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Short communication

Simplified method for measuring urinary leukotriene E_4

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Abstract

The conventional method for measuring urinary leukotriene E_4 (LTE4) is by reversed-phase high-performance liquid chromatography (RP-HPLC), followed by radioimmunoassay (RIA) or enzyme immunoassay (EIA). We measured urinary LTE4 levels by two methods, HPLC with EIA and EIA alone after initial crude extraction of urine using an octadecyl reversed-phase extraction cartridge (Sep-Pak). Ninety-three urine samples from normal subjects and patients with bronchial asthma and adult respiratory distress syndrome were tested. The results showed that urinary LTE4 levels measured by EIA significantly correlated with those measured by HPLC plus EIA in the three groups (r=0.88, 0.85, 0.68). The absolute values of urinary LTE4 measured by EIA without HPLC purification were higher than by EIA with HPLC purification. This suggests that HPLC may not be necessary for routine urinary LTE4 quantitation in different clinical situations.

Keywords: Leukotrienes

1. Introduction

Leukotrienes belong to a family of arachidonic acid metabolites with potent biological activities, and are implicated in many disease processes [1–6]. Leukotriene E_4 (LTE4) is the major metabolite of leukotriene metabolism, it is excreted in urine and is metabolically stable compared to other leukotrienes [7,8]. Hence urinary LTE4 level has been routinely used as a measure of leukotriene production in humans [9,10]. In earlier studies, urinary samples were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) prior to quantitation of the LTE4 levels by radioimmunoassay (RIA). Purification of the urinary samples was necessary for RIA, as the cross-reactivity of LTE4 with the other leukotrienes in RIA was reported to vary from 35% to 68% [11].

Recently, a commercial test system using enzyme immunoassay (EIA) for the quantitation of LTE4 was made available. This assay was reported to be specific for LTE4, with less than 10% cross-reactivity with the other leukotrienes. Thus, it is possible to omit the RP-HPLC purification step, for the quantitation of urinary LTE4 levels. In this study we have demonstrated that the urinary LTE4 levels can be directly quantitated using EIA. Purification and extraction of the urinary samples by RP-HPLC prior to quantitation is no longer necessary.

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2. Experimental

2.1. Chemicals

EIA LTE4 assay kit and synthetic LTB4, LTC4 and LTE4 were purchased from Cayman (Ann Arbor, MI, USA). ^{3}H-LTE4 was obtained from Amersham (Buckinghamshire, UK). All solvents used in HPLC were of HPLC grade (Merck, Darmstadt, Germany). All other chemicals and reagents used were of analytical grade (BDH, Poole, UK).

2.2. Patients and collection of urine samples

Urine samples collected from 19 normal subjects, 64 asthmatic patients, and 10 patients with adult respiratory syndrome (ARDS), were immediately stored at −70°C pending assay. At the time of assay, the urine samples were thawed and a 1.0-ml aliquot removed for the creatinine determination using the Kodak Ektach clinical chemistry slide.

2.3. HPLC optimization

Preliminary experiments were performed to determine the optimal conditions for separation of synthetic leukotrienes LTC4, LTB4 and LTE4. The column was a C_{18} reversed-phase column (Nova-Pak, 300 mm×3.9 mm I.D., Waters, Milford, MA, USA) and the HPLC system was a 600E Model from Waters. The separation procedure of Drazen et al. [10] for LTE4 purification was used with minor modifications. The final optimum condition was a methanol–water–acetic acid mixture (65:34.9:0.1, v/v) containing 0.05% EDTA (pH 5.0 with ammonium hydroxide) with a flow-rate of 0.5 ml/min at 22–24°C.

2.4. Comparison of two LTE4 assays

Urine samples were centrifuged at 1200 *g* for 10 min to remove particulates. A 20-ml volume of the supernatants, spiked with 11 000 dpm of ^{3}H-LTE4 (as internal standard for recovery estimation), was loaded onto Sep-Pak C_{18} cartridges to extract lipid materials. The Sep-Pak C_{18} cartridges were preconditioned with 5 ml of methanol, 5 ml of deionized water and 5 ml of 0.1% EDTA (pH 6.0). The loaded cartridge was washed with 5 ml of deionized water followed by 5 ml of petroleum ether. Leukotrienes were finally eluted with 5 ml of 90% methanol. The eluent was collected and divided into three aliquots (1 ml, 2 ml and 2 ml). The 1-ml aliquot was used for ^{3}H radioactivity measurement. The other two 2-ml aliquots were evaporated to dryness under vacuum. One of the dried aliquots was stored at −70°C pending EIA assay. The other dried aliquot was resuspended with 300 μl of the HPLC mobile phase and fractionated with HPLC. The fraction containing the highest ^{3}H-LTE4 activity (elution time of 26–30 min) was collected and subdivided into two portions (1 ml and 1.5 ml). The 1-ml portion was used for ^{3}H radioactivity measurement and the 1.5-ml portion was evaporated to dryness under vacuum and stored at −70°C pending EIA.

Quantitation of LTE4 by EIA was performed according to the instructions supplied with the Kit. EIA results were read by a Bio-Rad 3550 Model microplate reader. All LTE4 values were corrected for the amount lost and expressed as ng/mmol creatinine. Levels of urinary LTE4 with HPLC purification were compared with those without HPLC purification by examining the correlations and by using Student's paired *t*-test. Significance was assumed at $p<0.05$. The study was approved by the Hospital Ethical Review Committee.

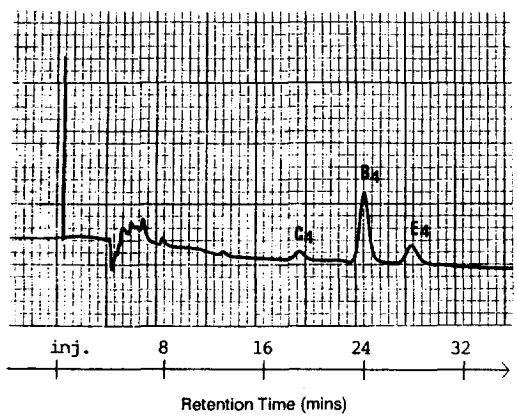

Fig. 1. Chromatogram of leukotrienes. Synthetic leukotriene mixtures consisting of LTC4 (10 ng), LTB4 (100 ng) and LTE4 (50 ng) were assessed by HPLC using a C_{18} RP-column. The mobile phase was methanol–water–acetic acid (65:34.9:0.1, v/v) containing 0.05% EDTA (pH 5.0). The flow-rate was 0.5 ml/min. The chart speed was 25 mm/min.

3. Results and discussion

3.1. HPLC optimization

The chromatogram of synthetic LTC4, LTB4 and LTE4 detected at 280 nm shows their separation under the conditions described above (Fig. 1).

3.2. LTE4 assays

Levels of urinary LTE4, measured by EIA, in samples crudely extracted with Sep-Pak C_{18} cartridge (s-LTE4) were significantly correlated with those samples subjected to additional purification using HPLC (h-LTE4). The correlation coefficients (r) for these two methods were 0.88 ($p<0.0001$), 0.84 ($p<0.0001$) and 0.64 ($p<0.029$) for normal subjects (Fig. 2a), asthmatic patients (Fig. 2b), and patients with ARDS (Fig. 2c), respectively.

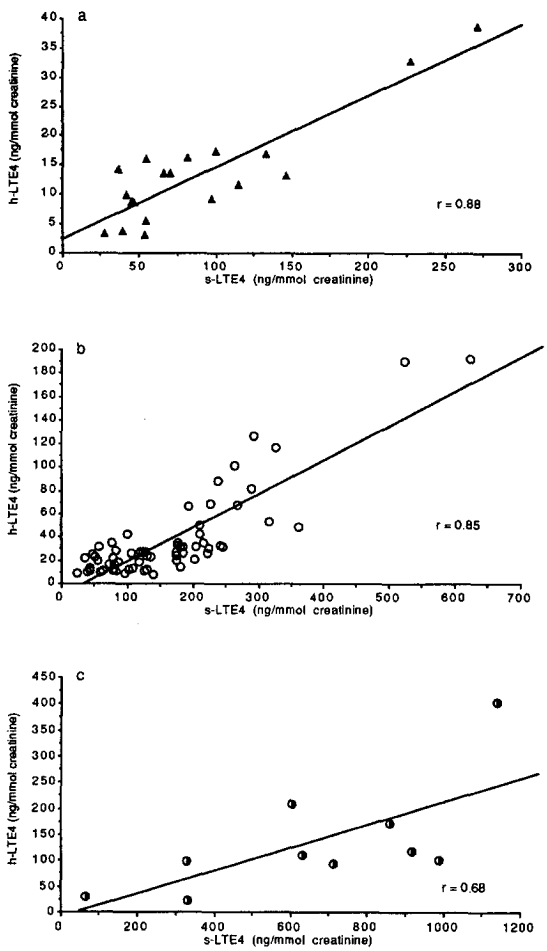

Fig. 2. Comparison of the urinary LTE4 levels from 19 normal subjects (panel a), 64 asthmatic subjects (panel b), and 10 ARDS subjects (panel c) as analyzed using EIA after purification by Sep-Pak alone (s-LTE4, x-axis) and by Sep-Pak plus HPLC (h-LTE4, y-axis).

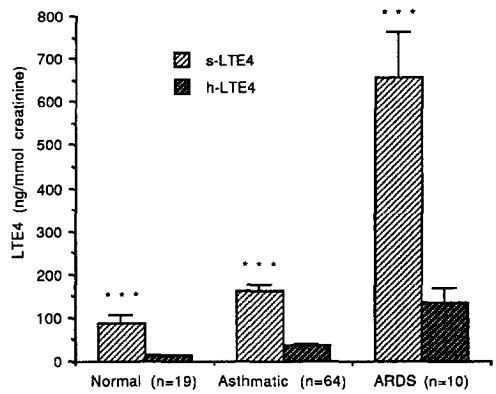

Fig. 3. The mean(±S.E.) urinary LTE4 levels measured by EIA of the samples without HPLC (s-LTE4) and with HPLC (h-LTE4) purification were compared. ***=$p<0.001$.

In all subjects, the concentrations of urinary LTE4 after Sep-Pak extraction were significantly higher than those subjected to both Sep-Pak extraction and HPLC purification (Fig. 3). The recovery (mean±S.E.) for the Sep-Pak procedure was 77.5±2.1%, 74.2±1.7% and 67.4±3.4%, and for the Sep-Pak plus HPLC purification it was 53.2±2.8%, 61.8±1.7% and 51.3±1.6% in normal subjects, asthmatic patients, and the patients with ARDS, respectively.

In most urinary LTE4 detections, HPLC was used to purify LTE4 prior to the quantitation. There were no studies which systematically evaluated the necessity for HPLC purification in the quantitation of LTE4. The new commercial LTE4 EIA is highly specific, and has much lower cross-reaction with other leukotrienes (<10%, manufacturer's instruction) compared to that in previous LTE4 RIA (35%–68%) [11]. In this study, our results clearly show that HPLC purification is not necessary in EIA quantitation of urinary LTE4.

We found that the levels of urinary LTE4 measured by EIA without HPLC purification were significantly higher than those measured after HPLC purification. There were two potential explanations for this observation. One explanation was that urin-

ary LTE4 could be lost during HPLC purification. Although a correction for this loss had been incorporated in the method by estimating the recovery, it was only an approximate calculation. Because the exact period of elution of ^{3}H-LTE4 internal standard and urinary LTE4 can vary 1–2 min in different samples, the fraction collected at the fixed time interval might not have contained the total amount of LTE4 in the original sample [12,13]. Another explanation was that urinary LTE4 itself could degrade during prolonged HPLC processing, a finding supported by several studies which clearly showed degradation of LTE4 both in vivo and in vitro [14,15].

Urinary LTE4 levels detected by the two methods in the study were significantly correlated. However, the correlation was lower for patients with ARDS (r=0.68) compared to that found for asthmatic (r=0.85) and normal (r=0.88) subjects. This difference could conceivably be due to: firstly, the confounding effect of very high levels of all leukotrienes and other structurally similar inflammatory mediators released in patients with ARDS. Although the cross-reaction between LTE4 and other leukotrienes is low in EIA, the magnitude of this in the presence of high levels of leukotrienes and structurally similar mediators might be large enough to account for the different results in the procedures (with and without HPLC). Secondly, the smaller sample size (n=10) could also influence the correlation.

In conclusion, urinary LTE4 remains useful for semi-quantitation of in vivo cysteinyl leukotrienes production. With the availability of highly sensitive and specific LTE4 monoclonal antibodies in EIA, HPLC extraction and purification of urine samples is no longer necessary for quantitation of urinary LTE4 levels. Furthermore, the additional purification using HPLC provides no apparent advantage over the omission of the HPLC purification step. Rather, considerable amount of time and costs can be saved as a result of the omission.

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