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Determination of leukotriene E_4 in human urine using liquid chromatography–tandem mass spectrometry

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Abstract

A liquid chromatographic–tandem mass spectrometric (LC–MS–MS) method was developed for the quantitation of urinary leukotriene E_4 (LTE_4). LTE_4 and its internal standard were extracted by solid-phase extraction and analysed using LC–MS–MS in the selected reaction monitoring (SRM) mode. A good linear response over the range of 10 pg to 10 ng was demonstrated. The accuracy of added LTE_4 ranged from 97.0% to 108.0% with a mean and SD of 100.6±2.4%. We detected LTE_4 (63.1±18.7 pg/mg creatinine, $n=10$) in healthy human urine. This method can be used to determine LTE_4 in biological samples.

Keywords: Leukotrienes

1. Introduction

Leukotrienes (LTs) are derived from arachidonic acid by the action of 5-lipoxygenase. The dihydroxy compound LTB_4 is a particularly chemotactic and chemokinetic agent for inflammatory cells. However, the cysteinyl LTs (LTC_4, LTD_4 and LTE_4) can cause a variety of biological effects; e.g., smooth muscle contraction [1], vasoconstriction [2] and increased mucus secretion [3]. The cysteinyl leukotrienes have pathophysiological effects, and their production has been implicated in a wide range of diseases, including allergic asthma [4], adult respiratory distress syndrome (ARDS) [5], inflammatory bowel disease [6], and systemic lupus erythematosus [7]. Therefore, establishment of a reliable microdetermination method is very useful for clinical appraisal of these diseases.

LTC_4 is rapidly metabolized in the lungs, being converted via LTD_4 to the less active LTE_4, which is excreted unchanged in the urine [8]. Urinary LTE_4 is, therefore, a useful index of systemic cysteinyl LTs biosynthesis.

The quantitation of LTE_4 in urine is often performed using a combination of solid phase extraction (SPE)/high-performance liquid chromatography (HPLC)/immunoassay techniques [9–13]. However, these techniques require complicated cleansing using HPLC to eliminate interfering substances from the urine, where the cross reactivity of antibodies can decrease the sensitivity of the immunoassay.

However, recent developments in mass spectrometry coupled with liquid chromatography have been made sufficiently dependable for reliable routine sample analysis. In particular, liquid chroma-

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tography–tandem mass spectrometry (LC–MS–MS) equipped with electrospray ionization (ESI) interface, is a powerful technique for highly specific and quantitative measurement of various compounds in biological samples [14].

We have established a method for measuring LTE_4 using simple solid-phase extraction and LC–MS–MS operating in the selected reaction monitoring (SRM) mode. In this study, we measured the urinary LTE_4 levels of healthy volunteers using the LC–MS–MS method.

2. Experimental

2.1. Materials

Leukotriene E_4 was purchased from Cayman Chemicals Co. (Ann Arbor, MI, USA). [20,20,20-$^2H_3$]Leukotriene E_4 (LTE_4-d_3) was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Empore C18 HD disk cartridges (7 mm/3 ml) were purchased from 3M Industrial and Consumer Sector (St. Paul, MN, USA). All solvents were HPLC grade obtained from Wako Pure Chemical Industries (Osaka, Japan).

2.2. LC–MS–MS analysis

The LC–MS–MS system was a Quattro II triple-quadrupole tandem mass spectrometer (Micromass, Manchester, UK). The ionization interface used was an electrospray operated in the negative-ion mode. A Nanospace SI-1 HPLC system (Shiseido, Tokyo, Japan) was used. Chromatography was performed on a C18 Capcell Pak UG120 (Shiseido, Tokyo, Japan; 1.5×150 mm, 3 μm) using isocratic elution with acetonitrile–water–acetic acid (45:55:0.02, v/v, adjusted to pH 5.6 with ammonium hydroxide) with a flow rate of 100 μl/min. The column was maintained at 40°C. Column effluent was introduced into the mass spectrometer using a fused silica capillary between 3.5 and 6 min after injection.

Full scan mass spectra were acquired by scanning MS1 from m/z 50–500 with a scan time of 1 s. The product ion mass spectra were obtained by choosing the molecular anions as the precursor ions and scanning MS2 from m/z 50–500 with a scan time of 1 s.

The selected reaction monitoring (SRM) was performed by monitoring the transitions between m/z 438 and m/z 333 for LTE_4 and between m/z 441 and m/z 336 for LTE_4-d_3. Collision gas (argon) thickness was 1.0×10^{-3} mBar. The capillary voltage was −3500 V. Cone voltage was −40 V with a collision energy of 20 eV. Source temperature was 180°C. Peak areas and the calibration curve were obtained using the MassLynx program (Micromass, Manchester, UK).

2.3. Sample preparation and extraction procedure

Human urine from healthy volunteers was investigated. After sampling, the urine was stored at −80°C until assayed.

To human urine (2 ml), LTE_4-d_3 (2 ng) was added as an internal standard. After centrifugation at 4000 *g* for 10 min to remove precipitates, the sample was adjusted to pH 4.0 with acetic acid and was passed through an Empore C18 HD disk cartridge, preconditioned with methanol (2 ml), followed by distilled water (2 ml). The cartridge was washed with distilled water (2 ml), 50% methanol (2 ml) and hexane (2 ml); peptides, salt, polar and non-polar interfering substances were eliminated. LTE_4 and LTE_4-d_3 were eluted with methanol (0.5 ml) in a siliconized glass tube to avoid absorption by the tube. After evaporating the solvent, the residue was reconstituted in mobile phase (20 μl) and sonicated for 30 s. Then, it was transferred to an autosampler vial; 5 μl was injected for LC–MS–MS.

2.4. Creatinine contents

Creatinine in human urine was determined using a Creatinine test kit (Wako Pure Chemical Industries, Osaka, Japan). The results are shown as corrected values.

3. Results

3.1. Mass and product ion mass spectra

The mass spectra of LTE_4 and LTE_4-d_3 are shown in Fig. 1. Each spectra revealed a base peak at m/z 438 and 441, respectively, corresponding to molecular anions ($[M-H]^-$). The product ion mass spectra

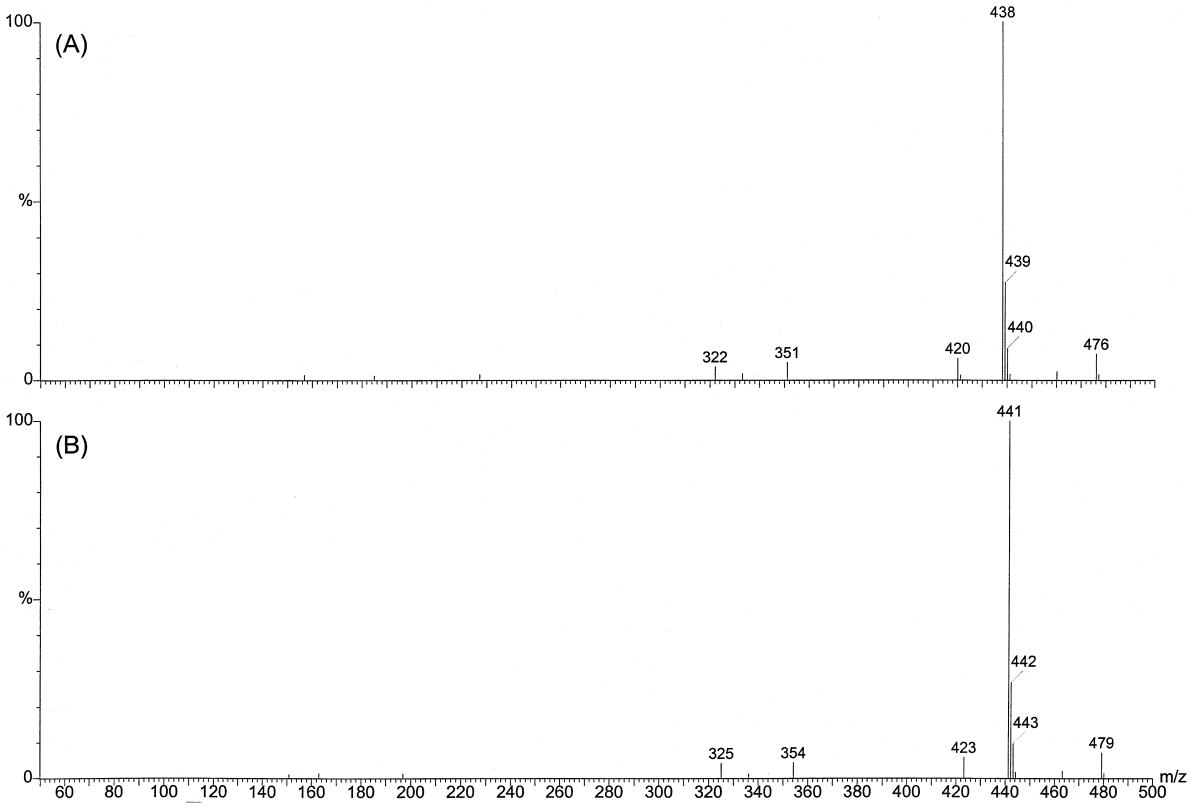

Fig. 1. Mass spectra of LTE_4 (A) and LTE_4-d_3 (B)

of both the analyte and its internal standard were obtained by choosing the molecular anions as the precursor ions (Fig. 2). The fragment ions observed at m/z 333 for LTE_4 and m/z 336 for LTE_4-d_3, which are believed to be derived from an initial rearrangement of the cysteine side-chain followed by dehydration [15], were most abundant at a collision energy of 20 eV.

3.2. Selected reaction monitoring (SRM)

Changes in the mobile phase composition can alter the sensitivity by influencing the ionization efficiency of LTE_4 on MS, in addition to separation on LC. Therefore, we first needed to optimize the mobile phase to obtain high sensitivity, good separation and short analysis time. High sensitivity on MS was compatible with good separation on LC by using the mobile phase of acetonitrile–water–acetic acid (45:55:0.02, v/v), and this composition also gave short analysis time. In addition, adjustment of pH to 5.6 with ammonium hydroxide led to a decrease in the noise level.

After optimization of the mobile phase, we tried to detect LTE_4 in human urine using LC–MS in the selected ion monitoring (SIM) mode. In the SIM mode, however, monitoring the molecular anion of LTE_4 failed to detect the peak because of the high background noise level. So, we used LC–MS–MS in the SRM mode. In this method, the background noise level was remarkably reduced and the peak of LTE_4 was sharply revealed. Moreover, since we used a microflow HPLC system, including a switching-valve unit to introduce the column effluent for a minimum time, we only required a small sample, which always kept the mass spectrometer clean and the background level of the chromatogram reduced.

The best sensitivities and minimum interferences were achieved by monitoring the transitions between m/z 438 and m/z 333 for LTE_4 and between m/z 441 and m/z 336 for LTE_4-d_3 in the SRM mode. Typical SRM ion current chromatograms of LTE_4

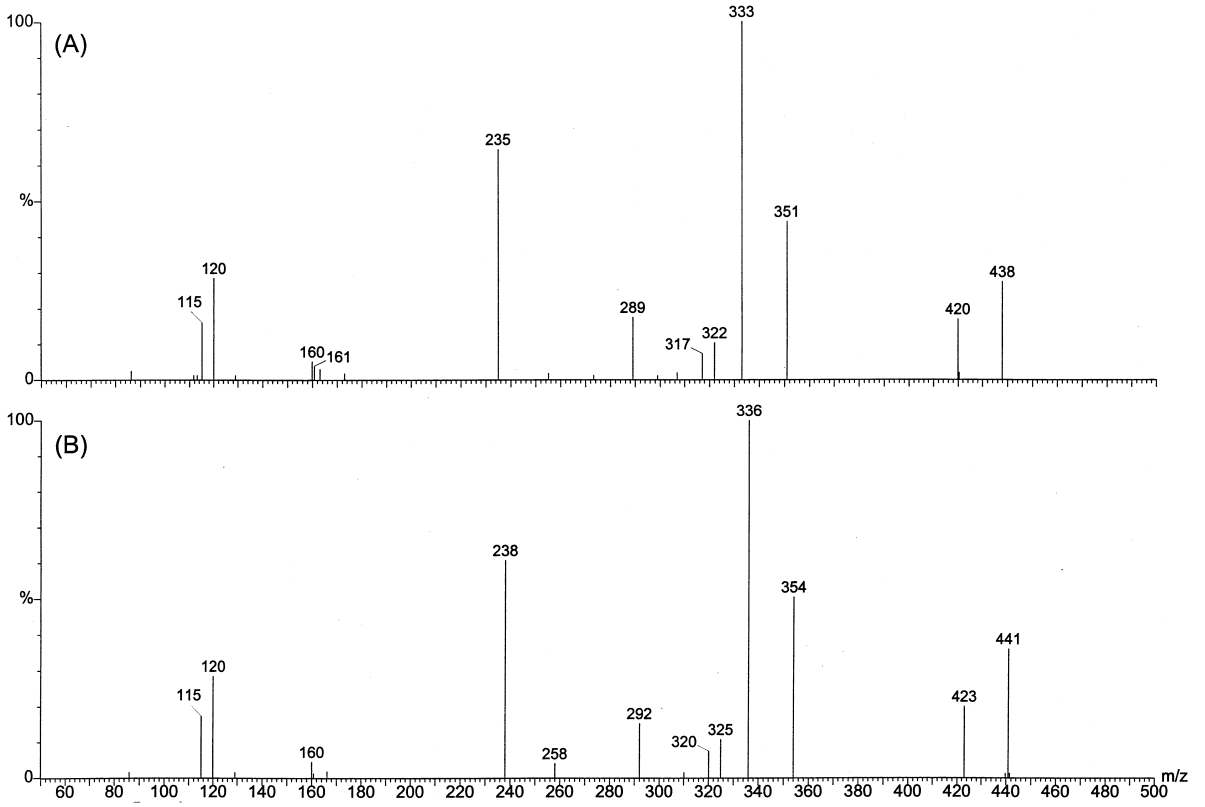

Fig. 2. Product ion mass spectra of LTE_4 (A) and LTE_4-d_3 (B). Molecular anions (m/z 438 for LTE_4 and m/z 441 for LTE_4-d_3) were chosen as the precursor ions in the MS–MS experiments.

and LTE_4-d_3, extracted from human urine containing LTE_4 of 42 pg/ml, are shown in Fig. 3. LTE_4 and LTE_4-d_3 had retention times of 4.7–4.8 min. When 10 pg of the LTE_4 was injected, SRM showed a single peak with a signal/noise (S/N) ratio of about 15:1.

3.3. Calibration graph

The calibration graph for LTE_4 was generated from SRM of increasing amounts of LTE_4 standard and it was spiked with constant levels of LTE_4-d_3 as the internal standard. Duplicate calibration standards were prepared. A linear calibration graph was constructed using least-squares regression of quantities versus peak area ratio to LTE_4-d_3. The linearity was good in the range of 10 pg to 10 ng ($r=0.998$). Using this curve, we determined the level of LTE_4.

3.4. Reproducibility

After addition of an internal standard to the urine of a healthy human adult, five samples were subjected to the sample preparation procedure. Thus, experimental reproducibility was investigated by analyzing these samples in triplicate. The findings are shown in Table 1. Statistical analysis was carried out according to one-way analysis of variance [16] to separate the analytical errors arising from two sources: sample preparation and LC–MS–MS in the SRM mode. The finding shown in Table 2a indicates that there was no significant contribution to the total variance from the sample preparation. The coefficient of variation in LC–MS–MS in the SRM mode was 3.8%.

To examine the accuracy and the precision of the present method, four urine samples spiked with LTE_4 at concentrations of 100, 200, 400 and 800

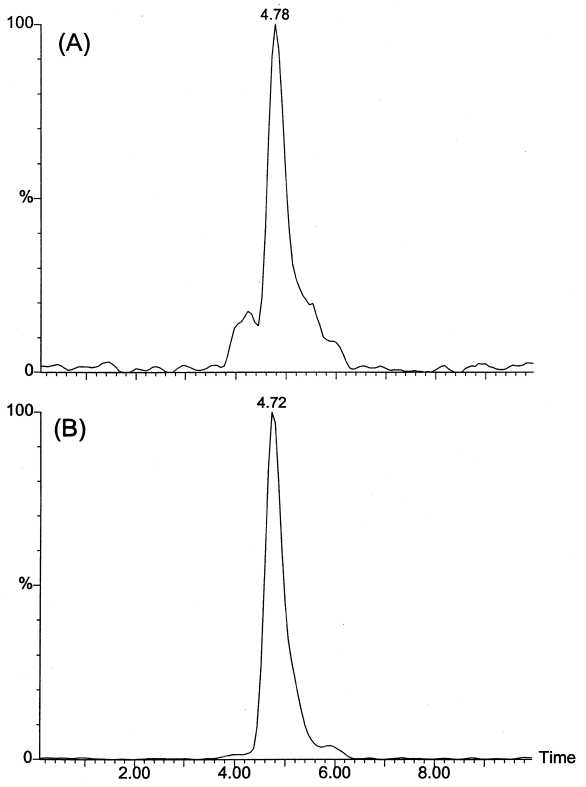

Fig. 3. SRM chromatograms of LTE_4 (A) and LTE_4-d_3 (B) extracted from human urine. SRM was performed by monitoring the transitions between m/z 438 and m/z 333 for LTE_4 and between m/z 441 and m/z 336 for LTE_4-d_3.

pg/2 ml-urine (35, 70, 140 and 280 pg/mg creatinine, respectively), were prepared. LTE_4 spiked concentrations correspond to approximately 30%–400% of endogenous LTE_4 found in healthy human urine [9–13]. These samples were analyzed in triplicate. The findings are shown in Table 1. The accuracy of added LTE_4 ranged from 97.0% to 108.0% with a mean and standard deviation of 100.6±2.4%. Statistical analysis was carried out according to two-way analysis of variance [16] to separate analytical errors between sources: concentration of LTE_4, sample preparation and LC–MS–MS in the SRM mode. The findings indicate that there was no significant contribution to the total variance from the spiking error and the sample preparation (Table 2B). Almost all of the variance in this experiment was attributed to LC–MS–MS in the SRM mode, because the errors from sample preparation and concentration of LTE_4 were negligible. The estimated urinary levels with the 95% confidence limit according to the simple linear regression analysis in this recovery test were calculated to be 153±3.7, agreeing with the concentrations of endogenous LTE_4 in non-spiked urine at the level of 153 pg per 2 ml of urine.

3.5. Application to human urine

LTE_4 levels in human urine from ten volunteers (eight males and two females, aged 21–39 years) were studied. None had taken medication at least two weeks prior to urine collection. LTE_4 and LTE_4-d_3 as the internal standard extracted from human urine were detected by SRM monitoring the transitions between m/z 438 and m/z 333 for LTE_4 and between m/z 441 and m/z 336 for LTE_4-d_3, and interfering substances were eliminated. The findings are shown in Table 3. LTE_4 was detected at 63.1±18.7 pg/mg creatinine in a range of 35.7–91.1 pg/mg creatinine in healthy adults.

4. Discussion

Mass spectrometry is a powerful technique for highly specific and quantitative measurement of extremely low levels of endogenous biological substances. There has also been a report on the use of a highly sensitive and specific, but rather complicated gas chromatography–mass spectrometry technique, to measure LTE_4 in urine [17].

Recently, liquid chromatography coupled with mass spectrometry has been successfully used for pharmaceutical analyses and quantitation. For determination of a wide variety of non-volatile or thermally labile molecules, such as LTE_4 [18], LC–MS and LC–MS–MS are especially useful. This study developed a method of determination of urinary LTE_4 using LC–MS–MS.

In this study, a microanalytical method of LTE_4 determination using LC–MS–MS after simple solid phase extraction was developed. Since LTE_4 is unstable in low pH conditions, but extraction efficients of LTE_4 from non-acidified urine were quite variable, careful adjustments to the urinary pH to 4.0 with acetic acid was made just prior to the ex-

Table 1
Accuracy of LTE_4 spiked human urine

Sample		Urinary levels (pg/2 ml)	LTE_4 recovered	
			pg/2 ml	Accuracy (%)
Non-spiked urine				
1		156, 158, 145		
2		148, 155, 159		
3		148, 155, 156		
4		148, 158, 156		
5		146, 158, 156		
Mean		153		
Spiked urine (2 ml)				
+100 pg	1	261, 256, 250	108, 103, 97	108, 103.0, 97.0
	2	250, 252, 252	97, 99, 99	97.0, 99.0, 99.0
+200 pg	1	347, 359, 352	194, 206, 199	97.0, 103, 99.5
	2	359, 354, 359	206, 201, 206	103.0, 100.5, 103.0
+400 pg	1	549, 559, 549	396, 406, 396	99.0, 101.5, 99.0
	2	546, 553, 555	393, 400, 402	98.3, 100.0, 100.5
+800 pg	1	963, 956, 946	810, 803, 793	101.3, 100.4, 99.1
	2	960, 951, 962	807, 798, 809	100.9, 99.8, 101.1
Mean±SD				100.6±2.4

traction. An Empore C18 disk cartridge was used for the extraction of LTE_4 from 2 ml of urine. After loading the sample, this cartridge was then washed with H_2O, 50% methanol and hexane. This choice, together with the use of minimal volumes of washing solvents, resulted in an excellent clean up. This

Table 2
Analysis of variance

Source	S	f	V	F_0
(A) Analysis of variance from the reproducibility test				
Sample preparation	3.067	4	0.767	0.022
LC–MS–MS	346.667	10	34.667	–
Total	349.734	14	–	–
F(4, 10, 0.05)=3.478				
(B) Analysis of variance from the accuracy test				
Spiking error (a)	5.025	3	1.675	0.279
Sample preparation (b)	1.354	1	1.345	0.226
a×b	35.228	3	11.743	1.959
LC–MS–MS	95.920	16	5.995	–
Total	137.527	23	–	–
F(3, 16, 0.05)=3.239				
F(1, 16, 0.05)=3.494				

[a] S=residual sum of squares; f=number of degrees of freedom; f_1=f sample preparation; f_2=f error; V=unbiased variance; F_0=observed value following F distribution variance ratio (V sample preparation/V error); $F(f_2, F_1, \alpha)$=density function of F distribution with f_1 and f_2 degrees of freedom.

Table 3
Determination of LTE_4 in urine from healthy human adults

Healthy volunteers	Sex	Age	LTE_4 pg/mg creatinine
A	male	23	64.4
B	female	24	47.1
C	male	34	35.8
D	male	23	72.6
E	female	24	37.1
F	male	24	91.1
G	male	38	66.9
H	male	25	60.8
I	male	22	86.5
J	male	22	69.1
Mean±SD		26	63.1±18.7

cartridge yielded good recovery even when a high flow was used during the sample loading and washing periods. It also required as little as 0.5 ml of elution solvent. Therefore, the time-consuming solvent evaporation time might become unnecessary. This feature is even more important for compounds that are thermally labile during the solvent evaporation process. Using this cartridge, quantitation of LTE_4 using enzyme immunoassay after extraction and purification using HPLC has been reported [19].

The concentration of LTE_4 in normal healthy volunteers was 63.1±18.7 pg/mg creatinine (n=10). The values of LTE_4 in human urine from normal subjects has been reported previously [9–13]. Analysis of our values indicated a normal distribution.

In conclusion, LC–MS–MS is a rapid, specific, sensitive and accurate method for quantitating LTE_4 in urine. The cysteinyl leukotrienes have been implicated in numerous pathophysiological conditions, and the method described here can be helpful in evaluating 5-lipoxygenase activation through measurement of LTE_4.

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