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BRIEF COMMUNICATION

Using high-performance liquid chromatography with UV detector to quantify exhaled leukotriene B_4 level in nonatopic adults

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This study aimed to evaluate the feasibility of the chemical method to analyze exhaled breath condensate (EBC) leukotriene B_4 (LTB_4) level in humans. High-performance liquid chromatography with a UV detector was applied to quantify the inflammatory biomarker. The LTB_4 concentration in the concentrated pooled EBC samples was 1.19 ng/μL, and the average LTB_4 concentration of each EBC sample was 15.38 ng/μL. This analytical technique was feasible to evaluate the levels of inflammatory mediators such as LTB_4 in human EBCs without any complicated sample pretreatment processes.
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Introduction

Leukotriene B_4 (LTB_4) is a potent chemokine and becomes the neutrophil chemotaxis once activated.[1,2] Increased LTB_4 and leukotriene E_4 (LTE_4) levels in specimens such as sputum, bronchoalveolar lavages, nasal capacity secretions, and exhaled breath condensates (EBCs) can reflect the severity of human airway inflammation.[2] Developing a reproducible, highly sensitive, and specific analytical method to measure EBC biomarkers even at low concentrations is important.[1,3,4] LTB_4 and LTE_4 in EBCs were measured by gas chromatography (GC)[5] and liquid chromatography (LC),[6–8] in addition to enzyme-linked immunoassays and fluorometric assays. The GC method needed derivatization prior to analysis.[7] Immunoassay methods often lacked specificity.[7] Cáp et al[5] measured the EBC LTB_4 and LTE_4 levels in children and adults with asthma and in healthy adults and children by GC/mass spectrometry (MS), and proved that EBC LTB_4 concentrations of the group with asthma were statistically higher than those of the healthy group. High-performance liquid chromatography (HPLC) was able to separate LTB_4, LTB_5, and 6-trans-LTB_4 of EBCs, being pooled from 22 patients with different lung diseases.[8] Pelclová et al[9] used LC/electrospray ionization–MS to quantify EBC leukotrienes in silicosis patients and healthy adults, and demonstrated that LTB_4 concentrations between the patients (26.8 ± 3.3 pg/mL) and healthy adults (29.4 ± 7.8 pg/mL) did not show any significant difference.

Until recently, no standardized chemical analytical methods have been established for the measurement of EBC biomarkers.[4,9–11] This study aimed to quantify EBC LTB_4 in healthy adults using a HPLC with UV detector (HPLC/UV).

Materials and methods

EBC collection

The study protocol was approved by the institutional review board of Chang Gung Memorial Hospital, Taipei, Taiwan. Prior to participating in the study, informed written consent was obtained from each individual. Sixty undergraduates with no history of allergies or pulmonary disease and no smoking behavior were recruited from Chang Gung University. Age range of recruited adults was 20–23 years (average 20.5 years). The percentages of female and male adults were 73.3% and 26.7%, respectively. The participants had no symptoms of airway infection and used drugs prior to EBC collection. Resulting EBCs (volumes ranging from 1 mL to 3 mL) were collected in a condensing chamber (Ecoscreen; Jaeger Toennies, Hoechberg, Germany) and stored at $-80^{\circ}C$.[12,13]

Standard preparation

The LTB_4 stock solution (10 ng/μL) was diluted from a 100 ng/μL LTB_4 solution (Cayman Chemical Co., Ann Arbor, MI, USA) with ethanol (purity ≥95%; Taiwan Sugar Co., Tainan City, Taiwan). Concentrations of calibration standards ranged from 1.0 to 10.0 ng/μl for LTB_4 were diluted from the stock solutions.

Sample pretreatment

Prior to HPLC analysis, all EBC samples were treated with argon at a flow rate of 3.5 L/min for 10 minutes to eliminate CO_2 and their water content was removed using a freeze dryer (Labconco Freeze Dryer 7750000; Labconco Corp, Kansas City, MO, USA). Ethanol (50 μL) was added to the dry EBC samples. To increase the amount of leukotrienes, 5 μL of each EBC–ethanol solution was taken and pooled to form a 300-μL sample. Ethanol was then removed under a nitrogen stream. Finally, ethanol was added up to a volume of 10 μL for HPLC quantitative analysis.

Analytical conditions

Liquid chromatographic separation was performed on an Agilent series 1200 HPLC system (Santa Clara, CA, USA). Chromatography was performed on an Agilent Eclipse XDB-C18 column (2.1×150 mm^2, 5 μm) using methanol (purity >95%, Mallinckordt Baker, Center Valley, PA, USA) and an aqueous solution of 10 mM ammonium acetate (purity >99%; Fluka Analytical, Buchs, Switzerland) (70:30, v/v) as the mobile phase and at a flow rate of 0.1 mL/min in an isocratic condition. The detection was accomplished with a UV–visible detector (HP-G1365D; Santa Clara, CA, USA). The wavelengths were set at 270 nm for LTB_4 and 280 nm for LTE_4. The retention times of LTB_4 and LTE_4 were 9.3 and 12.1 minutes, respectively. The injection volume was 2 μL.

Results

The peak areas for LTB_4 were plotted versus LTB_4 concentrations; good linearity was observed over the examination concentrations ranging from 1.0 ng/μL to10 ng/μL with an *r*-square (r^2) value of 0.9994. Using the calibration curve, the LTB_4 concentration of the pooled EBC sample was calculated. The chromatogram of the pooled EBC sample is shown in Fig. 1. The retention time was 9.2 minutes for LTB_4. The LTB_4 concentrations in EBC samples were calculated as follows:

The LTB_4 concentration in the sample (injected into the HPLC column), after being deducted from the calibration curve, was 1190 pg/μL.

1190 pg/μL × 10 μL (final concentrated pooled sample volume) = 11,900 pg (LTB_4 mass in concentrated pooled EBC samples)

11,900 pg × (50/5) (concentration factor) ÷ 128.94 mL (total original EBC volume) ÷ 60 (sample size) = 15.38 pg/mL (average mass per EBC sample)

The concentration of LTB_4 in the concentrated pooled sample was 1.19 ng/μL. The average EBC LTB_4 level of the adult participants was 15.38 pg/mL.

Discussion

This simple HPLC/UV method was able to detect the LTB_4 level in humans after concentrating EBC samples by

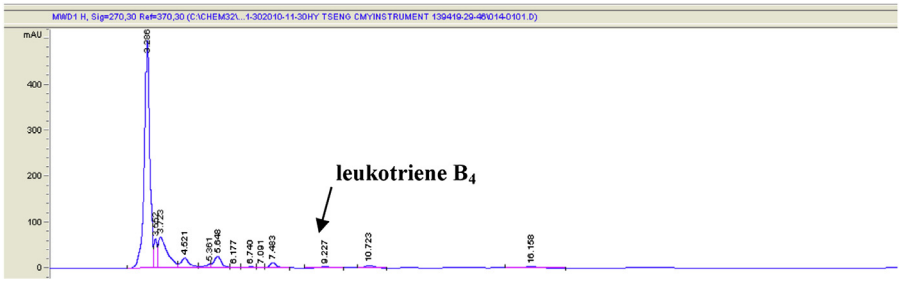

Figure 1 Chromatogram of the pooled exhaled breath condensate samples.

lyophilization and using an argon stream without interferences. No complicated chemical pretreatment[5] or filtration[8] was needed. A GC/MS study indicated that the EBC LTB_4 (205 pg/mL) and LTE_4 (135 pg/mL) levels in asthmatic patients were significantly higher than those in healthy adults (LTB_4 79 pg/mL and LTE_4 74 pg/mL).[5] Steroid-naive atopic children had a higher EBC LTB_4 level (255.1 pg) than healthy controls (87.5 pg), as found by LC/MS.[6] Those studies reported that the EBC LTB_4 and LTE_4 levels of asthmatic patients increased three-fold compared with the healthy group and would be detectable by the present method. The detection limits of this simple HPLC/UV method were higher than the MS methods[5,6] as the UV detection limit is generally 10-fold higher than the MS detection limit.[14] This explains why LTB_4 in a single EBC sample could not be detected in this study. The small allowable injection volume of this particular HPLC column was another limitation. Further studies should be conducted considering an alternative column allowing a large injection volume to increase the LTE masses on the column. Additionally, this study primarily evaluated the feasibility of HPLC/UV to analyze EBC LTB4 levels in healthy adults. Further studies are needed to evaluate the concentration distribution of EBC LTB4 levels in patients with allergic respiratory diseases using the HPLC/UV method.

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