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Review

LC/MS/MS analysis of leukotriene B_4 and other eicosanoids in exhaled breath condensate for assessing lung inflammation☆

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ABSTRACT

Leukotriene (LT) B_4 is a potent inflammatory lipid mediator that has been involved in the pathophysiology of respiratory diseases including asthma. Exhaled breath condensate (EBC) is a non-invasive method to sample secretions from the airways. LC/MS/MS techniques for measuring LTB_4 concentrations in EBC have been developed and are suitable for an accurate quantitative assessment of its concentrations in EBC. LC/MS/MS for other eicosanoids including 8-isoprostane, a marker of oxidative stress, and cysteinyl-LTs have been developed. This article, mainly focused on LTB_4, presents the analytical aspects of the LC/MS/MS techniques for measuring LTB_4 and 8-isoprostane in EBC, provides examples of their application to the assessment of airway inflammation in patients with asthma and other respiratory diseases, and discusses their potential utility for non-invasive monitoring of drug therapy.

Contents

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1. Introduction

Leukotrienes (LTs) are biologically active metabolites that are derived from arachidonic acid through the action of 5-lipoxygenase [1]. From the structural point of view, LTs are conjugated triene compounds containing a linear-20 carbon chain. Leukotriene (LT) B_4 (5S,6Z,8E,10E,12R,14Z)-5,12-dihydroxyeicosa-6,8,10,14-tetraenoic acid (Fig. 1), a potent chemoattractant for neutrophils, has been involved in the pathophysiology of inflammatory airway diseases such as chronic obstructive pulmonary disease (COPD) [2], cystic fibrosis [3], severe asthma and asthma exacerbation [1], whereas its role in less severe chronic stable asthma is less known [1]. Cysteinyl (Cys)-LTs are important inflammatory mediators in asthma and selective $CysLT_1$ receptor antagonists are currently used in asthma therapy [1,4]. The development of a robust analytical technique for the quantitative assessment of LTB_4 concentrations in biological fluids is essential for establishing the pathophysiological role of LTB_4 in patients with pulmonary diseases and the potential utility of its measurement for assessing the effects of pharmacological intervention. In patients with respiratory diseases, LTB_4 has been detected in plasma [5], bronchoalveolar lavage fluid [6], and sputum [2,3]. LTB_4 concentrations have been measured in exhaled breath condensate (EBC) [7–10], a non-invasive method for collecting airway secretions and studying the composition of airway lining fluid [11,12]. In most of these studies, LTB_4 concentrations in EBC have been measured using enzyme immunoassays (EIAs) [12]. Mass spectrometry techniques are considered the gold standard as they are highly specific and generally sufficiently sensitive. We have used gas chromatography/mass spectrometry (GC/MS) for measuring LTB_4 concentrations in EBC in adults and children with asthma and healthy controls [13]. However, definitive conclusions on the specificity of this method are precluded by the fact that arachidonic acid was used as internal standard [13]. Arachidonic acid is chemically different from LTB_4 and may behave differently during sample pre-treatment. Unlike GC/MS, liquid chromatography/mass spectrometry (LC/MS) does not require the two-step derivatization procedure resulting in three advantages: (1) increased recovery, (2) shorter time required for sample pre-treatment, and (3) lack of formation of incomplete derivatization by-products. We have developed two LC/MS/MS techniques for measuring LTB_4 concentrations in EBC using ion trap [14] and triple quadrupole mass spectrometer [15], respectively. Isoprostanes are prostaglandin (PG)-like substances that are produced in vivo independently of cyclooxygenase (COX) enzymes, primarily by free radical-induced peroxidation of arachidonic acid [16]. Measurement of F2-isoprostanes is one of the most reliable approaches to assess oxidative stress status in vivo, providing an important tool to explore the role of oxidative stress in the pathogenesis of human disease [16]. Using GC/MS, 8-isoprostane (15-F_{2t}-isoprostane or 8-iso-prostaglandin $F_{2\alpha}$) was measured in EBC in patients who were intubated for acute lung injury or acute respiratory distress sindrome [17]. A LC/ESI-MS/MS operated in multiple reaction monitoring (MRM) mode for measuring 8-isoprostane concentrations in EBC has been developed [18] and modified for parallel quantification of LTB_4 and cysteinyl-LTs [19]. This review presents the analytical aspects of these techniques, provides an example of their application to the assessment of airway inflammation in patients with respiratory diseases including asthma, asbestosis and silicosis, and discusses their potential utility for non-invasive monitoring of drug therapy.

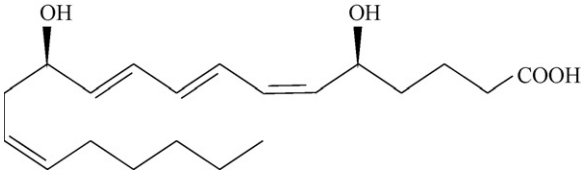

Fig. 1. Chemical structure of LTB_4.

2. Experimental

2.1. Materials

In the studies aiming at measuring LTB_4 concentrations in EBC, all solvents were high performance liquid chromatography grade (Merck, Darmstadt, Germany). Acetic acid extra pure was obtained from Riedel-de Haen, Sigma–Aldrich, Seelze, Germany. LTB_4 and LTB_4-d_4 were purchased from Cayman Chemicals Co (Ann Arbor, MI, USA) and stored at −20 °C until use. Chemical purity of LTB_4-d_4 was ≥97% and the deuterium incorporation was ≥1%. The working solutions were prepared daily using mobile phase as solvent [14,15]. In the study aiming at measuring 8-isoprostane concentrations in EBC, 8-iso-prostaglandin $F_{2\alpha}$ (8-isoprostane or 15-F_{2t}-isoprostane), [3,3′,4,4′, $^2H_4$] 8-iso-prostaglandin $F_{2\alpha}$ and 8-isoprostane affinity sorbent were obtained from Cayman Chemical Co, Ann Arbor, MI, USA; triethylamine (99.5%) and ammonium hydroxide (28% NH_3 solution in water) were obtained from Aldrich, USA; acetonitrile, water, methanol (LC/MS grade), acetic acid (99.9%) and formic acid (98–100%) were obtained from Riedel de Haen, Germany [18].

2.2. Study design

Two studies were performed for identifying and quantifying LTB_4 in EBC [14,15]. In the first study, the primary objective was to ascertain whether LC/MS and/or LC/MS/MS analysis could be used to measure LTB_4 in EBC and to establish the best analytical conditions [14]. Ion trap mass spectrometer was used for LTB_4 analysis. This study will be called methodological study throughout the text. In the second study, a modified LC/MS/MS technique was applied to the measurement of LTB_4 in EBC in atopic asthmatic children, atopic non-asthmatic children and healthy children [15]. A triple quadrupole mass spectrometer was used for LC/MS/MS analysis of LTB_4 in EBC. This study will be called observational study in children with asthma. Both studies were of cross-sectional design.

Between-day variations for LTB_4 measurements, expressed as intraclass correlation coefficient, were assessed by analysing three consecutive EBC samples obtained during 7 days from each child (20 children with asthma) [15].

Cross-sectional studies were performed to compare the concentration values of 8-isoprostane in EBC by group of non-smoking subjects with a long-term exposure to asbestos versus the control group [18] and to compare 8-isoprostane, LTB_4 and cysteinyl-LT concentrations in EBC in patients with silicosis and healthy subjects [19]. In these studies, a triple quadrupole mass spectrometer was used [18,19].

2.3. Subjects

In the methodological study, 8 patients with atopic asthma (6 adults and 2 children, age ranging from 7 to 42 years) and 2 healthy adults (age, 25 and 28 years) were studied [14]. Four out of 8 patients with asthma were not taking any regular treatment, but used inhaled short-acting β_2-agonists as needed for symptom relief. Three patients were on chronic treatment with oral montelukast, a cysteinyl-LT receptor antagonist, and 1 patient was on montelukast and fluticasone, an inhaled corticosteroid, for at least 4 weeks. In the observational study, 4 groups of children were included: 15 healthy children, 20 atopic non-asthmatic children, 25 steroid-naïve atopic children with mild intermittent asthma, and 22 atopic children with

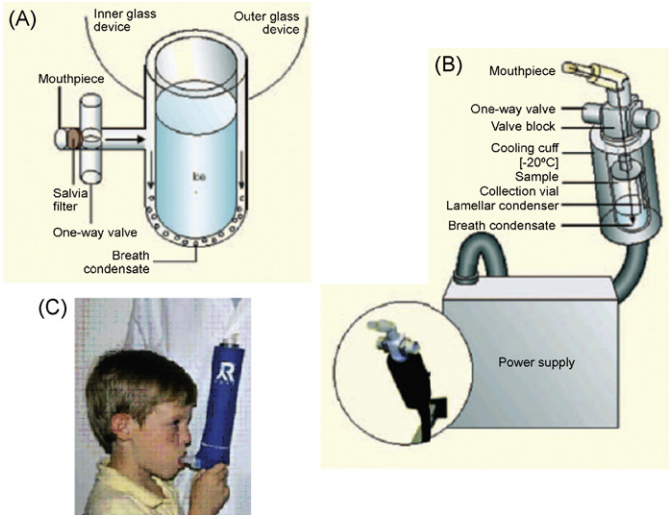

Fig. 2. Representation of EBC collecting systems. A homemade EBC collecting system (A) that consists of two glass containers which form of a double wall of glass. The inner side of the glass is cooled by ice. EBC is collected between the two glass surfaces. A commercially available condenser (EcoScreen®) (B). EBC is collected in the collecting vial as indicated by the arrow. A commercially available portable condenser (RTube®) (C). Reprinted with permission from Montuschi [12] (copyright 2007, Sage Publications).

persistent mild-to-moderate asthma who were receiving maintenance therapy with inhaled fluticasone at a constant dose for at least 8 weeks [either 100 μg/day (10 children) or 200 μg/day (12 children)] [15]. Atopy was confirmed by skin prick testing. Atopic non-asthmatic children had allergic rhinitis, a clinical history of atopy and positive skin test results. In this study, exhaled nitric oxide (NO), a marker of airway inflammation, was also measured using a chemiluminescence analyzer (NIOX, Aerocrine, Stockholm, Sweden) [15]. The diagnosis and classification of asthma was based on clinical history and examination and pulmonary function parameters assessed according to the "Guidelines for the Diagnosis and Management of Asthma" issued by the National Heart, Lung, and Blood Institute of the National Institutes of Health [20]. Study group subjects were excluded from the study if they had had upper respiratory tract infections in the previous 3 weeks or had used oral glucocoticosteroids in the previous 4 weeks [14,15].

8-Isoprostane was measured in 44 persons who were non-smokers with previous exposure to asbestos of 24 years in average and in healthy subjects who were non-smokers [18]. The number of subjects in the healthy control group and demographic characteristics (i.e., age and gender) of both study groups were not provided [18]. In another study, 8-isoprostane, LTB_4, and cysteinyl-LT concentrations were measured in EBC in 60 patients with silicosis (58 men and 2 women, age ranging from 48 to 79 years) and in 25 healthy subjects (23 men and 2 women, age ranging from 51 to 85 years) [19]. In both groups, smokers and non-smokers were included [19].

2.4. Exhaled breath condensate collection

In the studies described in this review, EBC was collected using a commercially available condenser (Ecoscreen, Jaeger, Hoechberg, Germany) as described previously (Fig. 2) [7]. Briefly, exhaled air entered and left the condensing chamber through one-way valves at the inlet and outlet, thus keeping the chamber closed. Subjects were instructed to breath tidally through a mouthpiece connected to the condenser for 15 min. An average of 1.5 ml EBC per subject was collected and stored at −80 °C. LTB_4 measurements were performed within 2 weeks from sample collection. To check for possible salivary contamination, α-amylase concentrations in all EBC samples were measured by in vitro colorimetric methods (Roche Diagnostics, Basel, Switzerland [14,15]; α-Amylase-Liquid BIO-LA-TEST kit, Pliva-Lachema, Czech Republic [18,19]).

In the methodological study, LTB_4 concentrations in EBC were expressed as pg/ml [14]. In the observational study performed in children, LTB_4 values in EBC were expressed as total amount (in pg) of leukotriene expired in the 15 min breath test (LTB_4 concentrations × volume of EBC). Concentration of LTB_4 in undetectable samples was arbitrarily considered 25 pg/ml that corresponds to 50% of the lower limit of quantification (50 pg/ml) [15].

2.5. Sample pre-treatment

In the methodological study, 1 ml of EBC samples was spiked with 1.5 ng/ml LTB_4-d_4 and concentrated 40-fold under a gentle nitrogen stream without any pre-treatment [14]. Then, 20 μl of the concentrate solution were directly injected into the sample loop. In the second study, 10 μl of EBC sample were directly injected into the liquid chromatograph with no pre-treatment [15].

In the study aimed at measuring 8-isoprostane in EBC with LC/ESI-MS/MS, EBC samples were pretreated with 50 μl of immunoaffinity sorbent for 60 min [18].

2.6. Analytical conditions

2.6.1. Methodological study

The stable isotope dilution method with LTB_4-d_4 as internal standard was used [14]. A Spectra System P4000 pump coupled with a LCQ Advantage ion trap mass spectrometer (Thermo Electron Corp., San José, CA, USA) was used [14]. This MS detector can operate in electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) modes. The tuning of the MS detector was optimized with the analyte in both ESI and APCI modes [14]. The mass spectrometer was operated at unit mass resolution. The mass spectra of LTB_4 and LTB_4-d_4 internal standard were acquired in full scan mode by infusion of a reference solution of 0.1 μg/ml using ESI and APCI in positive and negative ion polarity modes (m/z range 150–360 in negative ion mode and 150–420 in positive ion mode). The ESI mode was more sensitive than the APCI mode as the injection of 40 ng of LTB_4 provided a peak area in the range of 3×10^6 in the APCI mode and 1×10^7 in the ESI mode. The ESI product ion mass (MS/MS) spectra were obtained by choosing m/z 335 and 301 as precursor ions, in negative and positive polarity mode, respectively [14]. The optimized source parameters are shown in Table 1. Chromatography was performed on a Lichrospher 100RP-8 column (125 mm × 4 mm, 5 μm) (Merck, Darmstadt, Germany) at room temperature using a linear gradient, starting with acetonitrile/water/acetic acid 40:60:0.05 (v/v) adjusted to pH 5–6 which was changed to 100% acetonitrile over 15 min; the flow rate was 0.7 ml/min [14]. The column effluent was introduced into the mass spectrometer via a fused-silica capillary (0.1 mm i.d. × 0.190 mm o.d.). A sample loop of 20 μl was used.

Table 1
ESI-MS source parameters used for measuring LTB_4 in the methodological study.

Source parameters	Negative ion mode	Positive ion mode
Spray voltage	−4500 V	4500 V
Aux gas flow	16.5 l/min	16.5 l/min
Sheath gas flow	1.05 l/min	0.75 l/min
Capillary temperature	360 °C	360 °C
Capillary voltage	−9 V	5 V
Collision energy of MS^2 mode	36% of the maximum setting	36% of the maximum setting
Tube lens offset	−15 V	2 V
Skimmer voltage	8 V	10 V

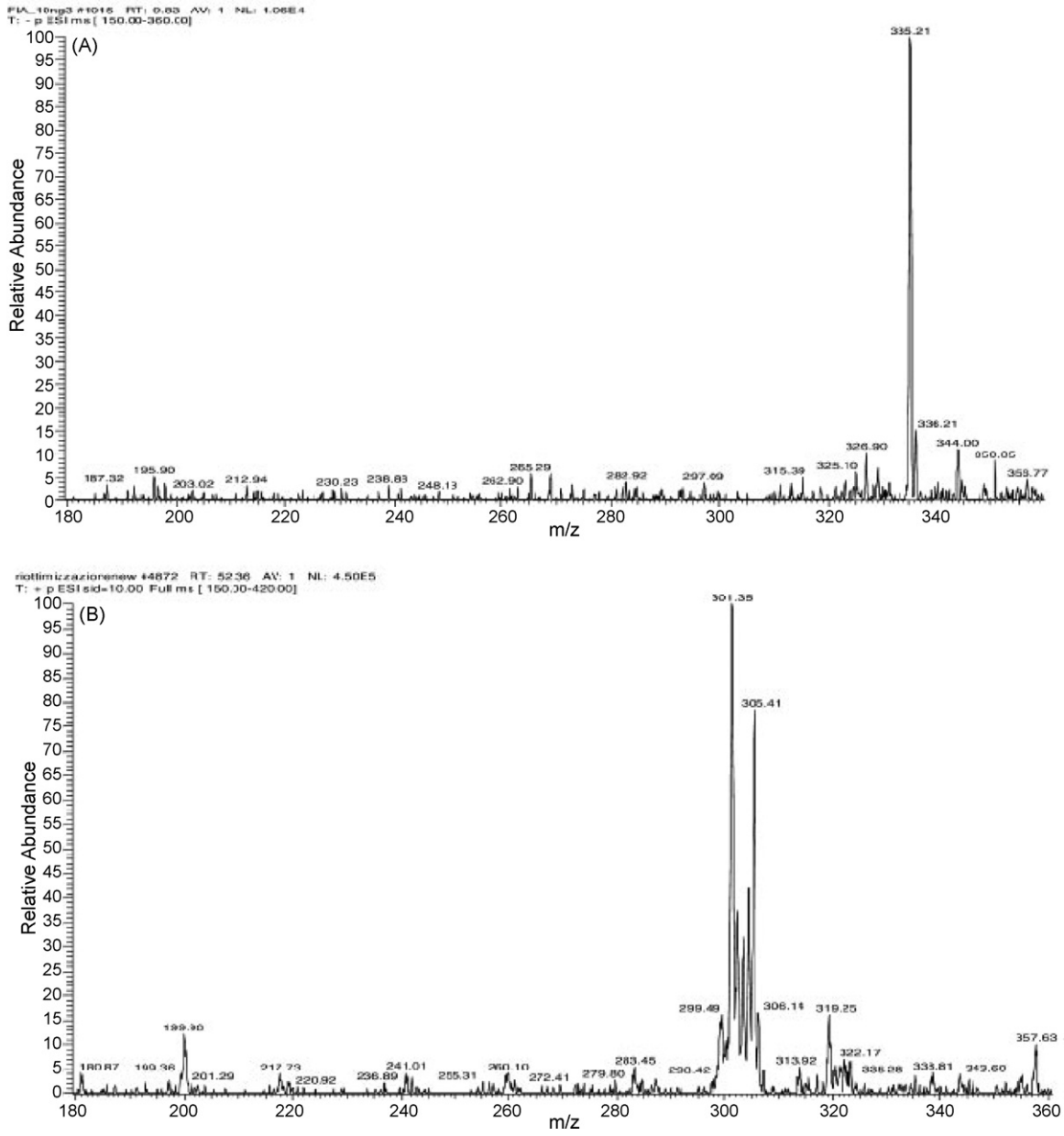

Fig. 3. ESI mass spectra of LTB_4 in negative (A) and positive (B) ion polarity mode. Reprinted with permission from Montuschi et al. [14] (copyright 2004, John Wiley and Sons).

2.6.2. Observational study in children with asthma

LTB_4 concentrations in EBC were measured with a Finnigan Surveyor® LC System pump coupled with a TSQ Quantum Ultra™ triple quadrupole mass spectrometer (Thermo Electron Corporation, San José, USA) [15]. LC/ESI-MS/MS analysis was performed in negative ion polarity mode by choosing *m*/*z* 335 as precursor ion [15]. The analytical conditions were similar to those described in the methodological study [14]. Chromatography was performed on a BetaBasic C18 column (15 cm × 2.1 mm internal diameter) (Thermo Hypersil-Keyston, Bellefonte, PA, USA) using a linear gradient with acetonitrile/water/acetic acid 30:70:0.05 (v/v) adjusted to at pH 5–6 which was changed to 100% acetonitrile over 4 min [15]. Flow rate was 0.25 ml/min. Source parameters were as follows: spray voltage, 4500 V; sheath gas pressure, 40 arbitrary units; aux gas pressure, 10 arbitrary units; capillary temperature, 300 °C; capillary offset, 30 V; tube lens offset, 110 V [15].

2.6.3. LC/ESI-MS/MS measurement of 8-isoprostane and other eicosanoids

A ProStar HPLC system (Varian, USA) with a Hypercarb Thermo 100 mm × 2.1 mm × 5 μm column connected to Hypercarb precolumn (Thermo Electron Corporation, San José, USA) was used [18]. Isocratic elution was performed using a mobile phase acetonitrile:water 70:30 (v/v) adjusted to pH 11 [18]. Flow rate was 250 μl/min. The injection volume was 20 μl. The LC system was coupled to the triple quadrupole mass spectrometer Varian 1200 L (Varian, USA) equipped with an electrospray ion source operated in the negative ion polarity mode (ESI^-) [18]. Full scan mass spectra were acquired using a continual infusion of the standard solutions at a concentration of 1 ng/ml and a flow of 50 μl/min [18]. 8-Isoprostane and 8-isoprostane-d_4 were measured using the MRM [18]. Selected product ion fragments corresponded to the maximum intensities for both the analyte and the deuterated internal standard ensuring maximum of sensitivity. The

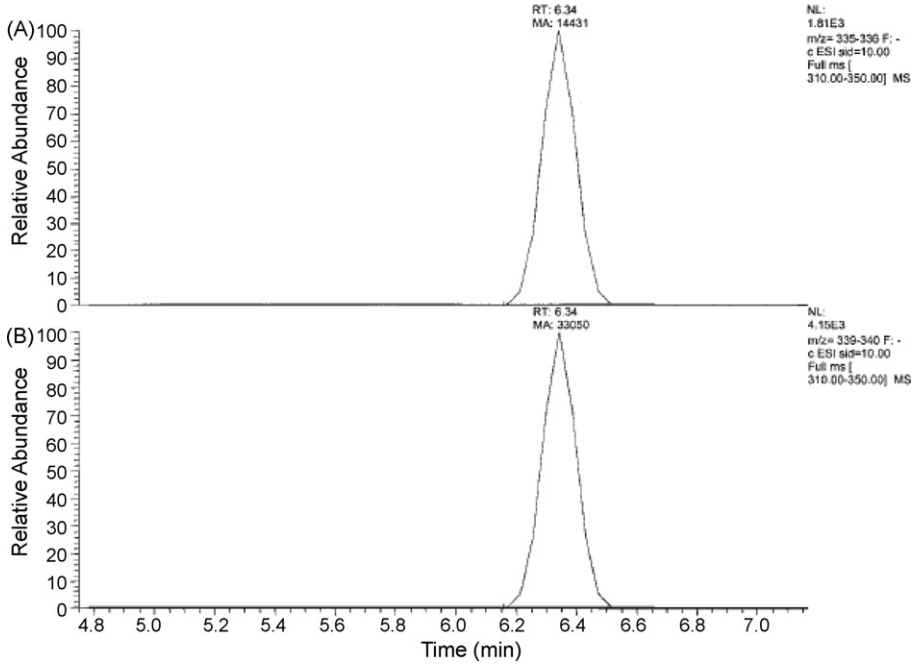

Fig. 4. Typical LC/MS extracted ion chromatograms for an EBC sample obtained from a patient with asthma. The $[M-H]^-$ ions m/z 335 (A) and m/z 339 (B) were used to measure LTB_4 concentrations. Reprinted with permission from Montuschi et al. [14] (copyright 2004, John Wiley and Sons).

scan monitoring reaction used for analyses were 353 → 193 for 8-isoprostane, and 357 → 197 for 8-isoprostane-d_4 [18]. Collision-induced dissociation was performed at 29 eV under 2.2 mTorr of argon pressure [18]. The capillary voltage was −70 V. The needle voltage was −4500 V. The temperature of ESI ion source was 300 °C [18]. This technique was adapted for parallel measurement of cysteinyl-LTs in EBC with immunoaffinity extraction and identical LC/ESI-MS detection in MRM mode developed for each eicosanoid [19]. LOD, LOQ and recovery for each eicosanoid were as follows: LTC_4, LOD = 2 pg/ml; LOQ = 16 pg/ml; recovery = 55%; LTD_4, LOD = 1 pg/ml; LOQ = 6 pg/ml; recovery = 61%; LTE_4, LOD = 1 pg/ml; LOQ = 5 pg/ml; recovery = 81% [19].

2.7. Statistical analysis

Linear regression analysis was used to assess the relationship between the LC peak area ratios for LTB_4/LTB_4-d_4 and LTB_4 values [14,15]. LTB_4 values in EBC were expressed as medians throughout with interquartile ranges (25th–75th percentiles) shown in parentheses [15].

Kruskal–Wallis test followed by pairwise Mann–Whitney *U*-tests were used to compare groups [15]. Correlations between variables were evaluated by Spearman's test and significance was defined as a value of $p < 0.05$.

3. Results

3.1. Methodological study

Preliminary experiments showed that the best results were obtained when operating the MS detector in the ESI mode [14]. To identify the best analytical conditions, we performed MS analysis of LTB_4 in ESI negative and positive polarity mode [14]. The negative ion ESI mass spectra of LTB_4 and LTB_4-d_4 internal standard revealed a base peak corresponding to molecular anions ($[M-H]^-$) at m/z 335 for LTB_4 (Fig. 3A) and m/z 339 for LTB_4-d_4 (internal standard) [14]. In the product ion mass spectrum of the carboxylate $[M-H]^-$ ion of LTB_4, the most abundant fragments were m/z 317 (water loss) and m/z 195. In positive ion polarity mode, the ESI mass spectra of LTB_4 and LTB_4-d_4 internal standard obtained revealed base peaks at m/z 301 for LTB_4 (Fig. 3B) and m/z 305 for LTB_4-d_4, corresponding to the loss of two molecules of water from the molecular ions ($[M+H]^+$). In the MS^2 spectrum of the m/z 301 $[M+H-2H_2O]^+$ precursor, the most abundant fragment ions were observed at m/z 241, 265 and 283 for LTB_4 [14].

For quantitative analysis of LTB_4 in EBC, we used ESI in negative MS^1 mode as the sensitivity of the MS^2 mode was insufficient [14]. The $[M-H]^-$ ions m/z 335 and m/z 339 (internal standard) were used to monitor endogenous LTB_4 (Fig. 4) [14]. In MS^1 mode, instrumental LOQ is 100 pg/ml with acceptable signal-to-noise ratios. MS^2 has a higher specificity, but a lower sensitivity, and can be used when LTB_4 concentrations in EBC are higher than 100 pg/ml [14].

The calibration curve ranged from 100 to 1000 pg/ml and was obtained by adding increasing amounts of LTB_4 to 1 ml of water and a constant amount of LTB_4-d_4 used as internal standard to each sam-

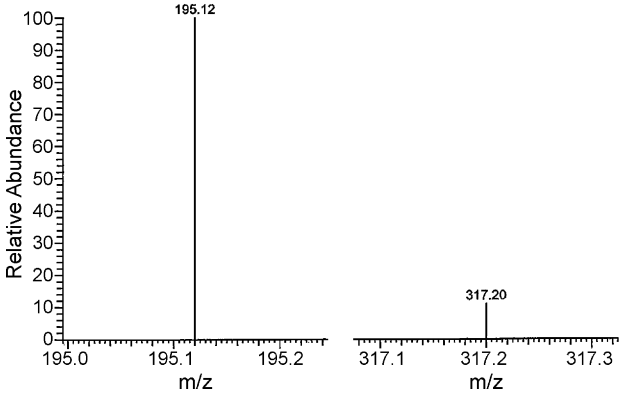

Fig. 5. Partial product ion mass spectrum of the $[M-H]^-$ ion derived from LTB_4 after ESI in negative ion mode using a triple quadrupole mass spectrometer (Thermo Electron Corporation, San José, USA).

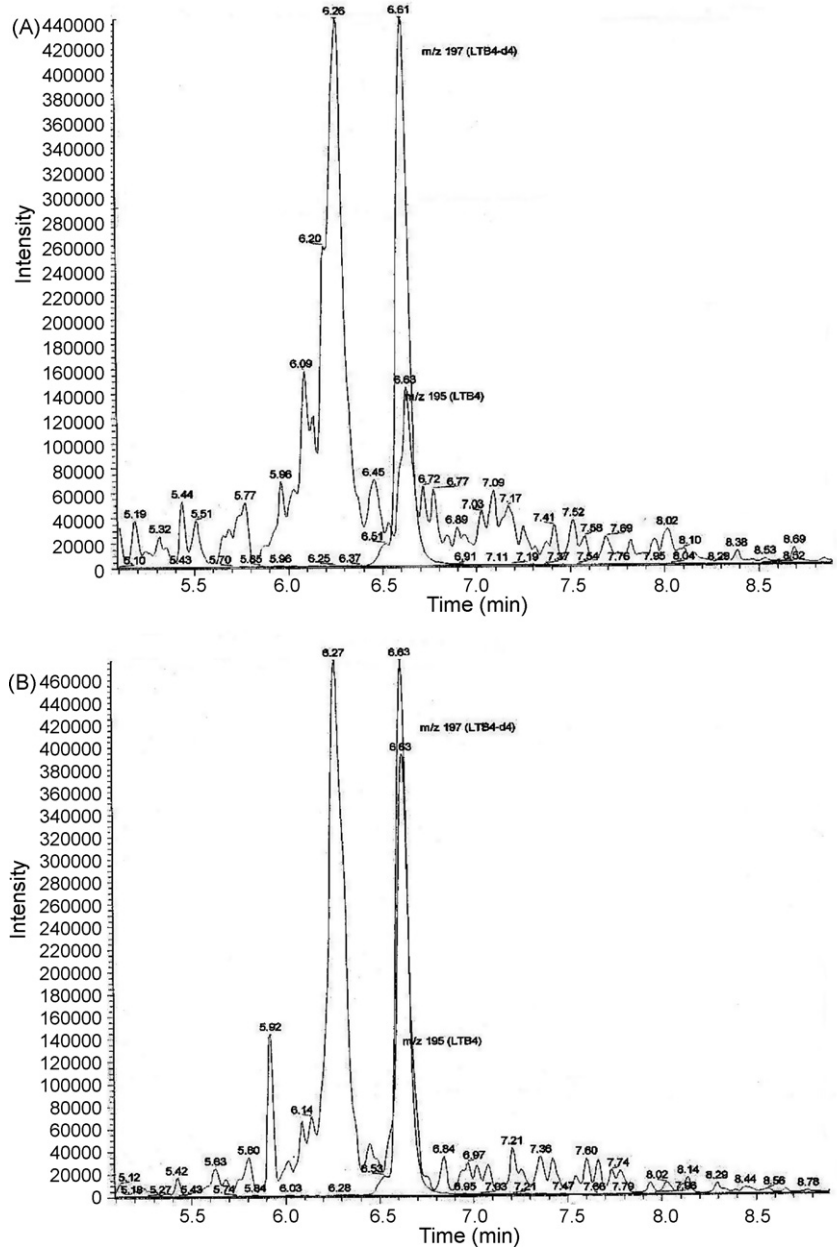

Fig. 6. LC/MS/MS chromatograms of EBC samples obtained from a healthy child (A) and from a child with asthma (B). The ions m/z 195 and m/z 197 were used to monitor endogenous LTB_4 and internal standard (LTB_4-d_4), respectively. LTB_4-d_4 was added to each sample to reach a final concentration of 1000 pg/ml. LTB_4 values, expressed as total amount (in pg) of leukotriene expired in the 15 min breath test and calculated with peak area ratios for LTB_4/LTB_4-d_4, were 450 pg/15 min in the child with asthma and 87 pg/15 min in the healthy child.

ple to reach a final concentration of 1500 pg/ml [14]. Each spiked solution was concentrated 40-fold under a nitrogen stream and 20 μl were directly injected into the LC/MS/MS system with no pretreatment [14]. The peak area ratios for LTB_4/LTB_4-d_4 were plotted versus LTB_4 concentrations. The linearity over the examined range was ($y = 0.0717x - 0.0033$, $r^2 = 0.99$) [14]. The coefficient of variation was ±16% ($n = 7$) at the lowest calibration point (100 pg/ml) [14].TB_4 concentrations in EBC were calculated using this calibration curve. α-Amylase concentrations were undetectable in any study samples, excluding significant salivary contamination [14]. LTB_4 in EBC was detected in 4 patients with asthma who were not being treated with anti-inflammatory drugs for asthma, whereas it was below the LOQ of the technique (100 pg/ml) in the 2 healthy subjects and in those 4 patients with asthma who were receiving anti-inflammatory drugs [14].

3.2. Observational study in children with asthma

Compared to the methodological study, the use of a triple quadrupole mass spectrometer made it possible to increase 10-fold the analytical sensitivity (10 pg/ml vs. 100 pg/ml) of the technique with a lower limit of quantification (LOQ) of 50 pg/ml. The calibration curve in the range 10–500 pg/ml had a good linearity with $r^2 = 0.9965$ [15]. Mean accuracy, calculated for LTB_4 concentrations ranging from 100 to 1000 pg/ml and expressed as percent relative error, was <10%. The intraclass correlation coefficient, expressing between-day variations in LTB_4 measurements in EBC, was 0.89. Quantitative analysis of LTB_4 in EBC was performed in MS^2 mode in order to increase specificity [15]. The mass spectra of LTB_4 and internal standard LTB_4-d_4 showed a base peak at m/z 335 for endogenous LTB_4 and m/z 339 for internal standard LTB_4-d_4, corresponding to

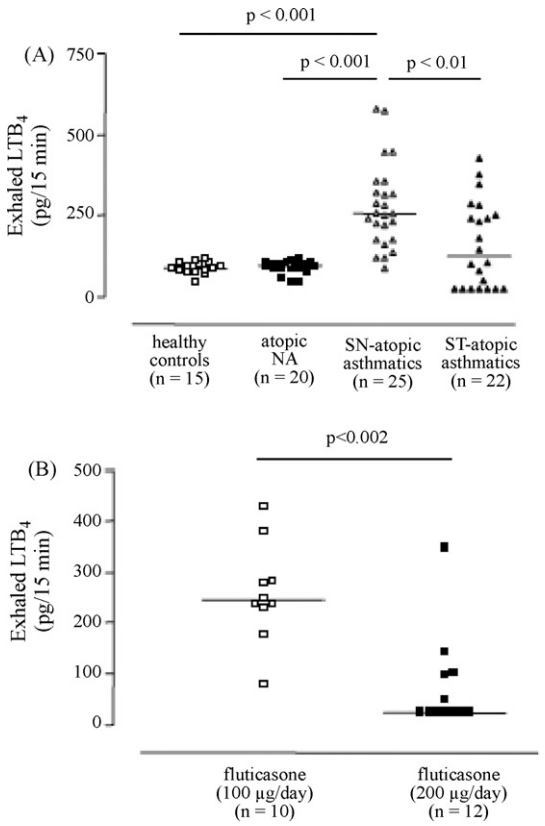

Fig. 7. LTB_4 concentrations in EBC in healthy children (open squares), atopic non-asthmatic children (NA) (filled squares), steroid-naïve children with atopic asthma (open triangles), and steroid-treated (ST) children with atopic asthma (filled triangles) (A). Exhaled LTB_4 values in atopic children with asthma who were receiving either 100 μg/day (open squares) or 200 μg/day (filled squares) of inhaled fluticasone at a constant dose for at least 8 weeks. LTB_4 values are expressed as picograms produced during 15 min of breathing. Median values are shown with horizontal bars. Reprinted from Montuschi et al. [15] (BioMedCentral Publisher, open access journal).

molecular ions [15]. The most abundant product ions obtained by collision-induced decomposition of the carboxylate anion $[M-H]^-$ derived from LTB_4 following ESI were *m/z* 195 and 317 (Fig. 5). To measure LTB_4 in EBC, after fragmentation of the parent ions *m/z* 335 (endogenous LTB_4) and *m/z* 339 (deuterated internal standard) with a collision energy of 17 and 19%, respectively, the transition 335 → 195 *m/z* and 339 → 197 *m/z* was monitored (Fig. 6) [15]. α-Amylase concentrations (<22 mU/ml) were not detected in any study sample, excluding significant salivary contamination. LTB_4 concentrations in EBC were detected in all study samples except for 7 steroid naive atopic children with asthma who were receiving the higher dose of inhaled fluticasone (200 μg/day) [15] (Fig. 7A).

Compared with healthy children [87.5 (82.5–102.5) pg] and atopic non-asthmatic children [96.5 (87.3–102.5) pg], exhaled LTB_4 was increased in steroid-naïve atopic children with asthma [255.1 (175.0–314.7) pg, $p < 0.001$] (Fig. 7A) [15]. Atopic non-asthmatic children and healthy children had similar LTB_4 values in EBC ($p = 0.59$) (Fig. 7A) [15]. Steroid-naïve asthmatic children had higher exhaled LTB_4 [255.1 (175.0–314.7) pg, $p < 0.01$] than children with asthma who were receiving inhaled glucocorticoids [125.0 (25.0–245.0) pg] (Fig. 7A) [15]. Steroid-treated atopic children with asthma had exhaled LTB_4 values similar to those in atopic non-asthmatic children ($p = 0.41$) and healthy controls ($p = 0.43$) (Fig. 7A) [15]. Among steroid-treated atopic children with asthma, children who were receiving the lower dose of inhaled fluticasone (100 μg/day at a constant dose for at least 8 weeks) had higher exhaled LTB_4 values [245.0 (235.0–282.5) pg, $p < 0.002$] than those who were treated with the higher dose of inhaled fluticasone (200 μg/day for at least 8 weeks) [25.0 (25.0–102.5) pg] (Fig. 7B) [15]. No differerence in median exhaled LTB_4 values was observed between asthmatic children who were treated with the higher dose of fluticasone, atopic non-asthmatic children ($p = 0.15$) and healthy controls ($p = 0.10$), whereas exhaled LTB_4 values were elevated in asthmatic children who were receiving the lower dose of inhaled fluticasone (atopic non-asthmatic children: $p < 0.005$; healthy children: $p < 0.001$) [15]. A major unknown peak that has to be identified was observed in all samples obtained from steroid-naïve asthmatic children (Fig. 6), healthy children (Fig. 6), and atopic non-asthmatic children (not shown), whereas it was not always present in steroid-treated children with asthma [15]. There was no correlation between exhaled LTB_4 and exhaled NO values in any study group. There was no correlation between exhaled LTB_4 or exhaled NO and age, sex, or lung function in any study group [15]. LC/MS/MS can also be used for measuring LTB_4 concentrations in EBC in adults with asthma as shown in Fig. 8.

3.3. *LC/ESI⁻-MS/MS measurement of 8-isoprostane and cysteinyl-leukotrienes*

In the ESI^- spectra, $[M-H]^-$ were observed at *m/z* 353 and *m/z* 357, respectively [18]. These precursor ions were used for the quantification of 8-isoprostane in the MRM mode [18]. In the ESI^--MS/MS spectra of 8-isoprostane and 8-isoprostane-d_4, the fragment ions at *m/z* 193 and *m/z* 197 were selected for MRM [18]. Calibration curves were obtained by adding increasing amounts of 8-isoprostane (1–1000 pg) and 250 pg of internal standard to 1 ml aliquots of acetonitrile:water solution (70:30) or 8-isoprostane-free EBC samples [18]. EBC samples obtained from 10 healthy subjects were pooled and 8-isoprostane was removed by immunoaffinity extraction [18]. The residual amount of 8-isoprostane was determined lower than 1 pg/ml EBC [18]. Both calibration curves were linear within the range of 1–1000 pg and correlation coefficients were higher than 0.998 [18]. In EBC, the LOD for 8-isoprostane was 1 pg/ml and the LOQ was 5 pg/ml [18]. Using this technique, 8-isoprostane concentrations were measured in EBC from patients with asbestosis who had higher mean 8-isoprostane concentrations (60 pg/ml) than healthy subjects (36 pg/ml) [18]. The mean 8-isoprostane (73.6 ± 9.9 pg/ml vs. 43 ± 10 pg/ml, $p = 0.0001$) and LTD_4 (21.1 ± 2.7 pg/ml vs. 14.7 ± 2.7 pg/ml, $p = 0.001$) concentrations were higher in patients with silicosis than in healthy subjects [19].

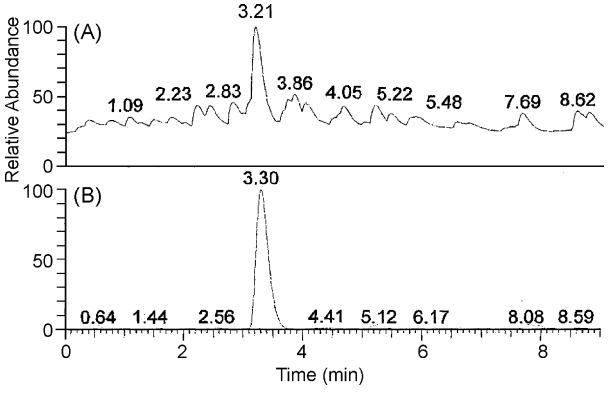

Fig. 8. LC/MS/MS chromatogram of an EBC sample obtained from an adult with asthma who was receiving 750 μg/day of inhaled fluticasone at a constant dose for at least 8 weeks. A Finnigan Surveyor® LC System pump coupled with a TSQ Quantum Ultra™ triple quadrupole mass spectrometer (Thermo Electron Corporation, San José, USA) was used. The ions *m/z* 195 and *m/z* 197 were used to monitor (A) endogenous LTB_4 and (B) internal standard (LTB_4-d_4), respectively. LTB_4-d_4 was added to each sample to reach a final concentration of 1000 pg/ml. LTB_4 concentration, calculated with peak area ratio for LTB_4/LTB_4-d_4, was 330 pg/ml.

4. Discussion

In most studies aimed at measuring LTB_4 and other eicosanoid concentrations in EBC, enzyme immunoassays (EIAs) were used. This analytical approach is suitable for large-scale studies and, due to its high sensitivity, for measuring exhaled eicosanoid concentrations that are usually in the picogram range. However, EIAs work well in buffer, but their behaviour in EBC and their possible matrix effects have not been formally studied. Reverse phase-high-performance liquid chromatography (RP-HPLC) coupled with UV spectroscopy can be used as a pre-analytical technique [21], but its sensitivity for a quantitative assessment of LTB_4 in EBC is generally inadequate. In a previous study, we showed that RP-HPLC peak of LTB_4 was clearly separated from that of the isomer 6-*trans*-LTB_4 [21]. In this study, EBC was subjected to RP-HPLC and LTB_4-like immunoreactivity was measured in the eluted fractions with a commercially available antiserum that has a cross-reactivity of 39% with 6-trans-LTB_4 [21]. No LTB_4-like immunoreactivity was detected in the eluted fractions correspnding to 6-*trans*-LTB_4 peak, no LTB_4-like immunoreactivity was detected. This provides in direct evidence that the concentrations of this LTB_4 isomer in EBC are negligible [21]. We developed LC/MS/MS techniques for measuring LTB_4 concentrations in EBC using LTB_4-d_4 as internal standard [14,15]. The best analytical conditions were obtained using ESI in negative ion polarity mode consistent with the fact that ESI is the most readily applied ionization technique for the analysis of eicosanoids and most of the applications of ESI to eicosanoid analysis has been in the negative ion polarity mode [22]. Similar results were obtaiend by a different group when a LC/MS/MS technique was applied to the measurement of 8-isoprostane and LTs in EBC [18,19]. In the methodological study, we identified the chromatographic behavior and the MS^2 fragmentation of LTB_4 in the EBC [14]. The ESI mass spectra in negative ion polarity mode revealed a base peak at m/z 335 for endogenous LTB_4 and m/z 339 for internal standard LTB_4-d_4, corresponding to $[M-H]^-$ ions [14]. To confirm the presence of LTB_4 in EBC, this eicosanoid was also detected in MS/MS mode which has a higher specificity. In line with previous studies [22], one of the most abundant product ion was observed at m/z 195 following collisional activation of the carboxylate anion $[M-H]^-$ of LTB_4 [14]. The origin of this ion involves double bond rearrangement initiated by the collisional process followed by carbon-carbon bond cleavage [22]. The proposed mechanism for LTB_4 fragmentation [22] involves a cyclization of the conjugated triene to a cyclic diene [23] followed by a complex series of rearrangements, abstraction of the carbon-5 proton by a carbon-12 alkoxide anion or even the carboxylate anion, and formation of anionic site at carbon-11 that undergo a proton shift to form the more stable oxygen-centered anion [22]. The resulting product has the structural characteristics required for a charge-driven α-hydroxy-β-ene fragmentation frequently observed for eicosanoids [22–24]. However, in our methodological study, the sensitivity of the LTB_4 measurement with the ion trap in MS^2 mode was insuffcient for quantitative purposes. For this reason, ion trap analysis of LTB_4 concentrations in EBC was performed in MS^1 mode. The LOQ was 100 pg/ml [14]. Under these experimental conditions, LTB_4 concentrations in EBC were detected only in the 4 patients with asthma who were not receiving anti-inflammatory drugs [14]. A limitation of the ion trap LC/MS measurement of LTB_4 in EBC is the high limit of quantification (100 pg/ml). This problem might be overcome using mass spectrometers with higher sensitivity.

In the observational study in children with asthma, due to the use of a triple quadrupole mass spectrometer the LOD was decreased from 100 to 10 pg/ml and the LOQ was 50 pg/ml [15]. This made it possibile to perform a quantitative analysis of LTB_4 concentrations in EBC in MS^2 mode with increased specifity of the technique [15]. In analogy to previous studies [22], the transition $335 \rightarrow 195$ m/z was used for measuring LTB_4 in EBC [15]. This study provides an example of the possibility to apply this technique to the assessment of airway inflammation in children with asthma [15]. Compared with healthy children, LTB_4 values in EBC were selectively elevated in steroid-naïve children with atopic asthma, but not in atopic non-asthmatic children and in atopic asthmatic children who were treated with inhaled glucocorticoids [15]. Higher degree of airway inflammation in steroid-naïve children with asthma was confirmed by elevated exhaled NO levels in these children [15]. These results indicate that measurement of LTB_4 in EBC can be used as a non-invasive marker of airway inflammation in steroid-naive children with asthma [15]. However, LTB_4 levels in EBC and exhaled NO are likely to reflect different aspects of airway inflammation as indicated by their different behaviour in atopic non-asthmatic children [15]. As asthma is mainly a chronic inflammatory airway disease, evidence of ongoing airway inflammation in asthmatic children with no current symptoms and well mantained lung function may suggest that pharmacological treatment is required. Children with asthma who were receiving inhaled glucococorticoids had lower exhaled LTB_4 values than steroid-naive asthmatic children, but this difference was significant only in children who were treated with the higher dose of fluticasone (200 μg/day for at least 8 weeks) [15]. These results might be explained by the fact that the lower dose of fluticasone was not able to decrease LTB_4 values in EBC in children with asthma, although the observational design of the study precludes definitive conclusions [15]. Large controlled studies are required to establish the effects of inhaled glucocorticoids and other anti-inflammatory drugs for asthma on LTB_4 concentrations in EBC in children with asthma.

Using LC/ESI^--MS/MS, other authors have shown that 8-isoprostane, a reliable marker of lipid peroxidation [25], can be accurately quantified in EBC in healthy subjects and in patients with asbestosis and silicosis [18,19]. Using this technique, this group reported elevated oxidative stress in patients with asbestosis and silicosis as reflected by 8-isoprostane concentrations in EBC [18,19]. The same authors have modified this technique for performing parallel measurement of cysteinyl-LTs [19], but there is a relative lack of information on the analytical procedures. Moreover, no chromatogram related to 8-isoprostane and cysteinyl-LT measurement in EBC was published [18,19]. Due to the relevance of this issue, more methodological details should be provided.

LC/MS/MS is the reference analytical technique for identifying and quantifying eicosanoid concentrations in the EBC. Another avantage of the LC/MS/MS analysis of LTB_4 in EBC is that this technique could be exploited for validation of immunoassays for LTB_4. When we used a triple quadrupole mass spectrometer, the LOQ for LTB_4 was 50 pg/ml [15]. Although substantially improved when compared with the ion trap technique [14], this LOQ could be insufficient for quantifying LTB_4 concentrations in EBC in some healthy subjects or in patients with a mild degree of airway inflammation. It is important to point out that in this study EBC samples were not concentrated [15]. Collecting large EBC volumes in order to increase sample concentration could overcome this problem. However, this may not be feasible, particularly in patients with severe airway inflammatory diseases or in children. Derivatization of EBC samples could also increase the sensitivity of LC/MS/MS techniques. Another limitation of MS techniques is the high cost.

5. Conclusions

LC/MS/MS has demonstrated that LTB_4, a potent inflammatory lipid mediator, is present in the EBC. This analytical methodology is suitable for an accurate quantitative assessment of LTB_4 concentrations in EBC. A LC/MS/MS technique for the measurement of 8-isoprostane concentrations in EBC has been developed and can be used as an alternative to GC/MS. The LC/MS/MS tech-

nique for 8-isoprostane has been adapted for parallel measurement of cysteinyl-LTs in EBC, although more methodological information is required. Measurement of LTB_4 and other eicosanoids in EBC with LC/MS/MS could be used for non-invasive assessment of airway inflmmation, is potentially useful for longitudinal studies in patients with inflammatory airway diseases and occupational exposure, and for monitoring the effect of pharmacological therapy.

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