup>, Mike-Qingtao Huang<sup>a</sup>, Anne Fourie<sup>b</sup>, Naidong Weng<sup>a</sup>

<sup>a</sup> Drug Safety Sciences, Janssen Research & Development, Johnson & Johnson, Raritan, NJ 08869, United States

<sup>b</sup> Drug Discovery, Immunology, Janssen Research & Development, Johnson & Johnson, La Jolla, CA 92121, United States

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### ABSTRACT

Leukotriene B<sub>4</sub> (LTB<sub>4</sub>) is a potent mediator of inflammation and has been recognized as an important target for therapeutic intervention for treatment of diseases such as asthma. In the current work, a highly selective and sensitive UPLC–MS/MS assay was developed for quantitation of LTB<sub>4</sub> in human sputum as a biomarker for LTB<sub>4</sub> biosynthesis inhibition. A fit-for-purpose strategy for method development, assay qualification, and study support was adopted for this biomarker project. A surrogate matrix (protein buffer) was used for preparation of calibration samples and certain levels of quality control (QC) samples to avoid interference from endogenous analyte, while the low QC was prepared in authentic matrix, human sputum. The analytical methodology utilized a liquid–liquid extraction procedure in 96-well plate format. Chromatographic separation was achieved with a reversed-phase ultra high pressure liquid chromatography (UPLC) column using gradient elution, and the run time was 4.5 min per sample. The lower limit of quantitation (LLOQ) was 0.2 ng/mL, and the calibration curve range was 0.2–20 ng/mL. Acceptable accuracy, precision, linearity, specificity, recovery, and matrix effect was obtained. Bench-top stability (6 h), freeze–thaw stability (3 cycles at –20 °C), and autosampler stability (97 h at ambient temperature) all met acceptance criteria. Frozen long-term stability for 166 days at –20 °C in sputum did not meet acceptance criteria by showing only ≥75% of nominal concentration and the information was taken into consideration for study support. Two important observations in the current work were: (1) LTB<sub>4</sub> was unstable in sputum in the presence of liquification reagent dithiothreitol (DTT). Therefore, a non-DTT treatment method for sputum processing was developed and applied to the bioanalytical assay and clinical study support; and (2) chromatographic separation of LTB<sub>4</sub> from its three non-enzymatically derived isomers, i.e. 6-trans-LTB<sub>4</sub>, 12-epi-LTB<sub>4</sub>, and 6-trans-12-epi-LTB<sub>4</sub>, was achieved. This assay was successfully applied to a Phase II clinical study for proof-of-concept of a LTA<sub>4</sub> hydrolase inhibitor for treatment of asthma.

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### 1. Introduction

Leukotrienes (LTs) are a group of potent pro-inflammatory mediators which are enzymatically generated from their precursor, arachidonic acid [1,2]. Upon stimulation of inflammatory cells such as polymorphonuclear leukocytes, monocytes, macrophages, and

mast cells, free arachidonic acid is released from membrane phospholipids by the action of phospholipase and then converted into Leukotriene A<sub>4</sub> (LTA<sub>4</sub>) through the 5-lipoxygenase/5-lipoxygenase-activating protein (5-LO/FLAP) pathway [3–8]. LTA<sub>4</sub> is further metabolized by LTA<sub>4</sub> hydrolase to form LTB<sub>4</sub>, which is a potent chemotactic agent and activator of inflammatory cells. Alternatively, conjugation of LTA<sub>4</sub> with glutathione and subsequent metabolism of the conjugated glutathione generate the series of cysteinyl leukotrienes, including leukotriene C<sub>4</sub> (LTC<sub>4</sub>), leukotriene D<sub>4</sub> (LTD<sub>4</sub>), and leukotriene E<sub>4</sub> (LTE<sub>4</sub>), all of which play an important role in the inflammation process and allergic response [1,2]. Due to their apparent critical role in diseases such as asthma and chronic obstructive pulmonary disease (COPD), various components in the leukotriene pathway are recognized as important targets for development of therapeutic intervention agents [9–13]. In the current work, an inhibitor of LTA<sub>4</sub> hydrolase is being

**Abbreviations:** BQL, below quantitation limit; COPD, chronic obstructive pulmonary disease; DTT, dithiothreitol; HQC, high QC; HSA, human serum albumin; LLE, liquid–liquid extraction; LLOQ, low limit of quantitation; LQC, low QC; LT, leukotriene; LTA<sub>4</sub>, leukotriene A<sub>4</sub>; LTB<sub>4</sub>, leukotriene B<sub>4</sub>; MQC, mid QC; MRM, multiple reaction monitoring; MS, mass spectrometry; MTBE, methyl-*t*-butyl ether; PBS, phosphate buffered saline; QC, quality control; UPLC, ultra high pressure liquid chromatography.

\* Corresponding author.

E-mail address: [wjian@its.jnj.com](mailto:wjian@its.jnj.com) (W. Jian).

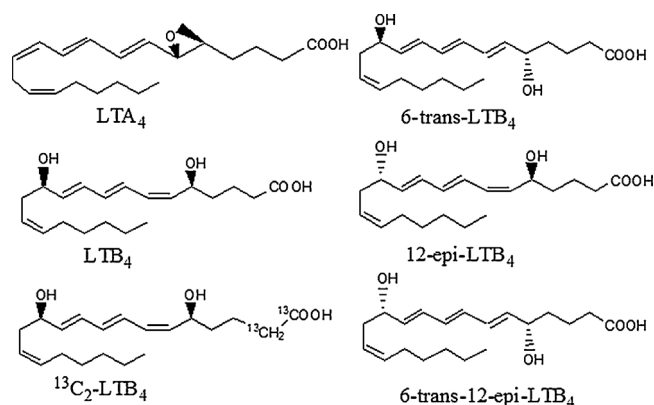


Fig. 1. Structure of LTA<sub>4</sub>, LTB<sub>4</sub>, <sup>13</sup>C<sub>2</sub>-LTB<sub>4</sub>, and the isomers.

developed as a drug candidate for treatment of asthma. A sensitive and selective method for measurement of LTB<sub>4</sub> in human sputum was needed as a biomarker to indicate the effectiveness of the drug candidate for inhibition of LTB<sub>4</sub> biosynthesis.

Analysis of sputum for cellular and soluble mediators is used increasingly as a noninvasive method of assessing airway inflammation [14]. Several analytical methods for quantitation of LTs in sputum have been reported, including HPLC/radioimmunoassay [15], radioimmunoassay (RIA) [10], and enzyme immunoassay (EIA) [16–21]. Although immuno-based assays are highly sensitive, it is difficult to eliminate cross-reactivity. Antibodies, especially when raised against lipids, sometimes do not provide specificity sufficient for discrimination of structurally similar but biologically different lipid mediators. As a result, the LT concentrations reported in literature show a broad range among different platforms and laboratories. Our internal investigation using commercial EIA kit also showed significant overestimation of LTB<sub>4</sub> spiked in sputum samples, especially at low ng/mL levels (data not shown), indicating non-specific detection. Therefore, a more specific platform such as mass spectrometry (MS) needed to be explored for quantitation of LTs in sputum. Recently, Chappell et al. reported an ultra high pressure liquid chromatography (UPLC)–mass spectrometry (MS) based method for quantitation of LTs in human sputum, which demonstrated excellent sensitivity (39.0 pg/mL for LTB<sub>4</sub>) and specificity against certain isomers of LTB<sub>4</sub> [22].

In this work, a highly selective and sensitive UPLC–MS method for quantitation of LTB<sub>4</sub> in human sputum was developed to support a clinical proof of concept study for a LTA<sub>4</sub> hydrolase inhibitor for treatment of asthma. Two unique aspects of the current work that had not been emphasized in previous reports are: (1) stability of LTs in the presence of sputum liquification reagent dithiothreitol (DTT) was thoroughly evaluated. Accordingly, a sputum processing method that preserved the stability of the analyte was developed. (2) Separation of the LTB<sub>4</sub> from its three non-enzymatically derived isomers (Fig. 1), including 6-trans-LTB<sub>4</sub>, 12-epi-LTB<sub>4</sub>, and 6-trans-12-epi-LTB<sub>4</sub> [5,23,24] was achieved, while separation from 12-epi-LTB<sub>4</sub> was not mentioned in previous literature. In addition, a fit-for-purpose strategy for biomarker assay development and assay qualification in support of a clinical study was adopted, and the details are discussed comprehensively in this manuscript.

## 2. Experimental

### 2.1. Chemicals and reagents

LTB<sub>4</sub> (5S, 12R-dihydroxy-6Z, 8E, 10E, 14Z-eicosatetraenoic acid, CAS number 71160-24-2), 6-trans-LTB<sub>4</sub> (5S, 12R-dihydroxy-6E, 8E, 10E, 14Z-eicosatetraenoic acid, CAS number 71652-82-9),

12-epi-LTB<sub>4</sub> (5S, 12S-dihydroxy-6Z, 8E, 10E, 14Z-eicosatetraenoic acid, CAS number 83709-73-3), and 6-trans-12-epi-LTB<sub>4</sub> (5S, 12S-dihydroxy-6E, 8E, 10E, 14Z-eicosatetraenoic acid, CAS number 71548-19-1), LTC<sub>4</sub> [5S-hydroxy-6R-(S-glutathionyl)-7E, 9E, 11Z, 14Z-eicosatetraenoic acid, CAS number 72025-60-6], LTD<sub>4</sub> [5S-hydroxy-6R-(S-cysteinylglycyl)-7E, 9E, 11Z, 14Z-eicosatetraenoic acid, CAS number 73836-78-9], LTE<sub>4</sub> [5S-hydroxy-6R-(S-cysteinyl)-7E, 9E, 11Z, 14Z-eicosatetraenoic acid, CAS number 75715-89-8], and <sup>13</sup>C<sub>2</sub>-LTB<sub>4</sub> (internal standard) were purchased from Cayman Chemicals (Ann Arbor, MI). Acetic acid, formic acid, ethanol, hexane, and human serum albumin (HSA) were obtained from Sigma (St. Louis, MO). Acetonitrile, ethyl acetate, and methanol were purchased from EMD Chemicals Inc. (Gibbstown, NJ). Dimethyl sulfoxide was obtained from Burdick and Jackson (Morristown, NJ). Phosphate buffered saline (PBS) was provided by Mediatech, Inc. (Manassas, VA). Human sputum was collected and processed by Drug Discovery, Immunology, Janssen Research & Development, Johnson & Johnson (La Jolla, CA). Sputolysin (DTT) reagent was purchased from Caldon Biotech (Vista, CA).

### 2.2. Standard and quality control sample preparation

LTB<sub>4</sub> was supplied in ethanol at concentration of 0.1 mg/mL. A standard spiking solution was prepared by diluting the 0.1 mg/mL stock solution in 50/50: acetonitrile/dimethyl sulfoxide (v:v) to a final concentration of 500 ng/mL. A quality control (QC) spiking solution was prepared by diluting a 0.1 mg/mL stock solution in 50/50: acetonitrile/dimethyl sulfoxide (v:v) to a final concentration of 1000 ng/mL. The stock solution and spiking solutions were stored in a freezer (−20 °C).

Due to presence of endogenous levels of LTB<sub>4</sub> in human sputum, and also the limited supply of human sputum as a matrix, the calibration standards, the mid QC (MQC), and high QC (HQC) samples were prepared in a surrogate matrix, 10 mg/mL of HSA in PBS. Calibration standard samples of LTB<sub>4</sub> were prepared at concentrations of 0.2, 0.4, 0.5, 1.0, 5.0, 10.0, 16.0, and 20.0 ng/mL fresh daily by serially diluting the 500 ng/mL standard spiking solution in 10 mg/mL HSA in PBS. The MQC and HQC samples were prepared at concentrations of 10.0 and 16.0 ng/mL by diluting the 1000 ng/mL QC spiking solution in 10 mg/mL HSA in PBS. Low QC (LQC) samples were prepared in pooled control human sputum (non-DTT treated) collected from normal subjects. The baseline concentration of LTB<sub>4</sub> in the sputum was determined in an initial analysis using calibration standard samples prepared in surrogate matrix. An appropriate amount of LTB<sub>4</sub> was then added to the sputum to achieve the nominal concentration of 0.6 ng/mL. The QC samples were stored frozen (−20 °C).

### 2.3. Sample preparation procedure

Two different procedures were initially evaluated for the sputum solubilization process: DTT (Sputolysin)–treatment, and non-DTT treatment. For DTT treatment, the Sputolysin stock solution (containing DTT at 65 mM) was diluted 10-fold with PBS, then mixed with an equal volume of sputum. The samples were kept at room temperature for 15 min (without mixing) and then centrifuged at 380 × g for 5 min. The supernatant was collected and stored at −20 °C. For non-DTT treatment, the sputum was mixed with an equal volume of PBS, shaken for 10 min, and then centrifuged at 380 × g for 5 min. The supernatant was collected and stored at −20 °C. Due to instability of the analytes in presence of DTT in sputum (see Section 3), non-DTT treatment was employed for the final sample collection protocol.

For LTB<sub>4</sub> analysis, the samples were processed using a liquid–liquid extraction procedure. Add 0.1 mL of sputum sample, 25 μL of internal standard spiking solution (100 ng/mL

of  $^{13}\text{C}_2\text{-LTB}_4$  in acetonitrile), and 50  $\mu\text{L}$  of 0.5% acetic acid into the wells of a 96-well polypropylene assay plate. Seal the plate with a 96-well plate cap and then vortex on an Eppendorf plate shaker (Eppendorf, Hamburg, Germany) at a setting of 1000 rpm for 1 min. Remove the seal, add 0.5 mL of the extraction solvent, 20/80:ethyl acetate/hexane (v/v), to each well using an automated liquid handler, Tomtec Quadra 3 SPE (Tomtec, Hamden, Connecticut). Seal the plate again and vortex on the Eppendorf plate shaker at a setting of 1700 rpm for 10 min, followed by centrifugation on a 96-well plate centrifuge (Eppendorf, 5810R) at a setting of  $1550 \times g$  for 3 min. Transfer 0.4 mL of the organic layer to a new 96-well plate using the Tomtec. Evaporate the contents to dryness on a TurboVap 96-well plate evaporator (Perkin Elmer, Waltham, MA) at a setting of  $30^\circ\text{C}$  and flow of 60 (arbitrary unit) for about 60 min. Reconstitute the samples with 100  $\mu\text{L}$  of 40/40/20: water/methanol/acetonitrile (v/v/v) and vortex the plate at a setting of 1000 rpm for 1 min. Inject 20  $\mu\text{L}$  for UPLC-MS analysis.

For analysis of  $\text{LTC}_4$ ,  $\text{LTD}_4$ , and  $\text{LTE}_4$ , a separate sample preparation procedure was used. Add 0.1 mL of sputum sample, 25  $\mu\text{L}$  of internal standard spiking solution (100 ng/mL of  $^{13}\text{C}_2\text{-LTB}_4$  in acetonitrile), and 50  $\mu\text{L}$  of 1.0% formic acid into the wells of a 96-well polypropylene assay plate. Seal the plate with a 96-well plate cap and then vortex on an Eppendorf plate shaker (Eppendorf, Hamburg, Germany) at a setting of 1000 rpm for 1 min. Remove the seal, add 2 mL ethyl acetate to each well using Tomtec. Seal the plate again and vortex on the Eppendorf plate shaker at a setting of 1700 rpm for 10 min, followed by centrifugation on a 96-well plate centrifuge (Eppendorf, 5810R) at a setting of  $1550 \times g$  for 3 min. Transfer 1.6 mL of the organic layer to a new 96-well plate using the Tomtec. Evaporate the contents to dryness on a TurboVap 96-well plate evaporator (Perkin Elmer, Waltham, MA). Reconstitute the samples with 100  $\mu\text{L}$  of 50:50: water/acetonitrile (v/v) and vortex the plate at a setting of 1000 rpm for 1 min. Inject 5  $\mu\text{L}$  for UPLC-MS analysis.

#### 2.4. Liquid chromatography and mass spectrometry

An Acquity UPLC system (Waters, Milford, MA) was used for chromatographic separation. For analysis of  $\text{LTB}_4$ , an Acquity BEH C18 (2.1 mm  $\times$  50 mm, 1.7  $\mu\text{m}$ ) column was used. UPLC mobile phase A was 1 mM ( $\sim 0.004\%$  by volume) formic acid in water, and mobile phase B was 35/65 acetonitrile/methanol (v/v). The weak and strong needle rinse solvents were 10/90 acetonitrile/water (v/v) and acetonitrile, respectively. The seal wash was 10/90 acetonitrile/water (v/v). The gradient started at 60% B, ramped to 90% B over 3 min, stayed at 90% for 0.5 min, and then returned to 60% B in 0.1 min. The run time was 4.5 min, and the UPLC flow rate was 0.65 mL/min. A post-column split of 1/3 MS/waste (v/v) was utilized.

For stability evaluation of  $\text{LTC}_4$ ,  $\text{LTD}_4$ , and  $\text{LTE}_4$ , the analysis was conducted using a Synergi Fusion-RP (2.1 mm  $\times$  50 mm, 4.0  $\mu\text{m}$ , Phenomenex, Torrance, CA) column. Mobile phase A was 5 mM ammonium acetate in water, and mobile phase B was 80/20 acetonitrile/methanol (v/v). The gradient started at 15% B, stayed at 15% for 0.3 min, ramped to 90% B over 1.7 min, stayed at 90% B for 0.5 min, and then returned to 15% B in 0.1 min. The run time was 3.5 min, and the UPLC flow rate was 0.6 mL/min.

The UPLC system was interfaced with an API 5000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). The operating parameters for analysis in negative Turbo Ion-spray mode were at the following values: curtain gas 20 psi, GAS1 15 psi, GAS2 40 psi, Ion-spray voltage  $-4500\text{ V}$ , source temperature  $500^\circ\text{C}$ , and CAD gas 4 (arbitrary units). Multiple reaction monitoring (MRM) transitions for  $\text{LTB}_4$ ,  $^{13}\text{C}_2\text{-LTB}_4$ ,  $\text{LTC}_4$ ,  $\text{LTD}_4$ , and  $\text{LTE}_4$  were  $m/z\ 335 \rightarrow 195$ ,  $m/z\ 337 \rightarrow 197$ ,  $m/z\ 624 \rightarrow 272$ ,  $m/z\ 495 \rightarrow 177$ ,

and  $m/z\ 438 \rightarrow 333$ , respectively. The analyte-dependent parameters were: entrance potential  $-10\text{ V}$ , and collision cell exit potential  $-8\text{ V}$ . Declustering potential for  $\text{LTB}_4$ ,  $^{13}\text{C}_2\text{-LTB}_4$ ,  $\text{LTC}_4$ ,  $\text{LTD}_4$ , and  $\text{LTE}_4$  were  $-90\text{ V}$ ,  $-90\text{ V}$ ,  $-50\text{ V}$ ,  $-60\text{ V}$ ,  $-50\text{ V}$ , respectively. Collision energy for  $\text{LTB}_4$ ,  $^{13}\text{C}_2\text{-LTB}_4$ ,  $\text{LTC}_4$ ,  $\text{LTD}_4$ , and  $\text{LTE}_4$  were  $-23\text{ V}$ ,  $-23\text{ V}$ ,  $-32\text{ V}$ ,  $-26\text{ V}$ , and  $-25\text{ V}$ , respectively.

The chromatographic peaks for the analytes and internal standards were integrated using Analyst 1.5.1. software (Applied Biosystems, Foster City, CA). The peak areas were imported to our Watson LIMS system (Innaphase, Philadelphia, PA) for regression analysis. The peak area ratios of analyte/internal standard were calculated and used to establish the calibration standard curve using linear regression (weighing factor,  $1/x^2$ ).

#### 2.5. Method development and assay qualification experiments

Stability of the analyte,  $\text{LTB}_4$ , as well as the cysteinyl leukotrienes in sputum in the presence and absence of DTT were evaluated during method development.  $\text{LTB}_4$ ,  $\text{LTC}_4$ ,  $\text{LTD}_4$ , and  $\text{LTE}_4$  were spiked together into the same lots of human sputum that were either with, or without, DTT pre-treatment at a concentration of 200 ng/mL for each analyte. The samples were aliquoted and stored either frozen or at room temperature for 18 h. The samples were then processed according to the extraction procedures described in Section 2.3 and analyzed according to the LC-MS conditions described in Section 2.4, and the peak area ratios were compared against those of freshly prepared non-DTT treated samples at the same concentration.

Recovery and matrix effects were determined during method development. Recovery was determined by comparing the peak area ratios ( $\text{LTB}_4/^{13}\text{C}_2\text{-LTB}_4$ ) in extracted sputum samples pre-spiked with  $\text{LTB}_4$  to those from extracted control sputum post-spiked with  $\text{LTB}_4$ . For both pre-spiked and post-spiked samples,  $^{13}\text{C}_2\text{-LTB}_4$  was post-spiked to same concentration to eliminate variations caused by different recovery and matrix effects. Matrix effect was determined by spiking  $\text{LTB}_4$  at the same concentration into extracted control human sputum (post-spike) and neat solution. The matrix factor was calculated by dividing the peak area of  $\text{LTB}_4$  in post-spiked sample by that obtained from neat. In both cases for recovery and matrix effect determination, the analyte was spiked at concentrations much higher than the baseline levels in control human sputum, therefore minimizing the impact of endogenous analyte.

In method qualification, the linearity, accuracy and precision of the assay were assessed in three separate analytical batch runs. Each batch included a standard curve and four replicates of the low, mid and high QC. The standard curve from each batch was regressed separately and the values for the QCs were calculated.

A specificity test was conducted to show separation of  $\text{LTB}_4$  from the other three isomers, 6-trans- $\text{LTB}_4$ , 12-epi- $\text{LTB}_4$ , and 6-trans-12-epi- $\text{LTB}_4$ . Individual neat solutions of each of the four isomers at a concentration of 10 ng/mL, and a mixed neat solution of the four isomers at 10 ng/mL for each, were injected to demonstrate the chromatographic separation.

Bench-top stability samples were prepared in four replicates at the LQC and HQC level and kept at room temperature for 6 h before extraction. Freeze-thaw stability was evaluated at the LQC and HQC level in four replicates by freezing the samples completely at  $-20^\circ\text{C}$  then allowing them to thaw completely at room temperature for three cycles. The samples were extracted with freshly prepared standard curves and QC samples. The mean concentrations of the tested samples were calculated and compared against the nominal concentrations. The processed sample/autosampler stability was evaluated by re-injecting extracted LQC and HQC samples that had been kept in the autosampler at ambient temperature for 97 h. The mean concentrations of the re-injected QCs were calculated using

**Table 1**  
Stability of LTs in human sputum. (A) Stability of LTs human sputum. The data were normalized to freshly spiked non-DTT samples for each analyte. (B) Stability of LTB<sub>4</sub> in different lots of human sputum. The data were normalized to freshly spiked non-DTT samples for each lot.

| A           | Non-DTT          |                  |                  |                  | DTT treated      |                  |                  |                  |
|-------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|
|             | LTB <sub>4</sub> | LTC <sub>4</sub> | LTD <sub>4</sub> | LTE <sub>4</sub> | LTB <sub>4</sub> | LTC <sub>4</sub> | LTD <sub>4</sub> | LTE <sub>4</sub> |
| Fresh spike | 100.0            | 100.0            | 100.0            | 100.0            | 107.1            | 41.0             | 45.2             | 26.6             |
| –20 °C 18 h | 99.4             | 30.5             | 36.4             | 20.8             | 72.6             | 57.1             | 54.6             | 57.8             |
| RT 18 h     | 96.7             | 15.6             | 47.5             | 19.6             | 44.8             | 13.1             | 31.3             | 26.9             |

| B           | Non-DTT |       |       |       |                | DTT Treated |       |       |       |                |
|-------------|---------|-------|-------|-------|----------------|-------------|-------|-------|-------|----------------|
|             | Lot 1   | Lot 2 | Lot 3 | Lot 4 | Protein buffer | Lot 1       | Lot 2 | Lot 3 | Lot 4 | Protein buffer |
| Fresh spike | 100.0   | 100.0 | 100.0 | 100.0 | 100.0          | 114.6       | 100.0 | 94.2  | 99.4  | 108.0          |
| –80 °C 18 h | 93.1    | 94.7  | 85.1  | 100.6 | 99.7           | 105.3       | 83.9  | 76.9  | 85.3  | 97.1           |
| –20 °C 18 h | 94.7    | 101.6 | 89.7  | 91.7  | 103.8          | 103.2       | 85.3  | 72.8  | 54.7  | 98.3           |
| RT 18 h     | 95.9    | 101.3 | 96.4  | 92.4  | 109.0          | 103.4       | 72.0  | 59.7  | 69.7  | 103.7          |

RT: room temperature.

a freshly prepared standard curve and were compared against the nominal concentrations. The long-term storage stability of each analyte in human sputum at –20 °C was evaluated up to 166 days. The LQC and HQC were extracted in four replicates with a freshly prepared standard curve. The mean determined concentrations for the QCs were compared against the nominal concentrations.

### 3. Results and discussion

#### 3.1. Method development

The first task was to choose a suitable process for generating the matrix for LTB<sub>4</sub> analysis. Sputum usually contains a high level of mucus glycoproteins, and therefore, it is viscous and sticky. A liquification process by adding a reducing mucolytic agent, such as DTT, to sputum is usually required to reduce the viscosity for easier handling and analysis [25,26]. DTT is a strong reducing reagent which can break the disulfide bonds present in mucus proteins and thus release the components in sputum for analysis. However, stability of leukotrienes in the presence of DTT was a potential issue. Previous research using an enzyme-linked immunosorbent assay (ELISA) to quantify LTB<sub>4</sub> has shown that sputum processing with DTT significantly reduces the detectable concentrations of LTB<sub>4</sub> [27]. The proposed mechanism was that DDT could directly interfere with the immunoassay, while instability was not mentioned as a potential factor. In this work, we evaluated the stability of LTB<sub>4</sub> in sputum in the presence of DTT, as well as the stability of other related molecules, including the cysteinyl leukotrienes LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub>. The stability experiment in human sputum in the absence and presence of DTT was conducted during method development as described in Section 2.5. The analytes were spiked at 200 ng/mL, a concentration much higher than the baseline concentrations that would be observed in human sputum, therefore eliminating the potential complications caused by endogenous levels.

As demonstrated in Table 1A, LTB<sub>4</sub> was stable in non-DTT treated sputum both at room temperature and under frozen (–20 °C) conditions, while showing a significant loss in DTT-treated sputum. In comparison, LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub> all experienced significant losses in both non-DTT and DTT treated sputum under both test conditions, as well as in freshly spiked DTT samples. In this experiment, all the analytes were spiked together. Since the biotransformation pathway of cysteinyl leukotrienes is from LTC<sub>4</sub> to LTD<sub>4</sub>, and LTE<sub>4</sub>, consecutively, the observed pattern of loss implied other degradation pathways. Due to the significant instability of LTC<sub>4</sub>, LTD<sub>4</sub>, and LTE<sub>4</sub> in sputum, it was decided that LTB<sub>4</sub> would be the primary target analyte for the current work. The experiment was further repeated for LTB<sub>4</sub> in four lots of human sputum, and a protein buffer (10 mg/mL HSA in PBS) as control. An additional storage condition

of –80 °C was also included. The results further confirmed that LTB<sub>4</sub> was relatively stable in the absence of DTT under all test conditions (Table 1B). In comparison, three of the four lots of sputum containing DTT showed a significant loss of LTB<sub>4</sub>, which was largely temperature-dependent. Minimal loss in lot 1 was speculated due to less protein/cellular contents in it, as this lot was observed to be much less sticky than the other three lots. Interestingly, in protein buffer containing DTT, LTB<sub>4</sub> was stable under all test conditions, implying that the instability was caused by a combination of DTT and the components in sputum. Given these results, it was decided that non-DTT treatment would be adopted for the sample collection procedure for the clinical study. Since the study was conducted for asthma patients, whose sputum is usually not very viscous, a non-DTT treatment (simply adding PBS as dilution buffer) was sufficient to achieve the desired state for handling and analysis of the sputum.

Different organic solvents and buffers were evaluated for optimal extraction recovery of LTB<sub>4</sub> from sputum. It was found that buffering using 0.5% acetic acid in a ratio of half the relative volume of sputum, followed by MTBE extraction, yielded a poor recovery of ~42%, even though the matrix factor was ~1.0. Then, different percentages of ethyl acetate in n-hexane (20%, 40%, 60%, and 80%) with combinations of acetic acid or formic acid were evaluated. It was found that recovery decreased as the proportion of ethyl acetate increased. In addition, use of formic acid rather than acetic acid compromised the recovery. Interestingly, the matrix factors obtained under all the tested conditions were close to 1, indicating very clean extracts and minimal ion suppression or enhancement. The highest recovery (92%) was achieved by using 0.5% acetic acid buffer followed by extraction using 20% ethyl acetate in n-hexane, which was used as the final condition (Table 2).

**Table 2**  
Recovery and matrix effect evaluation.

|               | Recovery (peak area ratio)          |            |
|---------------|-------------------------------------|------------|
|               | Post-spike                          | Pre-spike  |
| Replicate 1   | 2.380                               | 2.204      |
| Replicate 2   | 2.442                               | 2.251      |
| Replicate 3   | 2.358                               | 2.162      |
| Average       | 2.390                               | 2.210      |
| Recovery (%)  |                                     | 92.16      |
|               | Matrix effect (instrument response) |            |
|               | Neat                                | Post-spike |
| Replicate 1   | 6.26E+05                            | 6.45E+05   |
| Replicate 2   | 6.43E+05                            | 6.52E+05   |
| Replicate 3   | 6.52E+05                            | 6.46E+05   |
| Average       | 6.40E+05                            | 6.48E+05   |
| Matrix effect |                                     | 1.01       |

For chromatography method development, we focused on two important aspects: (1) separation of LTB<sub>4</sub> from its isomers; and (2) method sensitivity. It is known that there are three isomers of LTB<sub>4</sub> generated from non-enzymatic hydrolysis of the precursor, leukotriene A<sub>4</sub> (LTA<sub>4</sub>), including 6-trans-LTB<sub>4</sub>, 12-epi-LTB<sub>4</sub>, and 6-trans-12-epi-LTB<sub>4</sub> (Fig. 1). They share identical MRM transitions with authentic LTB<sub>4</sub>, and they may cause overestimation of its concentrations if not chromatographically separated. After extensive screening of columns and chromatography conditions, a reversed-phase UPLC column (BEH C18) was utilized to achieve the best chromatographic resolution. It was found that methanol in mobile phase B was the key for separation. However, acetonitrile helped improve sensitivity, elution time, and sharpness of the chromatographic peaks. A balance of the above factors was achieved by using a combination of 35/65:acetonitrile/methanol (v/v) in mobile phase B under gradient conditions. The total run time was only 4.5 min. Maintenance of separation was observed in multiple columns used for this project.

Increased sensitivity was addressed by using mobile phase additives and post-column splitting. LTB<sub>4</sub> was monitored under negative mode electrospray ionization, for which mobile phase pH and additives were found to be essential for high sensitivity. It was reported previously that 1 mM formic acid (~0.004%) in the mobile phase was optimal for negative electrospray ionization, even though it is counter-intuitive [28]. It was confirmed in our experiment to be better than some other commonly used buffer systems such as ammonium formate, ammonium acetate, formic acid at higher concentrations, or basic additives. Sensitivity was further improved by post-column splitting for reduction of mobile phase flow into the ion source, which enhanced efficiency of the electrospray ionization process. A split of 1:3 (MS:waste) was found to improve the signal intensity by 30% in comparison to no split; however, further reduction of the ratio of MS:waste caused a net decrease in signal. In the end, excellent sensitivity was achieved with an LLOQ of 200 pg/mL from an extraction of 0.1 mL of sputum.

In terms of the throughput of current method, 96-well plate format significantly improved the efficiency of liquid–liquid extraction in comparison to traditional format of individual tubes. In addition, the relative short run time (4.5 min) on the UPLC system contributed to the overall high speed of the method. It takes about 2 h to extract one 96-well plate of samples and about 8 h to analyze the whole plate on the LC–MS system. Two 96-well plates (192 samples) can be completed by one chemist within one day.

### 3.2. Bioanalytical assay strategy

One of the biggest challenges for quantitation of endogenous analytes, such as LTB<sub>4</sub>, originates from their endogenous nature. The analytes normally pre-exist in biological matrices used for preparation of calibration and QC samples, and the levels may be variable from lot-to-lot. This complicates the assay and may lead to compromised quantitation results. To overcome this challenge, two potential strategies could be used: (1) surrogate analyte in authentic matrix; and (2) authentic analyte in surrogate matrix [29,30]. Typically, a surrogate analyte is a stable isotope labeled version of the analyte, which will show nearly identical physical and chemical properties, LC behavior, and MS ionization efficiency to the authentic analyte, while being differentiated based on mass (i.e. MRM transitions). The concentration–instrument response relationship (the calibration curve) established using the surrogate analyte can therefore be applied to the instrument response of the authentic analyte to calculate concentrations. Very often, a second version of stable isotope labeled analyte is used as internal standard. This approach completely eliminates interference from the endogenous analyte at all concentration levels and enables preparation of QC samples in authentic matrix to cover the entire calibration range.

Importantly, it also enables quantification of endogenous analytes below their baseline concentration. Such would be the case when a therapeutic agent was designed to cause a net decrease in a biomarker or endogenous compound. For LTB<sub>4</sub>, the surrogate analyte approach was initially considered since two versions of stable isotope labeled analyte were commercially available: <sup>13</sup>C<sub>2</sub>-LTB<sub>4</sub>, and d<sub>4</sub>-LTB<sub>4</sub>. However, the authentic matrix, human sputum, was in short supply due to the difficult collection procedure. As a result, the alternative approach of authentic analyte in surrogate matrix had to be employed. A protein buffer containing 10 mg/mL of HSA in PBS, was used as a surrogate matrix for preparation of calibration samples. Due to the different nature of the surrogate matrix from authentic human sputum, bias could be introduced to LC–MS based assays in terms of matrix effects, specificity, stability, and recovery. Therefore, QC's should ideally be prepared in authentic matrix to fully reflect the assay performance in real matrix. However, our supply of sputum was only sufficient to prepare one level of QC sample. Since the baseline concentration of LTB<sub>4</sub> was very low in sputum (~0.26 ng/mL in a lot of pooled sputum), it was decided the LQC would be prepared in real matrix. The baseline concentration of LTB<sub>4</sub> in the sputum pool was estimated in an initial run. An appropriate amount of LTB<sub>4</sub> was then added to the sputum pool to achieve the nominal concentration of LQC (0.6 ng/mL). The MQC and HQC were prepared in surrogate matrix. The capability to measure the baseline level, and to accurately achieve the designated concentration of LQC, verified the performance of the assay at the lower end of the calibration curve, which was a confirmation of the reliability of the assay.

Currently, there is no specific regulatory guidance governing assay validation and study support for biomarkers in drug development. The consensus of the bioanalytical community is that a “fit-for-purpose” approach should be used for biomarker quantitation [31–34]. For biomarkers used in early drug development stages, such as discovery or Phase I and Phase II clinical trials, the data are generally used internally to make decisions about candidate selection or continuing the development of the drug. The extent of method validation is not considered of critical regulatory importance and the sponsor could incorporate whatever level of validation deemed appropriate [35,36]. Our application for this LTB<sub>4</sub> assay in a Phase II clinical study fell into this category, and therefore, it was characterized with experiments and parameters deemed scientifically sufficient to support the data generated by the assay and the decisions which needed to be made. The experiments considered critical for evaluation of assay performance were conducted, including accuracy and precision, specificity, and stability, which, to be differentiated from that for a routine drug assay, was named “qualification” rather than “validation”. Additional bio-analytical parameters, such as recovery and matrix effects, were evaluated and documented during method development, as discussed previously. Another practice was to adopt an acceptance criteria of 20% instead 15% in our assay qualification, considering the overall analytical challenges for a relatively unstable endogenous analyte.

### 3.3. Assay qualification

A lower limit of quantitation (LLOQ) of 0.2 ng/mL was achieved. Fig. 2A demonstrates a control human sputum sample that contains 0.26 ng/mL of LTB<sub>4</sub>. The chromatogram of a LQC prepared at concentration of 0.6 ng/mL by spiking additional LTB<sub>4</sub> into control human sputum is shown in Fig. 2B. A signal-to-noise ratio greater than 3 was achieved in both cases.

The linearity of the calibration curves was determined over the specified range of 0.2–20.0 ng/mL (Table 3). The calibration curve showed a coefficient of determination (R-squared) greater than 0.99. The %CV of the standards was ≤13.3% and the %Bias was within

**Table 3**  
Linearity, accuracy and precision of calibration standards ( $n=3$ ).

|                       | STD1  | STD2  | STD3  | STD4  | STD5 | STD6 | STD7 | STD8 | R-squared |
|-----------------------|-------|-------|-------|-------|------|------|------|------|-----------|
| Nominal conc. (ng/mL) | 0.20  | 0.40  | 0.50  | 1.00  | 5.00 | 10.0 | 16.0 | 20.0 | –         |
| Overall mean          | 0.202 | 0.401 | 0.491 | 0.964 | 5.19 | 10.4 | 16.1 | 19.1 | 0.99463   |
| %CV                   | 4.1   | 7.0   | 13.3  | 3.3   | 2.6  | 2.5  | 2.9  | 2.9  | 0.4       |
| %Bias                 | 1.0   | 0.3   | –1.8  | –3.6  | 3.8  | 4.0  | 0.6  | –4.5 | –         |
| $n$                   | 3     | 3     | 3     | 3     | 3    | 3    | 3    | 3    | 3         |

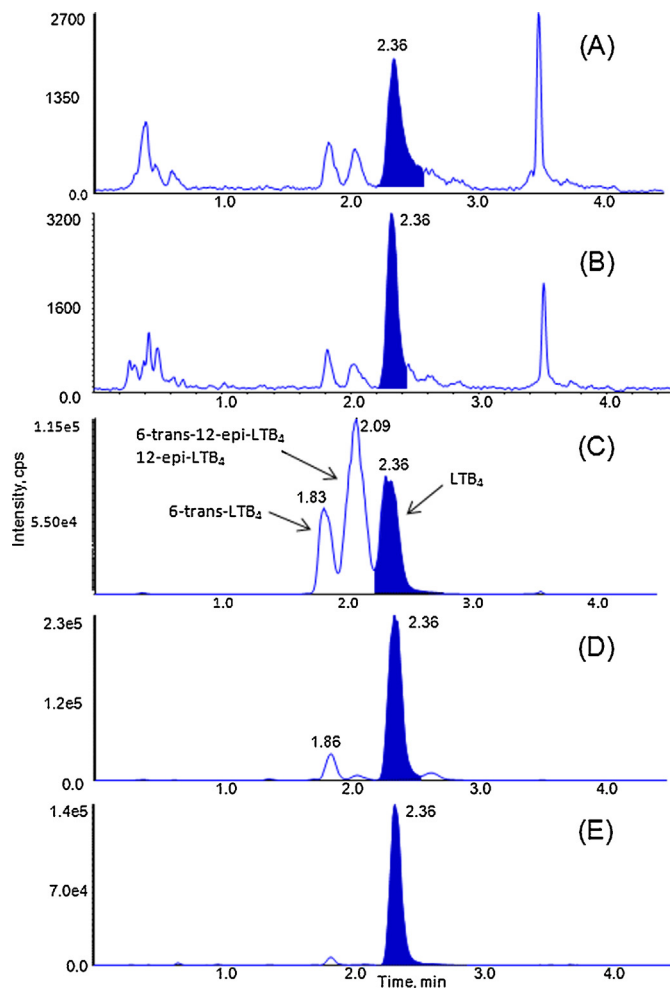


**Fig. 2.** LC-MS/MS chromatograms of LTB<sub>4</sub>. (A) Control human sputum; (B) LQC in human sputum; (C) neat solution of LTB<sub>4</sub> and its isomers showing separation; (D) an incurred human sputum sample; (E) internal standard in human sputum.

$\pm 4.5\%$  at all concentrations. Intra-assay and inter-assay accuracy and precision were assessed over three separate days by preparing a daily standard calibration curve along with 4 replicates of LQC, MQC, and HQC samples. Statistical analysis of the accuracy and precision is presented in Table 4. The intraday and interday %CV were  $\leq 12.4\%$  and  $\leq 12.6\%$ , respectively. The intraday and interday %Bias was within  $\pm 4.4\%$  and  $\pm 12.7\%$ , respectively.

**Table 4**  
Accuracy and precision results.

|     | Nominal conc. (ng/mL) | Intraday ( $n=4$ ) |      | Interday ( $n=3$ ) |      |
|-----|-----------------------|--------------------|------|--------------------|------|
|     |                       | %Bias              | %CV  | %Bias              | %CV  |
| LQC | 0.60                  | –2.5               | 12.4 | –12.7              | 12.6 |
| MQC | 10.0                  | –0.5               | 11.7 | –5.0               | 7.9  |
| HQC | 16.0                  | –4.4               | 6.1  | –6.9               | 5.6  |

The specificity for an endogenous analyte is very challenging to confirm due to constant presence of the analyte in the biological matrix. In the current work, careful comparison of the peak shape between injections of the neat analyte and extracted matrix samples under several different chromatographic conditions during method development provided evidence that the observed peak in the matrix samples was for the intended analyte, LTB<sub>4</sub>. More importantly, it was very critical to demonstrate specificity for LTB<sub>4</sub> against the three isobaric isomers. The specificity in method qualification was demonstrated by baseline separation of LTB<sub>4</sub> from the three isomers (Fig. 2C). In addition, the specificity of LTB<sub>4</sub> in the surrogate matrix was demonstrated by absence of analyte peak at the expected retention time from injection of an extracted surrogate matrix sample (data not shown).

The stability results are presented in Table 5. Overall, LTB<sub>4</sub> demonstrated acceptable stability under short term conditions in both authentic matrix, as observed for LQC prepared in sputum, and surrogate matrix, as observed for HQC in 10% HSA in PBS. The conditions evaluated included on the bench-top in matrix for 6 h, freeze–thaw in matrix for 3 cycles at  $-20^\circ\text{C}$ , and in the autosampler at ambient temperature for 97 h. The %Bias from nominal concentration was within  $\pm 15.3\%$  for both concentrations under all tested conditions. Since we routinely adopt (a priori) an acceptance criteria of 20% for all qualified assays in our laboratory, this was considered acceptable. However, the long-term freezer stability test did not meet acceptance criteria. The measured concentrations of QC samples stored at  $-20^\circ\text{C}$  for 166 days were lower than the nominal concentrations by 16.2% and 25.0%, in authentic matrix (LQC) and surrogate matrix (HQC), respectively. This was not an unexpected finding since LTB<sub>4</sub> is known to be chemically unstable over significant periods of time. The stability information was communicated to the clinical project team and was taken into consideration when interpreting the clinical data generated from incurred samples.

### 3.4. Assay application

During qualification, the assay was used to survey LTB<sub>4</sub> levels in sputum samples collected from normal subjects and patients diagnosed with COPD (or likely COPD). In general, the measured LTB<sub>4</sub> concentrations in normal subjects were lower than those in COPD patients (Table 6), consistent with the role of LTB<sub>4</sub> in inflammatory pulmonary diseases. Three out of five normal subjects showed concentrations below quantitation limit (BQL), except for one lot that was measured to be 4.18 ng/mL, much higher than the other samples for unknown reasons. The sputum lots from the normal subjects, excluding the one outlier, were then pooled and used as the matrix for preparation of LQC.

**Table 5**  
Stability evaluation. Results are expressed as mean %Bias from nominal concentration ( $n=4$ ).

|                                        | LQC (0.60 ng/mL) | HQC (16.0 ng/mL) |
|----------------------------------------|------------------|------------------|
| Room temp 6 h                          | –13.2            | –11.9            |
| Freeze/thaw 3 cycles                   | –10.2            | –13.1            |
| Autosampler 97 h                       | –15.3            | –8.1             |
| Long term $-20^\circ\text{C}$ 166 days | –16.2            | –25.0            |

**Table 6**LTB<sub>4</sub> concentration in sputum samples.

| Lot | Subject     | Concentration (ng/mL) |
|-----|-------------|-----------------------|
| 1   | Normal      | BQL (<0.2)            |
| 2   | Normal      | 4.18                  |
| 3   | Normal      | 0.56                  |
| 4   | Normal      | BQL (<0.2)            |
| 5   | Normal      | BQL (<0.2)            |
| 6   | Likely COPD | 1.05                  |
| 7   | COPD        | 0.21                  |
| 8   | COPD        | 0.74                  |
| 9   | COPD        | 0.89                  |

The qualified assay was applied in support of a Phase II clinical study. Sputum samples were collected from asthma patients treated with placebo or an LTA<sub>4</sub> hydrolase inhibitor. The patients were challenged by a bronchial allergen after drug or placebo administration. Fig. 2D and E are typical chromatograms of LTB<sub>4</sub> and the internal standard <sup>13</sup>C<sub>2</sub>-LTB<sub>4</sub> from an incurred sample, respectively. Baseline separation was shown for LTB<sub>4</sub> from its isomers, as well as from an interference peak eluting later than the analyte. The data indicated that treatment with the LTA<sub>4</sub> hydrolase inhibitor significantly reduced production of LTB<sub>4</sub> in asthma patients upon bronchial allergen challenge. The clinical study results are being published by the clinical project team separately as a poster [37] and manuscript (in preparation).

#### 4. Conclusion

A highly selective and sensitive UPLC–MS/MS method for quantitation of LTB<sub>4</sub> in human sputum as a biomarker was developed, qualified, and employed for support of a clinical study. A fit-for-purpose biomarker assay strategy was adopted for assay design, method development, and qualification. Important aspects of the bioanalytical assay, including stability of analyte in the presence of sputum liquification reagent DTT, recovery, and matrix effects were evaluated in method development. The linearity, accuracy, precision, stability and specificity was demonstrated in assay qualification. The assay was successfully applied in a Phase II clinical study for proof-of-concept of LTA<sub>4</sub> hydrolase inhibition for treatment of asthma.

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