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Quantitative measurement of cysteinyl leukotrienes and leukotriene B_4 in human sputum using ultra high pressure liquid chromatography–tandem mass spectrometry

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ABSTRACT

The role of leukotrienes (LTs) in airway inflammatory diseases, such as asthma, has been extensively reported. The measurement of LTs in sputum supernatants, which is commonly done via enzyme immunoassays (EIAs), may prove to be useful for assessing airway inflammation. Despite the many advantages of EIA, these methods suffer from a lack of selectivity. Therefore, a selective and reliable method for the analysis of LTs in human sputum is needed. In this study we developed and validated a sensitive and specific method using ultra high pressure liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS), to measure simultaneously cysteinyl leukotrienes (CysLTs) and leukotriene B_4 (LTB_4) in human sputum. Sputum supernatants obtained by ultracentrifugation were stabilized by protease inhibitors, spiked with stable isotopic internal standards, and subjected to solid phase extraction (SPE) and UHPLC separation. Multiple reaction monitoring (MRM) transitions were optimized and measured on a mass spectrometer. The limit of detection (LOD) for LTE_4 and LTB_4 was 9.8 and 19.5 pg/mL, respectively. The lower limit of quantitation (LLOQ) for LTE_4 and LTB_4 was 19.5 and 39.0 pg/mL, respectively. The dynamic range of the LTE_4 assay was from 9.8 to 5000 pg/mL, whereas for the LTB_4 assay was from 19.5 to 10,000 pg/mL. The intra- and inter-day % coefficient of variation (%CV) was <6.5% and <10%, for both LTE_4 and LTB_4, respectively. Spike recovery ranged from 105% to 111% for both analytes. In addition, twenty-two sputum samples were analyzed for cysLTs and LTB_4. Fourteen of these samples were purchased commercially and eight were collected during the course of a clinical trial. LTB_4 was detectable in all samples tested and it ranged from 79 to 7220 pg/mL. LTE_4 was detectable in most of the sputum samples (12.3–891 pg/mL), whereas LTC_4 and LTD_4 were below limit of detection for majority of sputum samples. The *in vitro* conversion of LTC_4 and LTD_4 into LTE_4 was observed. The measurement of LTB_4 was sensitive to low pH and high temperature. The use of UHPLC–MS/MS method will allow a more accurate and reliable quantitation of LTs in human sputum, which in turn, may lead to a better understanding of the role of LTs in airway disease pathways and the application in associated clinical treatments.

1. Introduction

Leukotrienes (LTs) are a group of potent lipid mediators of the inflammatory response derived from arachidonic acid. The structures of LTs are shown in Fig. 1. Biosynthesis and metabolism of LTs were studied and reported [1]. Briefly, arachidonic acid is released from phospholipids by the action of different phospholipase A_2 enzymes and converted into leukotriene A_4 (LTA_4) through the 5-lipoxygenase/5-lipoxygenase-activating protein (5-LO/FLAP) pathway. LTA_4 is subsequently metabolized by LTA_4 hydrolase into LTB_4 and, by LTC_4 synthase or different members of the membrane-associated proteins in the eicosanoid and glutathione metabolism

Abbreviations: LTs, leukotrienes; CysLTs, cysteinyl leukotrienes; LTB_4-d_4, deuterium labeled leukotriene B_4; LTE_4-d_5, deuterium labeled leukotriene E_4; UHPLC–MS/MS, ultra high pressure liquid chromatography–tandem mass spectrometry; SPE, solid phase extraction; MRM, multiple ion monitoring; 5-LO, 5-lipoxygenase; FLAP, 5-lipoxygenase-activating protein; COPD, chronic obstructive pulmonary disease; EIA, enzyme immunoassay; FEV_1, forced expiratory volume in one second; ISs, internal standards.

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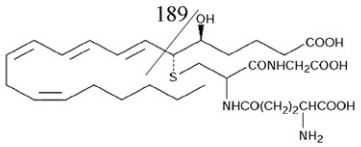
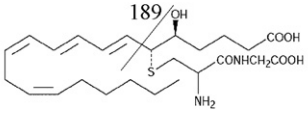
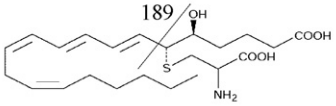
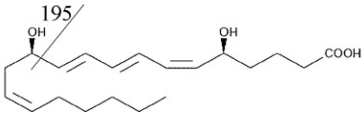
LTs	Chemical Structure	Precursor Ion	Product Ion
LTC_4		626.4 $[M+H]^-$	189.2 (100), 308.2 (100)
LTD_4		497.5 $[M+H]^-$	189.3 (100), 301.4 (36), 437.2 (17)
LTE_4		440.4 $[M+H]^-$	189.2 (100), 301.5 (38)
LTB_4		335.2 $[M-H]^-$	195.2 (100), 317.2 (33)

Fig. 1. Chemical structures of the LTs with the site of cleavages and the precursor ion, product ion optimized for MRM quantitation. Deprotonated or protonated precursor ion and product ion recorded are represented as *m/z* (relative abundance). Relative abundance in product ion has been normalized to the strongest MRM signals.

superfamily, such as microsomal glutathione transferase 2, into LTC_4 [1,2]. This is metabolized by a gamma-glutamyl transpeptidase into LTD_4, which is then metabolized by a dipeptidase into LTE_4. The role of LTs in various inflammatory diseases has been recognized by the successful development of selective leukotriene inhibitors and receptor antagonists for the treatment of disease [1,3].

Quantitative analysis of LTs has become an important tool when studying inflammatory airway diseases. Sputum cell analysis and measurement of biomolecules in sputum supernatants have been explored as a semi-invasive sampling method to study chronic lung diseases such as asthma, chronic obstructive pulmonary disease (COPD) and interstitial lung disease [4–6]. For instance, sputum CysLTs concentrations were found to be higher in patients with eosinophilic airway inflammation [7]. In an additional study, increased levels of CysLTs have been reported in sputum from patients with aspirin-intolerant asthma [8] in the diagnosis of aspirin-intolerant asthma. It has been reported that LTB_4 is a potent inflammatory mediator and can be detected in sputum supernatants [9]. CysLTs, including LTE_4 and LTB_4, have been detected in exhaled breath condensate [10,11], bronchoalveolar lavage fluid [12], and urine [13].

Several analytical methods for the quantification of CysLTs and/or LTB_4 in sputum have been reported, including HPLC/radioimmunoassay [14], radioimmunoassay [15], and enzyme immunoassay (EIA) [7,9,16,17]. LT concentrations in human sputum have been reported in wide ranges (from pg/mL to ng/mL) between labs and available technologies, which suggest a need for improved measurement of LTs in sputum. Mass spectrometry-based assays are normally considered the gold standard for the bioanalysis of small molecules based on specificity, sensitivity, and the capability of measuring multiple analytes simultaneously. In this report we describe the development and validation of a simple and robust analytical method using UHPLC–MS/MS to measure simultaneously CysLTs and LTB_4 in human sputum. Two important observations in this study were the instability of LTB_4 during sample processing and the conversion of LTC_4 and LTD_4 into LTE_4 *in vitro*.

2. Materials and methods

2.1. Chemicals and reagents

LTC_4, LTD_4, LTE_4, LTE_4-d_5, LTB_4, and LTB_4-d_4 were purchased from Cayman Chemical (Ann Harbor, MI, USA). HPLC-grade water containing 0.1% formic acid, acetonitrile, acetonitrile containing 0.1% formic acid, GC grade methanol, Optima LC/MS-grade water, 99% formic acid, and ammonium hydroxide (NH_4Ac) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Phosphate buffered saline (PBS), and Sigmacote were obtained from Sigma (St. Louis, MO, USA). Protease inhibitor cocktail was prepared from Complete Mini (EDTA-Free, Roche Diagnostics) by adding 1 protease inhibitor cocktail tablet in 10 mL 1 × PBS according to manufacture specification.

2.2. Preparation of standard stocks, calibration curve and quality controls

A calibration standard stock solution containing a mixture of 50 ng/mL of LTB_4, and 25 ng/mL each of LTE_4, LTD_4, and LTC_4, was prepared in methanol. The mixed internal standard (IS) stock solution containing 25 ng/mL of LTB_4-d_4 and 8 ng/mL of LTE_4-d_5 was made in methanol. The stock solutions were aliquoted and stored at −80 °C. Calibration standards were prepared by serial dilution in 15% methanol with protease inhibitor cocktail with concentration range of 9.8–5000 pg/mL for LTC_4, LTD_4, and LTE_4 and 19.5–10,000 pg/mL for LTB_4.

Quality controls (QC1, QC2) were prepared from pooled sputum supernatant following sputum ultracentrifugation. Due to the limited availability of sputum samples, three QCs (Low, Med, and High) were prepared by spiking three levels of LTE_4 and LTB_4 into human EDTA plasma diluted 1:50 in 1 × PBS with protease inhibitor cocktail. The diluted plasma was selected as the matrix that might mimic sputum supernatant in the total protein concentration. The QCs were aliquoted and stored at −80 °C until use.

Table 1
MRM conditions for LTs and deuterated internal standards.

LTs	MRM transition	Ion polarity	IS (V)[a]	DP (V)[b]	CE (V)[c]	EP (V)[d]	CXP (V)[e]	RT (min)[f]
LTD_4	497.5/189.3	(+)	5500	100	24	10	11	6.84
LTC_4	626.4/189.3	(+)	5500	130	35	10	15	7.11
LTE_4-d_5	445.5/194.4	(+)	5500	70	23	10	10	7.14
LTE_4	440.4/189.4	(+)	5500	70	24	10	15	7.18
LTB_4-d_4	339.2/197.0	(−)	−4500	−150	−22	−10	−10	8.17
LTB_4	335.2/195.0	(−)	−4500	−140	−23	−10	−15	8.22

[a] Ion spray voltage.
[b] Declustering potential.
[c] Collision energy.
[d] Entrance potential.
[e] Exit potential.
[f] Retention time.

2.3. Sputum samples

Fourteen spontaneous sputum samples were purchased from Bioreclamation (Hicksville, NY, USA). These spontaneous sputum samples were collected from fourteen patients under a physician's care. Patients' information was not provided by the vendor.

Eight induced sputum samples which had been collected from four asthmatic patients (A through D) on two separate visits over a 10-day period were also measured in this study. These induced sputum samples were the unused portion of samples from a completed pilot study in which other biomarkers of airway inflammation in induced sputum were measured. These samples were collected with Institutional Review Board approval and informed consent. They have been stored at −80 °C for approximately 6 years until use. Patients A and B were not on any oral or inhaled corticosteroids. Patient C was on 88 μg of Flovent in a metered-dose inhaler (MDI) which equated to a total daily dose of 88 μg of fluticasone. Patient D was on 2 inhalations of Advair 250/50 daily which equated to a total daily dose of 500 μg of fluticasone propionate. Sputum induction was performed as in previously described protocols [18,19].

2.4. Preparation of sputum supernatant

Sputum samples were thawed at room temperature, mixed by vortexing, aspirated using a pipette with wide-bore tips, and weighed. A protease inhibitor cocktail was then added at a final ratio of 50 μL of cocktail per 200 mg sputum. Sputum samples were ultracentrifuged at 51,000 × *g* for 90 min at 4 °C using a TLA-110 rotor in a Beckman Coulter Optima Max Ultracentrifuge (Fullerton, CA, USA). The sputum supernatants were transferred to microcentrifuge tubes without disrupting the pellet and stored at −80 °C.

2.5. Extraction of leukotrienes

Five hundred microliters (μL) of sputum supernatants, calibration standards, quality controls, and blank were diluted in siliconized glass centrifuge tubes with 500 μL of 15% MeOH in protease inhibitor cocktail. Twenty microliters of a mix of deuterated ISs was added to each tube except the blank. After vortexing, SPE was performed using an Oasis MAX cartridge (1cc, Waters, MA, USA) on a Rapid Trace workstation (Caliper Lifescience, MA, USA). The Oasis MAX cartridge was conditioned with 1 mL each of methanol and water, respectively. After the sample was loaded, the cartridge was washed sequentially with 1 mL of 5% NH_4OH in water, and then 1 mL of 70% methanol in water containing 2% formic acid. The retained LTs were eluted off the cartridge with 1 mL of methanol containing 0.1% formic acid. The eluant was dried down under nitrogen at 25 °C, reconstituted with 50 μL of 50% acetonitrile containing 0.1% formic acid, and then sonicated for 5 min.

2.6. UHPLC–tandem mass spectrometry system

Chromatographic separation was carried out on Acquity UHPLC (Waters, CA, USA) with a reversed-phase C-18 analytical column (BEH C18, 2.1 mm × 50 mm, 1.7 μm particle size, Waters CA, USA) protected by a Waters Van Guard pre-column (2.1 mm × 5 mm). The column oven temperature was set at 55 °C. Samples were injected in a 10 μL volume. Mobile phase A consisted of water with 0.1% formic acid. Mobile phase B consisted of acetonitrile with 0.1% formic acid. Flow rate was kept at 0.7 mL/min. Following a 0.5 min equilibration period with 18% mobile phase B, prostaglandin E_2 was separated by 4.5 minute isocratic elution with 27% mobile phase B (data not reported in this paper). CysLTs and LTB_4 were eluted by a 35% to 36% gradient of mobile phase B in the next 4.5 min.

An API 5000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA) coupled with an electrospray ionization (ESI) source was operated in MRM mode and switched between positive and negative ion modes. Gas settings were as follows: curtain gas 35 psi, collision gas 6 arbitrary units, nebulizer gas 50 psi, and heater gas 80 psi. Ion source was heated at 650 °C. Ultra pure nitrogen was used as curtain gas and collision gas. Dwell time per transition was set at 100 ms. Unit mass resolution was set in both quadrupole Q1 and Q3. Data were acquired and processed using Analyst 1.4.2. Other compound-specific operating parameters are shown in Table 1.

2.7. Assay performance characteristics

The UHPLC–MS/MS method performance characteristics were assessed for precision, accuracy, sensitivity, stability, extraction recovery, carryover, dilution linearity, and matrix effects. The analytical validation was performed in accordance with a workshop report issued from the Third AAPS/FDA Bioanalytical Workshop, "Quantitative Bioanalytical Methods Validation and Implementation: Best Practices for Chromatographic and Ligand Binding Assays" [20].

3. Results and discussion

3.1. Chromatograms—mass spectrometry

The mechanisms of electrospray ionization and the formation of product ions in tandem mass spectrometry of each LT in both positive and negative ions have been elucidated by Murphy *et al.* [21,22]. Our efforts aimed to optimize the mass spectrometric parameters to produce the greatest ionization efficiency in the MRM acquisition. Intensities of multiple MRM transitions were monitored simultaneously by direct infusion of 10 ng/mL of individual LT in 30% acetonitrile containing either 5 mM NH_4Ac or 0.1% formic acid on both positive and negative ion modes, at 10 μL/min rate.

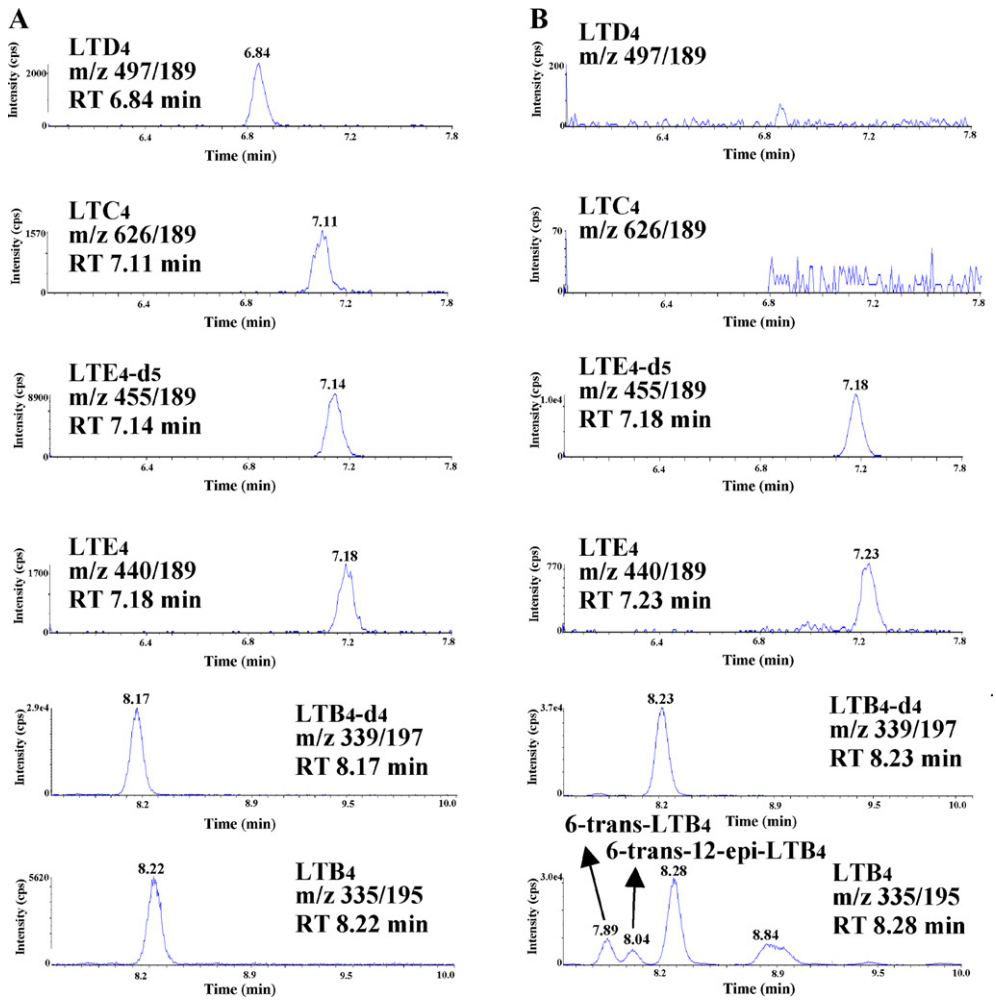


Fig. 2. Typical MRM chromatographic profiles of LTC_4, LTD_4, LTE_4, LTB_4, LTB_4 isomers and deuterium internal standards of (A) standard solution, corresponding to 78 pg/mL of CysLTs and 156 pg/mL of LTB_4; (B) a sputum sample.

For LTB_4, an MRM transition specific to the LTB_4 structure was only obtained in the negative ion mode with a deprotonated precursor ion at *m/z* 335.2 and a product ion at *m/z* 195.0. The similar fragmentation was reported by other authors when LTB_4 was measured in exhaled breath condensate [23]. No significant differences in the MRM intensity were observed by using either 5 mM NH_4Ac or 0.1% formic acid. The 6-*trans*-LTB_4 and 6-*trans*-12-*epi*-LTB_4 produced identical MRM transitions to LTB_4.

CysLTs produced strong MRM signals at *m/z* 624.2/272.0 for LTC_4, *m/z* 495.2/177.0 for LTD_4, and *m/z* 438.4/333.3 for LTE_4 in negative ion mode with 5 mM NH_4Ac. These protonated ions and primary fragmentations were in agreement with previous report [21]. In contrast, more intensive MRM signals were obtained in the positive ion modes with 0.1% formic acid at *m/z* 626.4/189.3 for LTC_4, *m/z* 497.5/189.3 for LTD_4, and *m/z* 440.4/189.3 for LTE_4, as shown in Fig. 1. A reduced LTC_4 signal was observed when the mobile phase containing 5 mM NH4Ac was utilized. Due to the instability of LTC_4 in NH_4Ac, 0.1% formic acid was chosen as pH control in the mobile phase with CysLTs measurement on positive ion mode.

The typical MRM chromatographic profiles of LTs obtained from standards and a sputum sample are shown in Fig. 2A and B, respectively. Generally, electrospray in negative ion mode was prone to have less interference. However as shown in Fig. 2B, the baseline of each CysLT from positive MRM was low and there were no interfering peaks for the CysLTs. It was notable that the optimized HPLC gradient generated an excellent separation for LTB_4 from LTB_4 isomers, 6-*trans*-LTB_4 and 6-*trans*-12-*epi*-LTB_4, that were eluted earlier.

3.2. Effects of pH and temperature on LTB_4 stability

Significant loss of LTB_4 signal was observed when the leukotrienes were eluted from SPE cartridge with 1 mL of methanol containing 2% formic acid, and then dried down at 40 °C. Coinciding with this decrease in signal was the appearance of 6-*trans*-LTB_4 and 6-*trans*-12-*epi*-LTB_4, neither of which was spiked. The same change occurred with LTB_4-d_4. Further investigation showed that both temperature and pH greatly affected the stability of LTB_4 without the presence of LTA_4. The effect of pH was tested by increasing the concentration of formic acid (0, 0.1, 0.5, 1.0, and 2.0%) in the SPE elution buffer (Fig. 3A). Compared to 0.1% formic acid in methanol (Fig. 3B), LTB_4 signal decreased 99% in the presence of 2.0% formic acid in methanol (Fig. 3C). Approximately 1% of LTB_4 was converted into 6-*trans* LTB_4 and 6-*trans*-12-*epi* LTB_4 (Fig. 3C). It is not known what other LTB_4 products are formed at this pH. Similarly when eluant was dried down at 40 °C for about 20 min, only 1% of LTB_4 signal remained and LTB_4 isomers were formed (Fig. 3D). It was critical to ensure LTB_4 stability in solution in order to measure LTB_4 and its isomers accurately and precisely. In light of the effects of pH and temperature on LTB_4 stability, SPE elution with 0.1% formic acid

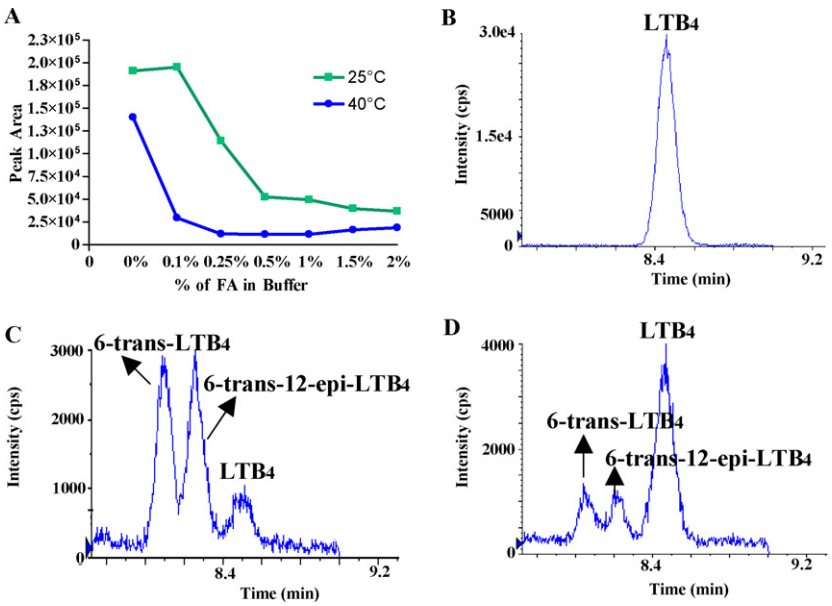


Fig. 3. Effect of pH and temperature on LTB4 stability of (A) pH and temperature effects; (B) eluted by methanol containing 0.1% FA and dried down at RT; (C) eluted by methanol containing 2% FA and dried down at RT; (D), eluted by methanol containing 0.1% FA and dried down at 40 °C.

and dry down at ambient temperature (~25 °C) were selected for the LTB_4 SPE step.

The nonenzymatic hydrolysis of LTA_4 *in vivo* leads to the formation of two LTB_4 stereoisomers, 6-*trans* LTB_4 and 6-*trans*-12-*epi* LTB_4 [24]. The mechanism for the conversion of LTB_4 into 6-*trans* LTB_4 and 6-*trans*-12-*epi* LTB_4 *in vitro* is unclear. The primary products of LTB_4 at high temperature and high acid are also unknown.

3.3. Effect of biological matrices on LTC_4 and LTD_4 stability

A summary of spike recovery in neat solvent and three different biological matrices is shown in Table 2. The percent recovery was calculated by spiking a mix of LTC_4, LTD_4 and LTE_4 and IS LTE_4-d_5 into buffer, plasma, unprocessed sputum, or the supernatant of sputum samples post ultracentrifugation ($n = 2$, from two subjects). LTC_4, LTD_4, and LTE_4 recovery in buffer was around 100%, respectively. However, when a mix of CysLTs was spiked into unprocessed sputum, ultracentrifuged sputum samples, or human plasma, the recovery of LTC_4 and LTD_4 was low and the recovery of LTE_4 was greater than 100%. Interestingly, the % recovery of the sum of LTC_4, LTD_4, and LTE_4 (total CysLTs) in each biological matrix was around 100%, respectively (90–96%) (Table 2). These results suggest that spiked LTC_4 and LTD_4 are converted into LTE_4 *in vitro* in sputum, and that LTE_4 may be a reasonable surrogate for total CysLTs in sputum. LTC_4 and LTD_4 appear stable in a buffered solution absent of a biological matrix. The greater instability of LTC_4 and LTD_4 in unprocessed sputum may be due to the higher concentration of proteins in the non-centrifuged matrix and/or the longer sample handing time needed as for the processing of the sputum.

The spiked recoveries for LTB_4 in two sputum supernatant samples were high (95% and 99%, respectively).

It is unknown whether the biosynthetic pathways of CysLTs *in vitro* are the same as *in vivo*. In our study, LTE_4 appeared to be the surrogate for total CysLTs in human sputum, since the conversion of LTC_4 and LTD_4 into LTE_4 *in vitro* was observed. Described later, the LTC_4 and LTD_4 levels were undetectable in 17 out of 22 sputum samples tested. LTE_4 has been shown to be present in the urine [25], sputum [26,27], and exhaled breath condensate [28,29]. It has been reported that LTE_4 is the only CysLTs, that is sufficiently stable to be detectable in biologic fluids, such as urine or bronchoalveolar lavage fluid [13,26].

We found that the endogenous LTC_4 and LTD_4 levels were below limit of detection in most of sputum samples. In addition, spiked LTC_4 and LTD_4 were converted into LTE_4 *in vitro* in sputum and plasma and also in a significant percentage of sputum supernatants. Due to the lack of QCs that contained stable LTC_4 and LTD_4, assay validation was only performed for LTE_4 and LTB_4, even though this assay was capable of measuring LTC_4, LTD_4, LTE_4, and LTB_4 simultaneously.

3.4. Calibration curves

For five sets of calibration standard curves, the concentrations were back-calculated and the accuracy and %CV were calculated for the back-calculated concentrations of each calibration standard

Table 2
Spiked recovery results of CysLTs in net buffer, plasma, unprocessed sputum and sputum supernatants.

Sample	LTC_4			LTD_4			LTE_4			%R of total cysLTs
	Base[a]	Spiked[b]	%R[c]	Base[a]	Spiked[b]	%R[c]	Base[a]	Spiked[b]	%R[c]	
Buffer	0	500	102	0	500	93	0	500	122	106
Plasma	0	500	0	0	500	139	0	500	130	90
Sputum	0	500	0	0	500	11	272	500	262	91
Sputum supernatant 1	0	250	74	0	250	40	10.5	250	160	90
Sputum supernatant 2	16.7	250	99	13.2	250	58	49.1	250	132	96

[a] Baseline measured in pg/mL.
[b] Spiked amount in pg/mL.
[c] Spike recovery percentage.

Table 3
Back-calculated concentrations of LTs from the calibration samples.

CysLT										LTB_4			
Nominal (pg/mL)[a]	LTC_4			LTD_4			LTE_4			Nominal			
	%Accu[b]	%CV	*n*	%Accu[b]	%CV	*n*	%Accu[b]	%CV	*n*	pg/mL[a]	%Accu[b]	%CV	*n*
9.8	99.4	0.6	2	104.8	6.1	4	104.9	1.9	3	19.5	103.5	1.9	5
19.5	100.5	5.8	4	101.6	8.4	5	98.6	4.8	5	39.1	93.6	6.5	5
39.1	99.8	9.4	4	97.0	11.1	5	96.0	5.5	5	78.1	95.3	3.0	5
78.1	87.6	0.6	2	88.8	4.9	4	89.2	2.7	5	156	93.4	3.8	5
156	93.6	6.0	5	98.0	7.3	5	98.0	3.3	4	313	98.8	4.7	5
313	107.5	5.1	5	102.9	6.8	5	101.8	2.0	5	625	103.6	3.1	5
625	99.8	11.2	4	101.5	10.2	5	101.1	2.8	5	1250	100.6	1.8	5
1250	95.6	12.2	3	107.1	8.6	5	102.8	2.0	5	2500	103.3	1.8	5
2500	96.0	8.1	5	97.0	6.2	5	105.3	2.5	5	5000	102.3	2.5	5
5000	98.8	10.4	5	102.7	6.6	5	102.9	2.5	5	10,000	102.4	1.1	5

[a] Nominal concentration in pg/mL.
[b] % accuracy.

Table 4
Summary of precision ($n = 5$) in pooled sputum supernatant QCs (QC1, QC2) and QC samples (low, med, high).

Sample	Intra-day		Inter-day		Intra-day		Inter-day	
	LTE_4 (pg/mL)	%CV	LTE_4 (pg/mL)	%CV	LTB_4 (pg/mL)	%CV	LTB_4 (pg/mL)	%CV
QC1	102	6.4	103	4.2	523	4.4	594	9.6
QC2	236	4.0	248	8.9	1800	3.7	1936	7.7
Low	67.3	3.2	65.2	3.3	69.9	6.3	72.8	6.7
Med	356	4.0	351	4.4	664	3.4	667	1.9
High	1536	4.2	1530	3.0	4390	4.1	4500	2.2

curve (Table 3). The standards with back-calculated concentrations greater than 15% of nominal (except 20% for LOD) were excluded from regression analysis. No %CV higher than 6.5% were observed for LTE_4 and LTB_4. %CV up to 12.2 was observed for LTC_4 and LTD_4. The assay was linear from 19.5 to 5000 pg/mL for CysLTs. The average correlation coefficients of LTC_4, LTD_4, and LTE_4 were 0.9969, 0.9989, 0.9983, respectively. The average slopes and intercepts were 0.0024 and 0.0040 for LTC_4, 0.0037 and 0.0045 for LTD_4, and 0.0036 and 0.0055 for LTE_4. For LTB_4 the standard curve was linear in the range of 19.5 to 10,000 pg/mL, with average correlation coefficients of 0.9991, slope of 0.0014, and intercept of 0.00021. The %CVs for the correlation coefficients and slopes for LT calibration curves were less than 0.1% and 3%, respectively.

3.5. Precision, accuracy and sensitivity

The precision of LTE_4 and LTB_4 was determined by calculating the %CV of repeat measurements of the pre-prepared, aliquoted, and stored QCs samples. Intra-day precisions were determined by analysis of five new samples from each of the QCs in one day. For inter-day precision, five QC samples were analyzed on consecutive days. As shown in Table 4, the CVs ranged from 3 to 6% for intra-day precision and 2–10% for inter-day precision. There were no apparent differences in precision between sputum supernatant samples versus diluted plasma QC samples.

The accuracy of the optimized methods was determined by spike recovery experiments. Spike recovery was measured in unspiked sputum supernatant samples and samples spiked with two levels of authentic LTB_4 and LTE_4, described in Table 5. Recoveries ranged from 105 to 111% with CV less than 6.5%.

The limit of detection (LOD), defined as the concentration at which signal/noise was $\geq$3, was determined from the lowest standard. Sensitivity of the mass spectrometer was monitored daily by performing a system suitability test. LOD was estimated conservatively to reflect possible signal fluctuation. During five inter-day analysis, the lowest standards on the standard curve provided an average S/N ratio of 8 (30.7 %CV) for 9.8 pg/mL of LTE_4 and an average S/N ratio of 8 (21.1 %CV) for 19.5 pg/mL of LTB_4. Thus the LOD was designated as 9.8 pg/mL of LTE_4 and 19.5 pg/mL of LTB_4. Any value at or below established LOD was reported as LOD. The lower limit of quantitation (LLOQ) was defined as the concentration at which signal/noise was $\geq$10. The LLOQ were 19.5 and 39.0 pg/mL for LTE_4 and LTB_4, respectively.

3.6. SPE extraction recovery, carryover and dilution

The efficiency of the SPE procedure was determined by the comparison of peak area obtained from spiking the same amount of LTE_4-d_5 and LTB_4-d_4 into sputum supernatants pre- and post-extraction. Under the present optimal SPE conditions, SPE extraction recovery was 88% and 97% for LTE_4-d_5 and LTB_4-d_4, respectively.

The amount of carryover between samples analyzed sequentially on the mass spectrometer was determined by running a blank solvent after a high concentration of standard. There was no detectable carryover.

Dilution linearity was assessed by diluting sputum supernatant serially with 15% MeOH in protease inhibitor cocktail in cases where the clinical sample concentrations were above the highest standard. A sputum supernatant containing 885 pg/mL of LTE_4 and 3450 pg/mL of LTB_4 showed linearity at dilutions up to 1:64 with a good correlation coefficient of 0.9992 for LTE_4 and 0.9994 for LTB_4 (Fig. 4).

Table 5
Summary of spike recovery in pool sputum supernatant.

LTs level	Baseline	Spiked amount	Observed amount	Recovery%
LTE_4 (pg/mL)	47.5	50.0	100.2	105
LTE_4 (pg/mL)	47.5	1000.0	1155	111
LTB_4 (pg/mL)	1180	400.0	1615	109
LTB_4 (pg/mL)	1180	2000.0	3310	107

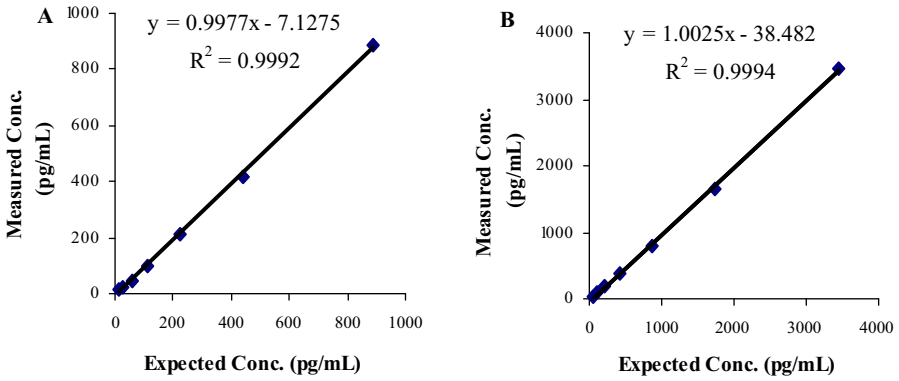


Fig. 4. Linearity of dilution results for one human sputum supernatant sample: (A) LTE_4; (B) LTB_4. Neat LTC_4 was 14.6 pg/mL. Neat LTD_4 was 11.8 pg/mL.

3.7. Matrix effects

Matrix effect was assessed by measuring the peak areas of the internal standards (ISs) LTE_4-d_5 and LTB_4-d_4 spiked into post-extracted sputum supernatant and dividing them by the peak areas of the same amount of ISs spiked directly into solvent. The matrix factor was calculated as 7.7 for LTE_4-d_5 and 2.3 for LTB_4-d_4, respectively. As suggested by the Crystal City III white paper [20], a matrix factor of greater than 1 may be due to ionization enhancement in the presence of a biological matrix. In a recent publication, Wang *et al.* reported that the analyte-to-IS peak area ratios decreased 18.9% for one out of two analytes, in one out of three lots plasma [30]. The authors hypothesized that IS was not able to effectively compensate for the matrix effect due to slight differences in retention times between the IS and analytes. The retention time of the deuterium labeled compound tends to be slightly shorter during reversed phase separations because of what is known as isotope effect, but this does not normally interfere with their ability to correct for extraction differences and/or matrix effects. In this study, the use of LTE_4-d_5 and LTB_4-d_4 seemed to have effectively compensated for the presence of the matrix effect as reflected in the high accuracy and precision data reported in this publication.

3.8. Stability

The storage stability of LTE_4 and LTB_4 was studied in sputum supernatants by analyzing samples: (1) on the day of preparation, (2) following storage at −80 °C for 75 days, (3) following 4 h at 4 °C and 4 h at room temperature, and (4) following up to 3 freeze/thaw (F/T) cycles. The variations of LTE_4 and LTB_4 were measured and expressed as the percentage of the starting levels. In addition, the stability of extracted sputum supernatant was also assessed in an auto-injector overnight at 4 °C and in freezer at −80 °C.

As summarized in Table 6, the sample storage conditions tested had minimal effects on LTB_4 (−1% to 6% change). Slightly larger effects were observed for LTE_4 (−9% to 14%).

Quantitation of LTs in post-extraction samples was still reliable after storage overnight at −80 °C. Neither concentrations nor absolute signals were changed significantly. But the absolute signal decreased by 50% at 4 °C overnight for all analytes including ISs (data not shown). The concentrations of LTs did not change remarkably due to the compensation of ISs. Since the reduction in signal could compromise assay sensitivity, a maximum of 50 sputum samples were processed per day. Half of the sample volume was stored at −80 °C in case re-injection was needed.

Table 6
Summary of pre- and post-SPE extraction stability in sputum supernatants.

	LTE_4		LTB_4	
	Sputum 1	Sputum 2	Sputum 1	Sputum 2
Pre-SPE extraction				
−80 °C 75 days	−7.7	0	13.7	9.4
4 °C 4 h	13	6	6	2
RT 4 h	7	13	0	−1
1 cycle F/T[a]	13	3	6	−1
2 cycle F/T	3	−9	−1	−1
3 cycle F/T	2	0	−1	−2
Post-SPE extraction				
−80 °C overnight	0	−2	0	1
4 °C overnight	−3	3	3	2

[a] Freeze/thaw.

3.9. Quantitative analysis of LTs in human sputum supernatants

In order to test the suitability of this new methodology as well to estimate concentration ranges of LTs in asthmatic patients, the measurement of total CysLTs and LTB_4 in available human sputum samples was carried out.

LTE_4 concentrations ranged from 12.3 to 891 pg/mL in 12 out of 14 purchased spontaneous human sputum samples, and LTB_4 concentrations ranged from 79.2 to 5280 pg/mL. Two sample contained undetectable levels of LTE_4 (<9.8 pg/mL). LTC_4 was undetectable in all spontaneous human sputum supernatants. LTD_4 concentrations were above LOD in only 3 of the 14 samples, ranging from 16.2 to 864 pg/mL.

For the induced sputum supernatants collected over two visits from four asthmatic patients, LTE_4 concentrations ranged from 11.9 to 705 pg/mL, and LTB_4 concentrations ranged from 333 to 7220 pg/mL (Table 7). For LTC_4 and LTD_4, only 2 samples showed detectable levels (from 13.2 to 99.6 pg/mL). These data were not used to assess the between- or within-patient variability of LTs in induced sputum because of the small sample size, unknown

Table 7
LTB_4 and LTE_4 concentration in induced sputum collected from four asthmatic patients over two visits.

Patient	Visit days	LTE_4 (pg/mL)	LTB_4 (pg/mL)
A	1	17.0	576
	3	18.3	333
B	1	145	1420
	3	705	7220
C	1	11.9	972
	10	77.9	4590
D	1	216	5330
	3	53.7	691

long term storage stability, unknown sputum sample quality, and unknown matrix effect variation in individual subjects.

There was a large discrepancy in sputum CysLTs levels reported in the literature. Pavord *et al.* [17] reported the median sputum CysLTs concentrations at 6.4 ng/mL in normal control subjects, 9.5 ng/mL in subjects with asthma, and 11.4 ng/mL in subjects with persistent asthma requiring inhaled corticosteroids. The sputum samples were treated by DTT and assay using an EIA. Aggarwal *et al.* [7] assayed LTE_4 in DTT treated sputum by EIA after extraction on a CysLT immunoaffinity sorbent and measured median sputum CysLT concentrations of 313 pg/mL in control subjects, 565 pg/mL in moderate asthmatic patients, and 324 pg/mL in severe asthmatic patients. Our LTE_4 data are more in line with the Aggarwal data. Pavord *et al.* speculated that DTT interference in the CysLTs assay was unlikely since concentrations were not significantly different in sputum treated with and without DTT, but could not exclude the possibility that the CysLTs concentrations in sputum from subjects with asthma might be increased by the effect of hypertonic saline on mast cells and other mediator-producing cells in the airway [17]. The results of this study, in which this SPE-LC–MS/MS technique was used, are similar to those obtained by Aggarwal *et al.* suggesting that sample clean-up may be necessary to remove interferences in the CysLTs assay.

Similar to CysLTs, LTB_4 levels in sputum have been reported in a wide range between labs and technologies. LTB_4 measurements in DTT treated induced sputum are taken as examples here. The LTB_4 levels were found as 72.8 ± 8.0 pg/mL for healthy subjects and 84.9 ± 11.6 pg/mL for COPD patients, when measured by a commercial EIA kit from Cayman [16]. Also by using the commercial EIA kit from Cayman, LTB_4 levels were reported as 313 ± 76 for healthy subjects and 550 ± 46 pg/mL for COPD patients [31]. Baseline sputum LTB_4 levels in patients with moderate COPD were 21.8 ± 12 pg/mL when measured by EIA (kit from DRG International) after purification over C18 columns [32]. In yet another paper, higher LTB_4 concentrations were reported in healthy subjects (0.3–1.1 ng/mL) and in COPD patient sputum (1.1–4.9 ng/mL) [33] by HPLC separation followed by EIA (kit from Amersham). Using our method, LTB_4 levels in asthma sputum ranged from 0.3 to 7.2 ng/mL, which was consistent with the last reference. It is not clear how much the differences in reported sputum LTB_4 levels are due to actual *in vivo* differences and how much are due to differences in the experimental conditions and the quality of sputum. Our data indicate the significant effects of pH and temperature on LTB_4 stability. Clearly a specific and reliable assay to measure LTs would be beneficial in understanding the role of LTs in airway disease pathways and their associated clinical treatments.

We have developed a mass spectrometry assay capable of measuring simultaneously CysLTs and LTB_4 in sputum with sufficient sensitivity and selectivity. Two important issues that need to be addressed are the chemical instability of LTB_4 and the biological instability of LTC_4 and LTD_4 *in vitro*.

4. Conclusions

In conclusion, the present assay for the determination of CysLTs and LTB_4 in human sputum samples is sufficiently specific, sensitive, and accurate for pathway study of these leukotrienes. It provides a suitable analytical platform for assessing pharmacodynamic changes in sputum and supports the suitability of using sputum as a minimally invasive biomarker of airway inflammation in further studies. The detection limits and biological ranges for LTB_4 and LTE_4 suggest that they could be useful in studies where changes in total CysLTs and LTB_4 are important pharmacodynamic readouts. LTE_4 appears to be a good surrogate for measurement of total CysLTs in sputum. This method can be easily modified for other biological matrixes.

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