Direct Injection Method for Quantitation of Endogenous Leukotriene E₄ in Human Urine by Liquid Chromatography/Electrospray Ionization Tandem Mass Spectrometry with a Column-switching Technique

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A new analytical method employing liquid chromatography/tandem mass spectrometry (LC/MS/MS) with a column-switching system was developed for quantitative determination of leukotriene E₄ (LTE₄) in human urine. A column-switching system using a trapping column, which concentrates the analyte and removes salts and other water-soluble contaminants, allowed direct injection of human urine. Because simultaneously eluted endogenous contaminants suppressed the ionization efficiency of LTE₄, good liquid chromatographic separation was very important for establishing this method, notwithstanding the high selectivity of MS/MS. The calibration curve was linear over the range from 10 to 3000 pg/mL, and the method showed good accuracy and precision. This method should therefore be very useful for determination of LTE₄ amounts in human urine in studies on leukotriene metabolism and the efficacy of antileukotriene drugs.

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Introduction

Leukotriene E₄ (LTE₄) is formed by enzymatic peptide cleavage reactions from leukotriene C₄. Peptide leukotrienes including leukotriene D₄ are well known to have various biological actions (*e.g.*, vasoconstriction and increase of vascular permeability),^{1,2} and are thought to play important roles in diseases such as asthma, cirrhosis, acute respiratory disease syndrome and systemic lupus erythematosus.³⁻⁷ A number of anti-leukotriene drugs, which act as biosynthesis inhibitors or receptor antagonists of the LTs, have proved to be of benefit to treat asthmatic reactions.^{8,9} Peptide leukotriene metabolites are excreted into urine as LTE₄ in humans,¹⁰ and urinary LTE₄ may therefore reflect the status of LTs in the whole body. Quantitative determination of LTE₄ in urine may thus be useful for evaluation of the effects of anti-leukotriene drugs in various diseases, and there is a need for an accurate and reproducible analytical method for determination of LTE₄ in urine.

The most widely utilized method to determine LTE₄ in urine consists of two steps. The first is solid-phase extraction (SPE) of LTE₄ and/or purification by high-performance liquid chromatography (HPLC), and the second is measurement by enzyme- or radioimmunoassay (EIA or RIA). During these complicated procedures, significant loss of analyte can sometimes occur by adsorption on the container walls and the solid phase, and by decomposition in the evaporation step because of the instability of LTE₄.¹¹⁻¹³ Although immunoassay is a highly sensitive and simple method for quantitating LTs, it

is difficult to eliminate cross-reactivity with related substances in urine. Therefore, immunoassay may often result in overestimation of basal eicosanoid level and the values obtained may vary from sample to sample. A method using gas chromatography/mass spectrometry (GC-MS) is the most sensitive and specific for determination of LTE₄.¹⁴ However, it also requires a complicated chromatographic purification procedure and derivatization of LTE₄ into the pentafluorobenzylester-trimethylsilylether derivative of 5-hydroxyeicosanoic acid by catalytic reduction/desulfurization. On the other hand, liquid chromatography/mass spectrometry (LC/MS) with an electrospray ionization (ESI) system can provide direct analysis of thermolabile molecules in biological fluids, and it has been reported to be the most suitable for structural characterization of leukotriene.¹⁵ Recently, LC combined with tandem mass spectrometry (LC/MS/MS), which is more selective, has been used to quantitate LTE₄ in human urine in combination with solid-phase extraction.¹⁶⁻¹⁸ However, extraction procedures including a concentration step of a labile compound may also result in variation of recovery from sample to sample, as noted above. Application of on-line solid-phase extraction using a simple column-switching technique should be more suitable for unstable substances such as peptide-bound leukotrienes.

In this paper, we describe a rapid, highly sensitive and specific on-line method for quantitation of LTE₄ in human urine by the use of LC/MS/MS. This method allows direct injection of urine without any pretreatment procedure, owing to the introduction of a column-switching technique.

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Experimental

Materials

LTE₄ and [20,20,20-³H₃]-LTE₄ (LTE₄-d₃) were purchased

Fig. 1 Product ion spectrum of LTE_4 in ionspray ionization using the positive ion detection mode.

Fig. 2 Effect of mobile phase additives on ionspray ionization efficiency.

from Cayman Chemicals (Ann Arbor, MI, USA) and BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA, USA), respectively. 4-Hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (HTEMPO) was obtained from Aldrich Chemical Co., (Milwaukee, WI, USA) and tetrasodium ethylenediaminetetraacetate (EDTA) was from Sigma (St. Louis, MO, USA). Ultrapure water was prepared by a Milli-Q SP TOC distillation unit (Millipore, Marlborough, MA, USA). Other organic solvents and reagents were of analytical grade.

Instruments

The HPLC system consisted of a system controller (SCL-10A, Shimadzu, Kyoto, Japan), three pumps (two LC-10ATs and one LC-9A, Shimadzu), an auto sampler (SIL-10A, Shimadzu), and two switching 6-port valves (FCV-12AH, Shimadzu). The Inertsil ODS-2 column (5 μm , 150 mm $\times$ 0.7 mm i.d.) and the Inertsil C-8 column (5 μm , 10 mm $\times$ 4.0 mm i.d.) were from GL Sciences (Tokyo, Japan). An API-III plus triple stage quadrupole mass spectrometer equipped with a turbo ionspray interface (Perkin-Elmer Sciex, Thornhill, Ontario, Canada) was employed. Eluate from the LC was introduced *via* a fused-silica capillary (50 μm i.d., GL Sciences) to the ionspray interface. It was maintained at 50°C with a nitrogen nebulization pressure of 50 psi, resulting in a nitrogen flow of 0.8 L/min. The curtain gas (nitrogen gas) flow was set at 1.2 L/min. The auxiliary gas for the turbo ionspray mode was nitrogen at a flow rate of 4 L/min and the temperature of the nebulizer gas was set at 500°C. All analyses were performed in the positive ion detection mode with an orifice potential of 65 eV and an ionspray voltage of 5000 V. The multiple reaction monitoring (MRM) mode was operated at a dwell time of 500 ms, with argon gas (2.5×10^{15} molecules cm^{-2}) as the collision gas for collision-activated decomposition (CAD). Monitoring ions were m/z 440 (precursor ion) – 301 (product ion) for LTE_4 , and m/z 443 – 304 for $\text{LTE}_4\text{-d}_3$.

Optimization of LC/MS conditions

In order to investigate the effect of mobile phase composition on the ionization of LTE_4 (10 $\mu\text{g/mL}$), injection was done using a Rheodyne 7520 injector with a 0.5 μL sample loop in flow injection mode at a flow rate of 10 $\mu\text{L/min}$. The mobile phase additives were trifluoroacetic acid (TFA), formic acid (HCOOH), acetic acid (AcOH), ammonium formate (HCOONH₄) and ammonium acetate (AcONH₄) at 10 mM concentrations in water/acetonitrile mixture (3:7, v/v).

Sample preparation

A standard stock solution of LTE_4 was prepared in ethanol at 100 $\mu\text{g/mL}$ and was stored at -27°C . Working standard solutions were prepared in methanol at 0.5, 1.5, 5, 15, 50 and 150 ng/mL. Calibration solutions were prepared in methanol at final concentrations of 10, 30, 100, 300, 1000, 3000 pg/mL prior to use. Urine of a healthy male volunteer was collected in a 50 mL polypropylene tube, to which had previously been added 1 mL of an antioxidant solution consisting of 0.5 mM HTEMPO and 0.1 mM EDTA. The collected urine was divided into 1 mL aliquots and stored at -27°C until analysis. Working standard solutions were added to the pooled urine to give final concentrations of 30, 100, 300 and 1000 pg/mL to prepare spiked urine samples. Working IS solutions of $\text{LTE}_4\text{-d}_3$ was prepared in methanol at the concentration of 50 ng/mL and stored at -27°C .

Accuracy and precision of the assay

Analytical errors were investigated by using spiked urine samples. A working IS solution (20 μL) was added to the spiked urine (1 mL). In order to evaluate the intraday variation in LC/MS/MS analysis, four samples of urine spiked at each concentration were analyzed on the same day. The interday variation was evaluated for 4 days. The relative error (RE, %) was calculated as [(found concentration) – (theoretical concentration)/spiked concentration] $\times$ 100 (%), and the precision was obtained in terms of the coefficient of variation (RSD, %).

Results and Discussion

The ionization behavior of LTE_4 , which has an amino group and a carboxyl group, was investigated. Intense protonated and deprotonated molecular ions were obtained with ESI in positive and negative ion detection modes, respectively. With CAD in MS/MS mode, the protonated ion (m/z 440) gave simple and more intense fragment ions in the positive ion detection mode, as shown in Fig. 1. In analysis by LC/MS/MS, both simplicity of the MS/MS spectrum and stability of the fragment ion monitored are very important for reproducibility and sensitivity of quantitative determination. We presumed that the most intense ion observed at m/z 301 was derived from dehydration followed by cleavage between fatty acid and cysteine, and it was therefore chosen for monitoring of LTE_4 at m/z 440 $\rightarrow$ 301.

Fig. 3 Schematic diagram of the LC/MS/MS system using the column-switching technique. Trapping column, 5 μm ODS (4.0 mm i.d. $\times$ 10 mm); analytical column, 5 μm ODS (0.7 mm i.d. $\times$ 150 mm); MP-A (mobile phase A), water/TFA = 1000:0.2 (v/v) at the flow rate of 1.0 mL/min; MP-B (mobile phase B), water/methanol = 9:1 (v/v) containing 10 mM acetic acid and 0.02% TFA; MP-C (mobile phase C), water/methanol (1:9, v/v) containing 10 mM acetic acid and 0.02% TFA. Elution was performed with 60–100% MP-C over 15 min at a flow rate of 230 $\mu\text{L}/\text{min}$.

in the MRM mode. The monitoring ion of $\text{LTE}_4\text{-d}_3$ was set at m/z 443 $\rightarrow$ 304.

In the ESI mode, the spray composition is also known to influence the sensitivity and the stability of the produced ion. The effect of mobile phase composition was therefore investigated on both LC separation and MS sensitivity. The ion intensities of LTE_4 monitored in the MRM mode were measured by flow injection analysis using several mobile phase additives; the results are shown in Fig. 2. Acetic acid as a mobile phase additive yielded the most intense ion of LTE_4 , which was 2.30-fold more intense than that obtained without acetic acid. Formic acid, ammonium formate and ammonium acetate also improved the ionization (1.60, 1.30 and 1.40-fold). However, TFA, a strong acid, slightly decreased the sensitivity (0.90-fold). The concentration of acetic acid in the spray also influenced the sensitivity of LTE_4 . The ion intensity of LTE_4 was greatest at the concentration of 10 mM (data not shown). However, LTE_4 contains a peptide moiety, and gave a broad peak with tailing in the chromatogram using the mobile phase chosen in the above experiment. We therefore added a very small amount of TFA (0.02%) to the mobile phase to avoid adsorption of the peptide moiety on residual silanol moieties of the solid phase. This small amount of TFA improved the peak shape of LTE_4 (data not shown) and did not influence the ionization efficiency.

Hopfgartner *et al.* reported that the ionspray interface is a concentration-sensitive device.¹⁹ The use of a column with a smaller diameter, such as a microbore column, should increase the concentration of the analyte in the spray liquid. However, injection volume on a microbore column is limited to a few microliters because of the low bed volume. To overcome this problem, Oda and coworkers have used a column-switching system with precolumn concentration.²⁰ Figure 3 shows a schematic diagram of a linear gradient system using the column-switching technique. This system is equipped with a trapping column, which functions for concentration of analytes and for desalting of urine samples, allowing injection of urine without any pretreatment procedure. In the analysis of

endogenous biologically active substances or drugs and their metabolites by LC/MS/MS, rapid analysis is often achieved at the cost of poor separation from endogenous contaminants. Moreover, ionization in the ESI mode is easily influenced by contaminants which are eluted simultaneously. When the urine sample was analyzed in a step gradient mode with a simple column-switching system, the ionization was strongly suppressed (data not shown). Therefore, good LC separation is very important for highly sensitive and reproducible quantitative determination, notwithstanding the high selectivity of MS/MS. In this system, the total flow was delivered using two pumps and was divided with a coaxial splitter and a resistant column (split ratio, 1:10). Elution was performed in a linear gradient mode. LTE_4 gave a sharp, symmetrical peak on the chromatogram of the calibration solution (1000 pg/mL), as shown in Fig. 4 (A). A chromatogram of urine spiked at the concentration of 1000 pg/mL is shown in Fig. 4 (B). The peak area of LTE_4 in the spiked urine was almost equal to that of the calibration sample and the calibration curve obtained from urine samples spiked various amounts of LTE_4 paralleled that obtained from calibration solutions containing the same amounts of LTE_4 . Those results show that this system can separate LTE_4 from large amounts of endogenous contaminants, and, using a linear gradient mode, can detect LTE_4 without significant suppression of ionization efficiency.

However, large amounts of endogenous contaminants accumulated around the orifice of the ionspray interface during continuous analysis for long time, and the sensitivity gradually decreased. To overcome this problem, we placed a six-port valve (V2) in front of the ionspray interface (Fig. 3). When the contaminants were eluted from the analytical column, the effluent was drained so that they were not introduced into the ionspray interface, and the mobile phase C, which consisted of water/methanol mixture (3:7, v/v), was introduced into the ionspray interface by an infusion pump to maintain a stable ionspray. Just before the retention time of LTE_4 , V2 was switched to introduce the analytes into the ionspray interface. Just after elution of LTE_4 , V2 was returned to its original

Fig. 4 Representative MRM chromatograms in the linear gradient system. (A) MRM chromatogram of calibration solution (1000 pg/mL); (B) The chromatogram of spiked human urine (1000 pg/mL).

Fig. 5 Representative MRM chromatograms of LTE₄ in urine spiked at the concentration of 100 pg/mL. (A) without the six-port valve (V2); (B) with the six-port valve (V2).

position and the line was cleaned up with the mobile phase C. In order to confirm the validity of this technique, a urine sample spiked at the concentration of 100 pg/mL was injected into this system. A chromatogram of the spiked urine without V2 switching is shown in Fig. 5 (A). Large amount of hydrophilic contaminants were eluted before the appearance of LTE₄. The chromatogram of the spiked urine with V2 switching is shown in Fig. 5 (B). The results suggest that the switching removed the hydrophilic contaminants and maintained the stability of the ionspray. This system permits continuous analysis for 30 h without decrease of the sensitivity.

Next, we conducted quantitative validation of this new determination method to confirm its performance and usefulness. The calibration solutions were analyzed five times. Table 1 shows the results. Excellent correlation coefficients (more than 0.999) were obtained for LTE₄, and the curves were linear in the range of 10–3000 pg/mL. Linear calibration curves were constructed by weighted (1/y) least-squares regression of concentration *versus* peak area ratio (analyte/internal standard) of the calibration solutions. The linear range and R.S.D. values were appropriate for measuring the biological release and production of LTE₄.

We then analyzed spiked urine samples, which were prepared by adding known amounts of working standards to human urine.

Table 1 The precision and accuracy in analysis of calibration samples ($n = 5$)

	Concentration/pg mL ⁻¹					
	10	30	100	300	1000	3000
Found	10.6	30.9	103.6	325.5	1021.3	2973.0
R.S.D.(%)	29.4	6.6	5.7	5.5	1.0	1.8
R.E.(%)	6.0	3.0	3.6	8.5	2.1	-0.9

Table 2 Intra- and interday variation in analysis of LTE₄ in human urine samples ($n = 4$)

	Standard added/pg mL ⁻¹				
	0	30	100	300	1000
Intraday					
Found	157.6	186.2	250.5	433.0	1122.9
R.S.D.(%)	3.42	4.40	5.28	1.33	4.91
R.E.(%)	—	-4.7	-7.1	-8.2	-3.5
Interday					
Found	153.4	187.8	247.7	428.7	1123.9
R.S.D.(%)	2.14	4.78	2.14	5.23	6.00
R.E.(%)	—	14.7	-5.7	-8.2	-2.9

Table 2 shows the results of interday and intraday accuracy and precision. These data suggest that the method is accurate and reproducible for analysis of endogenous LTE₄ in human urine. The concentration of LTE₄ in urine of a healthy subject was measured, and the value, 155.5 pg/mL ($n = 1$), coincided well with that reported previously. Thus, this simple, accurate and reproducible method should be very useful for quantitative determination of endogenous LTE₄ in human urine.

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