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Validation of leukotriene B_4 measurements in exhaled breath condensate

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Abstract. *Objective:* To qualitatively validate an enzyme immunoassay to measure leukotriene B_4 in exhaled breath condensate. Exhaled breath condensate is a new non-invasive method to monitor airway inflammation.
Subjects: Twenty-two subjects with different lung diseases attended the outpatient clinic on one occasion for exhaled breath condensate collection.
Methods: Samples were pooled together and purified by reverse-phase high-performance liquid chromatography. The fractions eluted were assayed for leukotriene B_4 by enzyme immunoassay.
Results: A single peak of leukotriene B_4-like immunoreactivity co-eluting with leukotriene B_4 standard (retention time: 24 min) was identified by enzyme immunoassay. Reverse phase-high performance liquid chromatography peak of leukotriene B_4 was clearly separated from those of 6-*trans*-leukotriene B_4 (retention time: 14 min) and leukotriene B_5 (retention time: 18 min) for which the antiserum used in the enzyme immunoassay had the highest cross-reactivity. Leukotriene B_4 recovery was 64%.
Conclusions: This study provides evidence for the presence of leukotriene B_4 in the exhaled breath condensate and the specificity of the enzyme immunoassay used.

Key words: Exhaled breath condensate – Leukotriene B_4 – Enzyme immunoassay – Reverse-phase high-performance liquid chromatography – Airway inflammation

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Introduction

Airway inflammation plays an important pathophysiological role in lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) [1]. Airway inflammation may precede the mechanical lung damage and is not necessarily correlated with lung function tests [2].

Exhaled breath condensate (EBC) analysis is a new method to sample secretions from the airways [3]. This method is completely non-invasive and is potentially useful for long-term monitoring of patients, including children, with inflammatory airway diseases [4, 5]. Measurement of Leukotriene $(LT)B_4$, a potent neutrophil chemoattractant, in EBC may provide a useful approach for monitoring airway inflammation in patients with lung diseases. In previous studies, we have shown that LTB_4 is detected in EBC in healthy subjects and is increased in patients with asthma [6, 7]. In these patients, LTB_4 levels were correlated with exhaled nitric oxide, a marker of airway inflammation [7]. We have recently measured LTB_4 in EBC in patients with COPD [8] who had higher levels of this eicosanoid than healthy subjects [5]. In these studies, LTB_4 in EBC was measured by commercially available enzyme immunoassays leaving open the issue of the specificity of this analytical approach. Although the mass spectrometry methodology is highly sensitive, specific, and accurate, it requires expensive instrumentation and it is time consuming. Quantitative high performance liquid chromatography (HPLC) is a valid alternative to mass spectrometry, but it may not be suitable for routine use particularly when a large number of samples need to be assayed. In this regard, the major limitation for the wide-spread use of measurements of LTB_4 in EBC by investigators in airway inflammation has been the issue of reliability of immunoassays for this eicosanoid.

The aim of this study was to qualitatively validate an enzyme immunoassay for LTB_4 in EBC by reverse-phase high-performance liquid chromatography (RP-HPLC) analysis of the immunoreactive material.

Materials and methods

Subjects and study design

EBC was obtained from a group of 22 patients with different lung diseases: sixteen men and six women, age 57.9 ± 3.0 yr (mean ± SEM), FEV_1 80.8 ± 5.1% predicted value, FVC 98.5 ± 4.7% predicted value, ten with stable COPD, two with exacerbated COPD, two with COPD and lung cancer, two with recurrent bronchitis, two with stable asthma, one with chronic cough, one with lung cancer, one with bronchopneumonia, and one healthy smoker. Eleven patients were current smokers (> 10 pack-year), six were ex-smokers (> 10 pack-year), and five were non-smokers. Eight patients were treated with inhaled corticosteroids (fluticasone: 0.5–1 mg/day; beclomethasone: 1.2–1.6 mg/day) and three patients with oral corticosteroids (methylprednisolone: 8–16 mg/day). The diagnosis of asthma and COPD was based on the criteria for the American Thoracic Society [9].

Samples were pooled together to increase the amount of LTB_4. A total of 35.5 ml EBC was collected.

Subjects attended on one occasion for EBC collection and lung function measurements. Subjects attended the outpatient clinic at Respiratory Pathophysiology Unit in the University Hospital "A. Gemelli" in Rome. Informed consent was obtained from all subjects. This study was approved by the Ethics Committee of the University Hospital "A. Gemelli".

Experimental procedure

The experimental procedure included the following steps: 1) collection of EBC samples which were pooled together; 2) extraction of the EBC sample pool and of 5 ng standard LTB_4; 3) RP-HPLC purification of LTB_4-like immunoreactivity and standard LTB_4; 4) enzyme immunoassay analysis of LTB_4-like immunoreactivity and LTB_4 in the HPLC eluted fractions.

Collection of exhaled breath condensate

EBC samples were collected using a specially design condensing chamber (Ecoscreen, Jaeger, Hoechberg, Germany) as described previously [10]. Briefly, exhaled air entered and left the chamber through one-way valves at the inlet and outlet, thus keeping the chamber closed. Subjects breathed tidally through a mouthpiece connected to the condenser for 15 min while wearing noseclips. A temperature of –20 °C inside the condensing chamber throughout the collection time produced sample cooling down. An average of 1.54 ml EBC per patient was collected. Samples were stored at –70 °C before eicosanoid extraction, RP-HPLC purification, and enzyme immunoassay measurements which were performed within 2 weeks after the collection of the EBC samples. In preliminary experiments, we measured α-amylase concentrations in EBC samples obtained from the same group of subjects. α-Amylase concentrations in EBC were measured by an in vitro colorimetric method using maltotriose with the indicator group, 2-chloro-p-nitrophenol (Roche Diagnostics, Basel, Switzerland). Consistent with previous studies [11], no α-amylase concentrations were detected in any sample suggesting no contamination of EBC with saliva.

Extraction of exhaled breath condensate samples

LTB_4 was extracted from a pool of EBC samples (35.5 ml total). One ml aliquots of EBC sample pool was measured unextracted to evaluate the total endogenous LTB_4 content and to calculate recovery after extraction and RP-HPLC purification. LTB_4 was extracted following a procedure previously described for 8-isoprostane [12]. Briefly, the EBC sample pool (34.5 ml) was extracted on Sep-Pak C18 cartridges (Waters Associates, Milford, Massachussetts, USA) and eluted with 10 ml ethyl acetate. The eluate was subjected to silicic acid column chromatography, further eluted with benzene/ethyl acetate/methanol (60 : 40 : 30, by volume), and vacuum-dried in a Speedvac evaporator linked with a Savant-refrigerated condensation trap. Recovery for LTB_4 was calculated by two different criteria: (1) by extracting 5 ng of LTB_4 standard added to an equal volume of distilled water and by measuring the total LTB_4 amount in aliquots of fractions eluted from the HPLC by enzyme immunoassay and (2) by calculating the recovery of endogenous LTB_4 which was obtained dividing the total LTB_4-like immunoreactivity measured in the RP-HPLC eluted fractions by the total amount of LTB_4 extracted. The latter was calculated multiplying the mean LTB_4-like immunoreactivity in the pool of EBC samples before extraction and RP-HPLC purification by the total sample volume extracted.

HPLC purification and enzyme immunoassay analysis

This procedure included: 1) RP-HPLC ultraviolet profiling of standard LTB_4, 6-*trans*-LTB_4, LTB_5, and LTE_4; 2) RP-HPLC purification of the solvent system (blank) to exclude carry-over after injection of the standard into RP-HPLC; 3) RP-HPLC purification of LTB_4-like immunoreactivity in the extracted EBC sample pool; 4) a second blank; 5) RP-HPLC purification of the extracted standard LTB_4 (5 ng); 6) enzyme immunoassay for LTB_4 in the HPLC eluted fractions.

1) The peak of LTB_4 eluted at 24 min and was identified by ultraviolet profiling of standard (0.5 µg). The peak width ranged from 5 to 6 min. Considering that the anti-LTB_4 serum used to measure exhaled LTB_4 in the enzyme immunoassay has a significant cross-reactivity with 6-*trans*-LTB_4 (39%) and LTB_5 (100%), the peaks of these LTB_4 analogues were also determined in a similar way. LTE_4 peak was also identified by ultraviolet profiling of standard (0.5 µg). Retention times for 6-*trans*-LTB_4, LTB_5, and LTE_4 were 14 min, 18 min, and 32 min, respectively.
2) The solvent system (blank) was injected into RP-HPLC and purified. The eluted fractions were collected every min for 45 min at a flow rate of 1 ml/min for enzyme immunoassay analysis of LTB_4-like immunoreactivity to exclude possible carry-over after standard ultraviolet profiling.
3) A single eluate obtained from the EBC sample pool was recovered with 100 µl of methanol and then subjected to RP-HPLC (C18, 125 × 4.6 mm, 5 µm, LiChrospher column) with the solvent system acetonitrile/water/acetic acid (65 : 35 : 0.02, by volume); 1 min samples were collected for 45 min.
4) A second blank was performed to exclude carry-over after EBC sample pool purification.
5) Standard LTB_4 (5 ng) was subjected to RP-HPLC and the eluted fractions were collected as above to calculate recovery. All HPLC purifications were carried out isocratically.
6) Finally, each RP-HPLC fraction was vacuum-dried as described above, recovered in 0.5 ml enzyme immunoassay buffer, diluted 1 : 10 and tested in the enzyme immunoassay system. LTB_4 was measured by a commercially available enzyme immnunoassay kit following the instructions of the manufacturers (Cayman Chemicals, Ann Arbor, Michigan, USA). The detection limit for LTB_4 was 10 pg/ml with an IC_{50} of 35 pg/ml. LTB_4 antiserum cross-reactivity is shown in Table 1. Details on anti-LTB_4 serum preparation are not provided by the manufacturers. The intra-assay (n = 6) and inter-assay (n = 8) coefficients of variation of LTB_4 were 5 to 10% and 10 to 15%, respectively, across the range of values measured (10–250 pg/ml).

Materials

6-*trans*-LTB_4 standard was purchased from Cayman Chemicals. LTB_4, LTB_5, LTE_4 standards were purchased from Sigma Chemical Co (St. Louis, Missouri, USA). All solvents HPLC grade were from Merck (Darmstadt, Germany).

Table 1. Specificity of anti-LTB_4 serum used for EIA.

Ligand	Cross-reactivity of antiserum (%)
LTB_4	100.0
LTB_5	100.0
6-*trans*-LTB_4	39
6-*trans*-12-epi-LTB_4	0.7
20-hydroxy-LTB_4	0.5
5(S)-HETE	0.03
20-carboxy-LTB_4	< 0.01
5(S),12(S)-DiHETE	< 0.01
12(S)-HETE	< 0.01
15(S)-HETE	< 0.01
LTC_4	< 0.01
LTD_4	< 0.01
LTE_4	< 0.01

Cross-reactivity was determined after addition of either homologous (LTB_4) or heterologous (other eicosanoids) ligands to the antibody-tracer complex. Displacement of 50% of initial binding was determined for different compounds and relative % was expressed as concentration of homologous/concentration of heterologous ligand × 100.
Definition of abbreviations: EIA = enzyme immunoassay; HETE = hydroeicosatetraenoic acid; LT = leukotriene.

Results

Qualitative validation of the enzyme immunoassay method to measure LTB_4 in EBC was sought by RP-HPLC separation of a pool of samples and subsequent enzyme immunoassay analysis. A single peak of immunoreactivity co-eluting with standard LTB_4 was identified, indicating that the unknown LTB_4-like immunoreactivity in EBC has an identical chromatographic behaviour with authentic LTB_4 (Fig. 1A and 1B). LTB_4-like immunoreactivity was detected only in the following RP-HPLC eluted fractions (flow rate: 1 ml/min): fraction 22 (353 pg/ml), fraction 23 (545 pg/ml), fraction 24 (680 pg/ml), fraction 25 (358 pg/ml), fraction 26 (320 pg/ml), fraction 27 (280 pg/ml) (Fig. 1B). Moreover, RP-HPLC peak of LTB_4 (retention time: 24 min) was clearly separated from those of 6-*trans*-LTB_4 (retention time: 14 min) and LTB_5 (retention time: 18 min) for which the antiserum used in the enzyme immunoassay had the highest cross-reactivity (39% and 100%, respectively) (Fig. 2). Finally, no LTB_4-like immunoreactivity was detected in the eluted fractions corresponding to 6-*trans*-LTB_4 and LTB_5 peaks indicating that the concentrations of these LTB_4 analogues in the EBC, if any, are negligible (Fig. 1B). After RP-HPLC purification of the standard, LTB_4 concentrations were detected only in the following eluted fractions: fraction 22 (275 pg/ml), fraction 23 (1001 pg/ml), fraction 24 (1018 pg/ml), fraction 25 (371 pg/ ml), fraction 26 (248 pg/ml), and fraction 27 (289 pg/ml) (Fig. 1A). Recovery of LTB_4 was 64.0% after extraction and RP-HPLC purification of the standard compound (5 ng) (Fig. 1A). Mean LTB_4-like immunoreactivity concentration measured in an unextracted aliquot (1 ml) of the EBC sample pool was 108.7 pg/ml. An estimated total amount of 3,760 pg of LTB_4-like immunoreactivity was extracted and the calculated recovery for endogenous LTB_4 was 67.5% (Fig. 1B).

LTB_4-like immunoreactivity was undetectable in all the RP-HPLC eluted fractions of blanks thus excluding the possibility of carry-over after standard and sample purification.

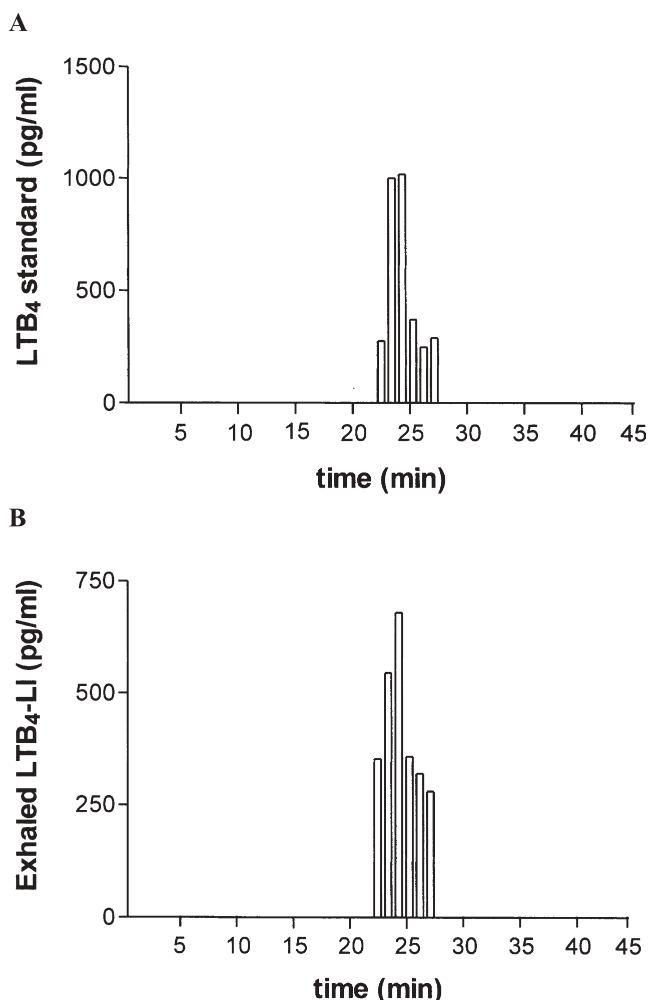

Fig. 1. Reverse phase-high performance liquid chromatography (RP-HPLC) analysis of leukotriene (LT) B_4 in exhaled breath condensate (EBC). **A**) LTB_4 concentrations in the fractions eluted measured by enzyme immunoassay after extraction and RP-HPLC purification of the standard compound (5 ng). Retention time was 24 min. Recovery was 64%. **B**) LTB_4-like immunoreactivity in a pool of EBC samples. Eluted fractions were collected every min for 45 min at a flow rate of 1 ml/min (5 min intervals are shown in x-axis). Aliquots were measured by enzyme immunoassay. A total of 3,760 pg of LTB_4-like immunoreactivity was extracted and purified by RP-HPLC. Recovery for endogenous LTB_4 was 67.5%. All RP-HPLC purifications were carried out isocratically. Details of the solvent systems used are described in the text.

Discussion

In this study, we performed qualitative validation of an enzyme immunoassay method to measure LTB_4 in EBC through studies of chromatographic behaviour of the measured material. We demonstrated for the first time identical chromatographic behaviour of LTB_4-like immunoreactivity in EBC with the respective standard. These results strongly suggest that immunoreactive material detected in EBC is rep-

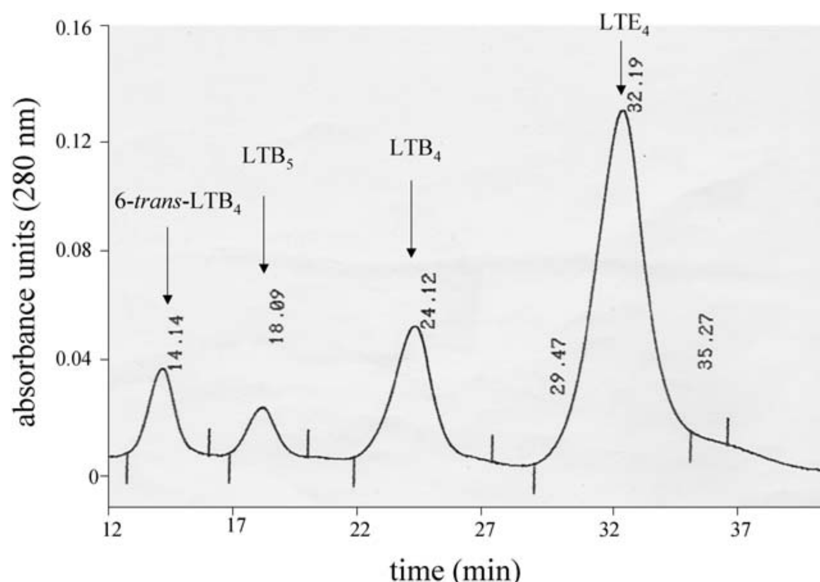

Fig. 2. Reverse phase-high performance liquid chromatography (RP-HPLC) peaks of leukotriene (LT) B_4, 6-*trans*-LTB_4, LTB_5 and LTE_4 identified by ultraviolet profiling of corresponding standards (0.5 µg). Retentions times for LTB_4, 6-*trans*-LTB_4, LTB_5 and LTE_4 were 24 min, 14 min, 18 min, and 32 min, respectively. RP-HPLC was performed from 0 to 45 min (only the interval ranging from 12 to 40 min is shown, x-axis). Details of the solvent systems used are described in the text.

resented by authentic LTB_4, although the possible existence of different compounds behaving in an indistinguishable fashion in certain solvent systems cannot be definitively ruled out. However, this is likely to have limited biological relevance. The anti-LTB_4 serum we used in the enzyme immunoassay has the highest cross-reactivity with 6-*trans*-LTB_4 (39%) and LTB_5 (100%), whereas cross-reactivity with other LTB_4 analogues is <1%. Differences in the retention times between LTB_4 (24 min), 6-*trans*-LTB_4 (14 min), and LTB_5 (18 min) and lack of LTB_4-like immunoreactivity in the RP-HPLC fractions in which these compounds are expected to elute indicate that the amount of these LTB_4 analogues in EBC, if any, is negligible.

Recovery for LTB_4 after extraction and RP-HPLC purification of the standard compound (5 ng) (64%) was similar to the recovery for endogenous LTB_4 (67.5%).

We also measured the mean concentration of LTB_4-like immunoreactivity in an unextracted aliquot from the EBC sample pool (108.7 pg/ml). However, comparisons with previous studies were not possible since EBC samples in our study were obtained from patients with different lung diseases and, probably, with a different degree of airway inflammation. On the other hand, the aim of this study was the RP-HPLC qualitative validation of LTB_4 measurements in EBC by an immunoassay and not the quantification of exhaled LTB_4 concentrations in inflammatory airway diseases.

In conclusion, our study provides evidence for the presence of LTB_4 in EBC and the specificity of its measurements by the enzyme immunoassay discussed above. However, only the comparison with an independent assay method such as gas chromatography/mass spectrometry (GC/MS) can provide definitive evidence for the identification of LTB_4-like immunoreactivity in EBC. Moreover, quantitative comparisons between immunoassays and GC/MS or HPLC are required. On the other hand, GC/MS is a very expensive and time-consuming method, not suitable for routine use. A potential implication of our study is the large scale use of this enzyme immunoassay to measure exhaled LTB_4 which may contribute to the development of EBC analysis in respiratory medicine. Validation of immunoassays for LTB_4 in EBC is important for proposing exhaled LTB_4 as a non-invasive marker for monitoring airway inflammation in different lung diseases.

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