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Short communication

Efficient method for the quantitation of urinary leukotriene E_4: extraction using an Empore C_{18} disk cartridge

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Abstract

We describe here an efficient procedure for the precise quantitation of leukotriene E_4 (LTE_4) in a small volume of urine, which was achieved mainly by the use of an Empore extraction disk cartridge. After addition of [^{3}H]LTE_4 to 2 ml of urine, an Empore C_{18} cartridge was used for initial extraction of the urine, which resulted in the extraction of LTE_4 in a small volume of solvent. The eluate could then be injected onto a high-performance liquid chromatography column without further concentration. After separation by high-performance liquid chromatography, LTE_4 was extracted from the effluent using an Empore C_{18} cartridge. The concentration of LTE_4 was subsequently quantified by enzyme immunoassay. LTE_4 can be recovered from urine with sufficient efficiency (69.9±4.7%, mean±S.D., $n=101$). The coefficient of variation of the assay procedure was less than 10%. When urine was spiked with different amounts of LTE_4, the recovery of LTE_4 added to the urine specimen was less than 120%. The concentration of LTE_4 in urine from normal healthy subjects was 48.0±15.3 pg/mg creatinine ($n=15$).

Keywords: Leukotrienes

1. Introduction

Evaluation of the function of peptidyl leukotrienes (LTs) in pathophysiological and physiological processes requires information on their synthesis and metabolism in vivo. The initial product, LTC_4, is rapidly metabolized into LTD_4, LTE_4 and further metabolites. After intravenous administration of [^{3}H]LTC_4 to humans or monkeys, [^{3}H]LTE_4 was a predominant metabolite in their urine and about 5% of the total radioactivity was recovered as LTE_4 in the urine within 4 h [1–4]. A substantial amount of radioactivity was associated with more polar compounds. Urinary LTE_4 concentrations increased during the first 3 h after allergen inhalation in atopic patients [5–7]. These observations suggest that measurement of urinary LTE_4 concentrations represents a specific, non-invasive approach to assess LTC_4 biosynthesis, although there is a possibility that the increased urinary concentration of LTE_4 may result from attenuated degradation of LTE_4 to further metabolites rather than from the increased biosynthesis of LTC_4 [8]. Increased urinary concentration of LTE_4 has been reported for patients with many other diseases [8–11].

Since LTE_4 is present in extremely small quan-

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tities in urine, it has been quantitated by radioimmunoassay or enzyme immunoassay after several extraction, concentration and purification by high-performance liquid chromatography (HPLC) steps in most studies [5,8,12,13]. Some workers have extracted and separated LTE_4 using a fully automated robotic system [14,15], but it is expensive to assemble such an apparatus. There has also been a report on the use of a gas chromatograph–mass spectrometer to quantitate LTE_4 in urine [16]. We report here on a more convenient method for LTE_4 quantitation, which avoids tedious evaporation of eluates from extraction cartridges and from HPLC columns and thereby minimizes losses in these steps. This method is rapid, yields reproducible results and is more readily applicable to clinical studies.

2. Experimental

2.1. Reagents and chemicals

Reagents were purchased from the following suppliers: 4-hydroxy-TEMPO (Aldrich, Milwaukee, WI, USA); [14,15,19,20-^{3}H(N)]leukotriene E_4 ([^{3}H]LTE_4, specific radioactivity 4884 GBq/mmol, New England Nuclear, Boston, MA, USA); LTE_4 (Cascade Biochemical, University Park, UK); LTC_4/D_4/E_4 enzyme immunoassay kit (Amersham, Amersham Place, UK). The antiserum is reported to have the following cross-reactivities: LTC_4, 100%; LTD_4, 100%; LTE_4, 70%; LTB_4, 0.3%; NOVA-PAK C_{18} column (15 cm×3.9 mm I.D., 4 μm, Millipore, Milford, MA, USA). Empore C_{18} extraction disk cartridge (column size 7 mm/3 ml, 3M, St. Paul, MN, USA).

2.2. Apparatus

The HPLC system consisted of a Shimadzu LC-6A pump, a Shimadzu SPD-6A UV detector and a Rheodyne Model 7125 injector equipped with a 0.5-ml loop. Separation by reversed-phase HPLC was performed isocratically on a NOVA-PAK C_{18} column using a mobile phase composed of methanol–distilled water–acetic acid (65:35:0.1, v/v) containing 0.1% EDTA, and with the pH adjusted to 5.4 with ammonium hydroxide, at a flow-rate of 1.0 ml/min at 35°C.

2.3. Sample collection

Most urine samples were collected from asthmatic patients who were in a stable condition at the time of collection and from normal healthy subjects. In one experiment, the results of which are depicted in Fig. 2, some urine samples were collected during a severe asthmatic attack. Asthmatic patients with aspirin sensitivity were selected on the basis of positive past history and aspirin provocation results. After the free radical scavenger, 4-hydroxy-TEMPO, was added, to a final concentration of 1 m*M*, urine samples were stored at −35°C until the assay.

2.4. Measurement of urinary LTE_4 concentration

We used siliconized glass tubes, polypropylene tubes and polypropylene pipettes throughout the study. Urine samples were thawed and centrifuged at 1600 *g* for 5 min at 4°C to remove precipitates. An aliquot of urine was removed for the determination of creatinine concentration by the Jaffé reaction. Approximately 13 000 dpm of [^{3}H]LTE_4, which corresponds to about 18 pg, were added to 2 ml of urine as an internal standard and the urine (without pH adjustment) was applied to an Empore C_{18} cartridge that had been preconditioned using methanol followed by distilled water. The column was rinsed with 3 ml of distilled water, 3 ml of methanol–0.01 *M* acetate buffer containing 0.01% EDTA, adjusted to pH 5.6 with ammonium hydroxide (1:9, v/v), and 3 ml of *n*-hexane. LTE_4 was then eluted with 0.3 ml of a mixture of methanol and the same acetate buffer as described above (mixture A, 95:5, v/v). The eluate was mixed with 0.15 ml of 17 m*M* acetate buffer containing 0.1% EDTA (pH adjusted to 5.4 with ammonium hydroxide), to give the same composition as the elution buffer used in HPLC, and the solution was injected onto the HPLC column. The column effluent was collected at about 2 ml intervals using a fraction collector, and the fraction that contained ^{3}H-radioactivity and also corresponded to the retention time of authentic LTE_4 was diluted with two volumes of distilled water. This solution was applied to an Empore C_{18} cartridge.

After the cartridge was washed with 3 ml of distilled water, the fraction eluted with 0.5 ml of mixture A was transferred to a siliconized glass tube or to a polypropylene tube. The eluate was concentrated under reduced pressure or a stream of nitrogen and then was dissolved in 0.5 ml of the assay buffer that was supplied in the commercial enzyme immunoassay kit. A 0.05-ml aliquot of the solution was assayed in duplicate for LTE_4 concentration determination using the enzyme immunoassay kit according to the manufacturer's instructions. A standard curve was constructed using authentic LTE_4. Two samples of the solution (0.15 ml each) were used for duplicate measurements of radioactivity in a liquid scintillation spectrometer. The values obtained were subtracted from the amount derived from [^{3}H]LTE_4 to give the amount of endogenous LTE_4 in urine. The concentrations of urinary LTE_4 (pg/2 ml) were calculated as follows:

$$(10 \times A - B/C \times 500/150) \div (B/D \times 500/150)$$

where A is the urinary LTE_4 concentration, as measured by the enzyme immunoassay (pg/0.05 ml), B is the amount of radioactivity in 0.15 ml of the solution, C is the specific radioactivity of [^{3}H]LTE_4 and D is the amount of ^{3}H-labelled radioactivity that was added initially as an internal standard. The concentrations were expressed as pg per mg of creatinine.

3. Results and discussion

Peptidyl-LTs are potent constrictors of the smooth muscle of airways and may contribute to the bronchial hyperresponsiveness observed in asthmatic patients. In humans, 12–20% of radioactivity from intravenously administered [^{3}H]LTC_4 appears in the urine and 4–6% of the total infused dose is identified as LTE_4 [2–4]. Measurement of the urinary concentration of LTE_4 therefore provides a whole-body index of LTC_4 production. There has been evidence suggesting that peptidyl-LTs play a role in the pathogenesis of asthma. Allergen challenge is associated with increased excretion of urinary LTE_4 in sensitized subjects [5–7]. Of particular interest are the findings that aspirin-sensitive asthmatics have a higher basal level of production of LTE_4 than other asthmatics [17,18]. In addition to the increased basal level of production of LTE_4, aspirin provocation is accompanied by an increase in urinary LTE_4 excretion in these patients [19,20].

Chromatographic particles are immobilized within a matrix of polytetrafluoroethylene fibers in Empore C_{18} extraction disk cartridges. This cartridge has several advantages over similar cartridges: (a) it requires as little as 0.3 ml of solvent to elute LTE_4 and the extract can be injected onto a HPLC column without further concentration; (b) the packing particles do not pass into the eluate. After an initial extraction step, it is not necessary to remove particles from the eluate prior to injection onto an HPLC column. In addition, the latter feature has another advantage. When a conventional solid-phase extraction column was employed for the extraction of LTE_4 from the HPLC effluent, the recovery was occasionally remarkably low. This was attributed to the fact that LTE_4 was adsorbed on particles, which had passed through the column to various degrees, when exposed in an aqueous solution. Since it is time-consuming to evaporate the HPLC effluent under reduced pressure or a nitrogen stream, we used an Empore C_{18} cartridge for the extraction of LTE_4 from the effluent.

After extraction of LTE_4 from urine using an Empore cartridge, the extract was separated by HPLC. Fig. 1 shows a typical chromatogram. Authentic LTE_4 was eluted at 12.7±0.7 min in ten different experiments and the retention times for authentic LTC_4, LTD_4 and N-acetyl-LTE_4 were about 6.6, 10.5 and 10.0 min, respectively. When ultraviolet absorption of the column effluent was monitored at 280 nm, contaminating material of a brown color was eluted in the first several minutes. The absorbance declined subsequently and almost returned to the baseline level by 15 min. Thus, extracts could be processed by HPLC every 15 to 20 min and we have purified up to twenty samples a day.

Since [^{3}H]LTE_4 in large amounts could affect the ultimate precision of the enzyme immunoassay for endogenous LTE_4, we spiked with about 18 pg of [^{3}H]LTE_4, which is equivalent to nearly half the concentration of endogenous LTE_4 present in the

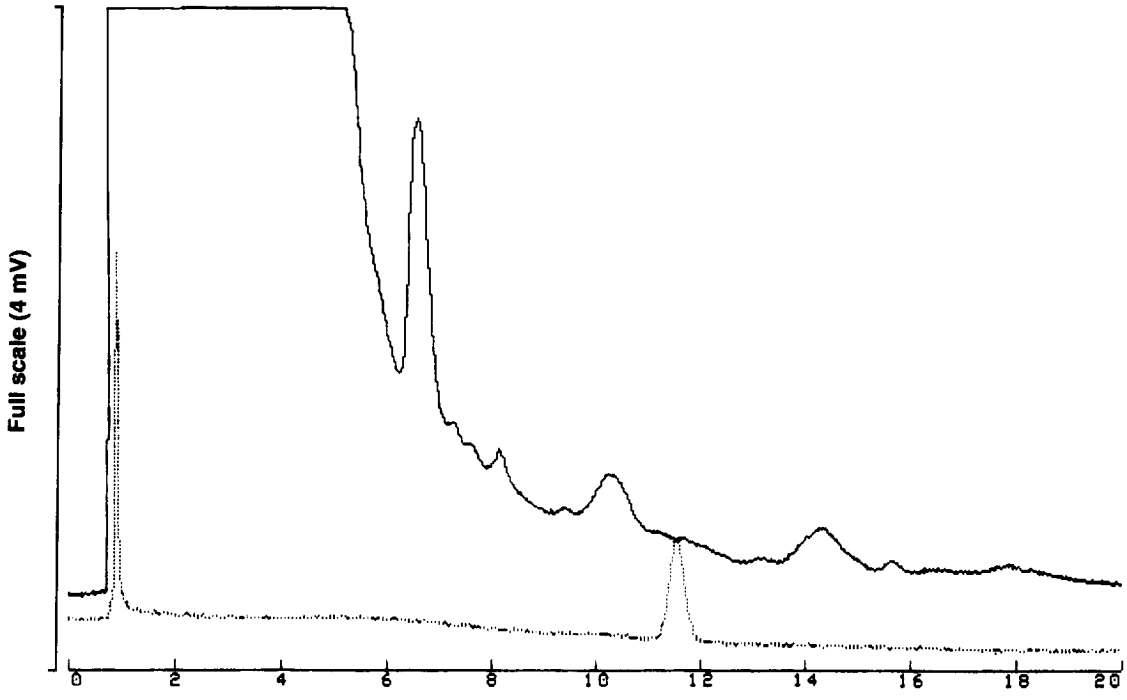

Fig. 1. Separation of the Empore cartridge extract of urine by HPLC. Absorbance was measured at 280 nm. The dotted line shows the elution pattern of authentic LTE_4 (5 ng).

urine of most normal subjects, to allow assessment of recovery of LTE_4. As estimated from ^3H-labelled radioactivity studies, the overall recovery of LTE_4 was 69.9±4.7% (mean±S.D., $n=101$).

We used a urine specimen collected from an aspirin-sensitive asthmatic to check the precision of our method. The precision of the procedure was evaluated by analyzing samples of the same urine specimen containing low, medium or high concentrations of LTE_4. As shown in Table 1, the urine from the aspirin-sensitive asthmatic contained a higher concentration of LTE_4 than the urine samples of asthmatic patients not exhibiting aspirin sensitivity. The coefficient of variation was less than 5% at higher concentrations of LTE_4. However, the coefficient of variation was about 10% at concentrations of LTE_4 of around 20 pg/2 ml, a concentration that was encountered frequently in routine analysis. The variation may be predominantly associated with the precision of the enzyme immunoassay, of which the intra-assay variation has been suggested to be about 10% according to the kit information leaflet.

Accuracy of the method was determined by adding a known amount of LTE_4 to four aliquots of the same urine specimen. As shown in Table 2, the addition of 30 pg of LTE_4 to 2 ml of urine resulted in a net calculated concentration of 55.6±3.6 pg/2

Table 1
Precision of quantitation of urinary LTE_4 by enzyme immunoassay in combination with HPLC

LTE_4 (pg/2 ml)	Average LTE_4 (pg/2 ml)
20.2	20.7±1.8 (8.7)
18.5	
22.8	
21.3	
49.3	45.0±3.4 (7.6)
41.1	
45.1	
44.4	
243.2[a]	228.0±11.4 (5.0)
217.8	
220.8	
230.3	

Figures in parentheses are the coefficients of variation (%).
[a] Collected from an asthmatic patient who exhibited aspirin sensitivity.

Table 2
Accuracy of quantitation of urinary LTE_4 by enzyme immunoassay in combination with HPLC

Amount of LTE_4 added to 2 ml of urine (pg)	Total amount of LTE_4 detected (mean±S.D.) (pg)	Recovery (%)
0	27.8±2.7	–
30	55.6±3.6	92.7
60	86.7±2.7	98.2
120	166.0±7.1	115.2

ml, resulting in an accuracy of 92.7%. The recoveries of LTE_4 were 98.2 and 115.2% when urine samples were spiked with 60 and 120 pg of LTE_4, respectively. The accuracy of this method was within acceptable limits.

The concentration of endogenous LTE_4 in urine from normal subjects was 48.0±15.3 pg/mg creatinine ($n=15$). The basal level of LTE_4 in human urine from normal subjects has been reported in several papers: 35±10 pg/mg creatinine, as determined by radioimmunoassay with HPLC [9], 83.8±38.2 pg/mg creatinine, as determined by enzyme immunoassay with HPLC [21] and 63±38.8 pg/mg creatinine, as determined by gas chromatographic–mass spectrometric assay [16]. The values we obtained were in accordance with these literature values.

Some investigators have recently reported that LTE_4 could be quantitated by immunoassay without any purification of urine [22–24]. We have compared LTE_4 levels between samples assayed after an initial extraction using a solid-phase column and those assayed after subsequent HPLC purification. As shown in Fig. 2, LTE_4 levels measured by enzyme immunoassay without an additional purification step were substantially higher than those measured by enzyme immunoassay in combination with HPLC in all urine samples, with the exception of the urine from one patient. Only the urine samples represented by blank circles showed comparable concentrations of LTE_4, irrespective of purification with HPLC. There appears to be a correlation for urine samples from some patients. However, it is apparent from the data that a substance(s) other than LTE_4 that reacts with the antibody to LTE_4, thereby affecting the determination of LTE_4 concentration, is present in most samples that were not purified by HPLC. There

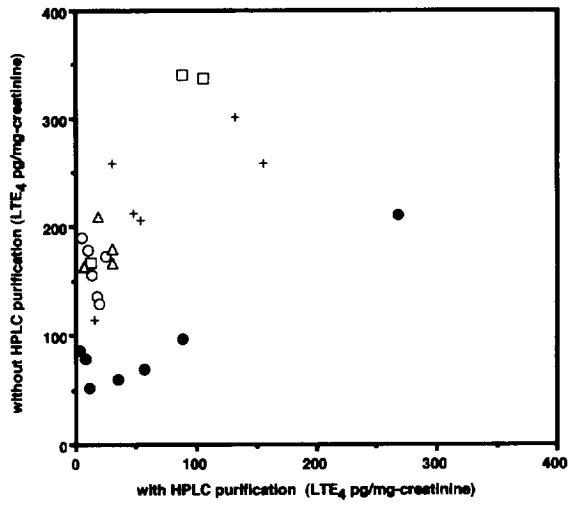

Fig. 2. Comparison of LTE_4 concentrations in urine samples determined by enzyme immunoassay after purification by HPLC, as described here, and enzyme immunoassay without purification by HPLC. Urine was collected from five different patients with asthma (●, ○, □, △, +) on several occasions.

is no proof that the degree of cross-reactivity is constant among the different urine samples. Consequently, we believe that solid-phase extraction of LTE_4 from urine is insufficient for obtaining reliable data and additional chromatographic steps are required before quantitation of urinary LTE_4.

The method reported here is much less labor intensive than currently available methods and will provide a valid assessment of systemic LTC_4 production.

References

[1] C. Denzlinger, A. Guhlmann, P.H. Scheuber, D. Wilker, D.K. Hammer and D. Keppler, J. Biol. Chem., 261 (1986) 15601.
[2] N.H. Maltby, G.W. Taylor, J.M. Ritter, K. Moore, R.W. Fuller and C.T. Dollery, J. Allergy Clin. Immunol., 85 (1990) 3.
[3] A. Sala, N. Voelkel, J. Maclouf and R.C. Murphy, J. Biol. Chem., 265 (1990) 21771.
[4] M. Huber, J. Müler, I. Leier, G. Jedlitschky, H.A. Ball, K.P. Moore, G.W. Taylor, R. Williams and D. Keppler, Eur. J. Biochem., 192 (1990) 309.
[5] P.J. Manning, J. Rokach, J.-L. Malo, D. Ethier, A. Cartier, Y. Girard, S. Charleson and P.M. O'Byrne, J. Allergy Clin. Immunol., 86 (1990) 211.

[6] J.Y. Westcott, H.R. Smith, S.E. Wenzel, G.L. Larsen, R.B. Thomas, D. Felsien and N.F. Voelkel, Am. Rev. Respir. Dis., 143 (1991) 1322.
[7] C.M. Smith, P.E. Christie, R.J. Hawksworth, F. Thien and T.H. Lee, Am. Rev. Respir. Dis., 144 (1991) 1411.
[8] M. Huber, S. Kästner, J. Schölmerich, W. Gevok and D. Keppler, Eur. J. Clin. Invest., 19 (1989) 53.
[9] G.R. Bernard, V. Korley, P. Chee, B. Swindell, A.W. Ford-Hutchinson and P. Tagari, Am. Rev. Respir. Dis., 144 (1991) 263.
[10] S.P. Allen, A.P. Sampson, P.J. Piper, A.H. Chester, S.K. Ohri and M.H. Yacoub, Coronary Artery Dis., 4 (1993) 899.
[11] J. Fauler, A. Thon, D. Tsikas, H. von der Hardt and J.C. Frölich, Arthritis Rheum., 37 (1994) 93.
[12] P. Tagari, D. Ethier, M. Carry, V. Korley, S. Charleson, Y. Girard and R. Zamboni, Clin. Chem., 35 (1989) 388.
[13] K. Sladek, R. Dworski, G.A. Fitzgerald, K.L. Buitkus, F.J. Block, S.R. Marney, Jr. and J.R. Sheller, Am. Rev. Respir. Dis., 141 (1990) 1441.
[14] D. Nicoll-Griffith, R. Zamboni, J.B. Rasmussen, D. Ethier, S. Charleson and P. Tagari, J. Chromatogr., 526 (1990) 341.
[15] D. Nicoll-Griffith and R. Zamboni, Prostaglandins, 43 (1992) 523.
[16] D. Tsikas, J. Fauler, F.M. Gutzki, T. Röder, H.J. Bestmann and J.C. Frölich, J. Chromatogr., 622 (1993) 1.
[17] H.R. Knapp, K. Sladek and G.A. Fitzgerald, J. Lab. Clin. Med., 119 (1992) 48.
[18] T. Lee and P.E. Christie, Thorax, 48 (1993) 1189.
[19] P.E. Christie, P. Tagari, A.W. Ford-Hutchinson, S. Charlesson, P. Chee, J.P. Arm and T.H. Lee, Am. Rev. Respir. Dis., 143 (1991) 1025.
[20] E. Israel, A.R. Fischer, M.A. Rosenberg, C.M. Lilly, J.C. Callery, J. Shapiro, J. Cohn, P. Rubin and J.M. Drazen, Am. Rev. Respir. Dis., 148 (1993) 1447.
[21] K. Asano, C.M. Lilly, W.J. O'Donnell, E. Israel, A. Fischer, B.J. Ransil and J.M. Drazen, J. Allergy Clin. Immunol., 96 (1995) 643.
[22] M. Kumlin, F. Stensvad, L. Larsson, B. Dahlén and S.E. Dahlén, Clin. Exp. Allergy, 25 (1995) 467.
[23] M. Kumlin, J. Chromatogr. B, 725 (1996) 29.
[24] D.W. Qui, K.P. Hui, C.W. Lee, T.K. Lim and W.C. Tan, J. Chromatogr. B, 677 (1996) 152.