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A highly sensitive and selective method for the determination of Leukotriene B_4 in human plasma by negative ion chemical ionization/gas chromatography/tandem mass spectrometry

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Abstract

We have developed a highly sensitive and highly selective method for the determination of Leukotriene B_4 (LTB_4) in human plasma using negative ion chemical ionization/gas chromatography/tandem mass spectrometry (NICI/GS/MS/MS) analysis. The developed method was summarized as follows. Deuterated LTB_4 (d_4-LTB_4) was added to human plasma samples as an internal standard, and samples were extracted by a Sep-pak C18 column. Extracted LTB_4 was derivatized into the pentafluorobenzyl ester of bis-trimethylsilyl ether (PFB-TMS-LTB_4) and quantified on the basis of selected reaction monitoring (SRM) at m/z 299 of $[M\text{-PFB-2TMSOH}]^-$ by NICI/GC/MS/MS analysis, which was the product ion of $[M\text{-PFB}]^-$. The detection limit for the quantification of LTB_4 in human plasma was 10 pg ml^{-1}, sufficiently sensitive to determine the concentrations of endogenous LTB_4 in human plasma. The plasma level of LTB_4 measured in healthy male volunteers was 33.85 ± 33.91 pg ml^{-1} (mean $\pm$ S.D. in six volunteers). The technique of MS/MS used in this method offers much greater sensitivity and selectivity than single-stage mass spectrometry. The developed method showed good reproducibility with a simple and rapid extraction procedure, and would be useful for examining the relationship between various disease states and the levels of LTB_4 in biological fluids.

Keywords: Leukotriene B_4 (LTB_4); Human plasma; Tandem mass spectrometry (MS/MS); Selected reaction monitoring (SRM) technique

1. Introduction

Leukotriene B_4 (LTB_4) is a dihydroxy metabolite of arachidonic acid via a 5-lipoxygenase pathway. LTB_4 is well known as a mediator of inflammation owing to its potent chemotactic and chemokinetic activity for polymorphonuclear leukocytes (PMNs) and venular permeability. There have been many methods reported for the determination of LTB_4 in biological fluids, by radioimmunoassay (RIA) [1–9], radioreceptorassay (RRA) [10,11], high-performance liquid chromatography (HPLC) [12] and gas chromatography/mass spectrometry (GC/MS) [13,14]. However, the RIA methods have the problem of cross-reactivity of antibody with other endogenous substances, such as isomeric dihydroxyeicosatetraenoic acids in biological fluids. The RRA methods also have the drawback of cross-reactivity of receptor with other ligands. HPLC methods have merit for purification, but lack the degree of sensitivity required for our purpose. The GC/MS method, reported by Blair et al. [14], was carried out by negative ion chemical ionization/gas chromatography/single-

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stage spectrometry (NICI/GC/MS) analysis. In this method, LTB_4 in human serum was derivatized into the pentafluorobenzyl ester of bistrimetylsilyl ether (PFB-TMS-LTB_4) and quantified on the basis of the selected ion monitoring (SIM) technique; the extraction and purification procedures by a solid-phase cartridge column Sep-pak C18, thin-layer chromatography (TLC) and further TLC purification after the derivatization step would be required. In general, complex extraction and purification procedures are not only time-consuming, but result in a low recovery. Further, the concentration of endogenous LTB_4 in biological fluids is generally extremely low, typically in the picogram range, and there may be interference from other endogenous substances. Accordingly, higher sensitivity and higher selectivity are required for the determination of LTB_4 in biological fluids.

This paper describes the development of a method for the determination of LTB_4 in human plasma by negative ion chemical ionization/gas chromatography/tandem mass spectrometry (NICI/GC/MS/MS) analysis [15] on the basis of a selected reaction monitoring (SRM) technique. In the developed method, one extraction procedure was performed by a solid-phase cartridge column. We also compared NICI/GC/MS/MS analysis with NICI/GS/MS analysis for the determination of LTB_4 in human plasma.

2. Experimental

2.1. Materials

LTB_4 (5(*S*),12(*R*)-dihydroxy-6,8,10,14(*Z*,*E*,*E*,*Z*)-eicosatetraenoic acid) (Fig. 1) was purchased from Sigma (St. Louis, MO, USA). 6,7,14,15-d_4-LTB_4 was purchased from Cayman Chemical (Ann Arbor, MI, USA); its isotopic concentration was more than 98 atom% D. Pentafluorobenzylbromide (PFBB) and *N*,*N*-diisopropylethylamine were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). *N*,*O*-bis-trimethylsilyltrifluoroacetamide (BSTFA) and a Sep-pak C18 column were obtained from Pierce (Rockford, IL, USA) and Waters (Milford, MA, USA), respectively. The solvents of HPLC grade and the reagents of analytical grade were purchased from Wako Pure Chemical (Osaka, Japan) and Katayama Chemical (Osaka, Japan).

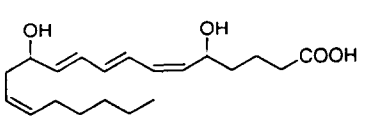

Fig. 1. Chemical structure of LTB_4.

2.2. Human plasma samples

Human plasma samples from healthy male volunteers were obtained by centrifugation of blood treated with the anticoagulant heparin sodium. The samples were stored at −20°C until analysis.

2.3. Extraction and derivatization procedure

The extraction procedure was as follows. Deuterated LTB_4 (d_4-LTB_4, 1 ng) was added to human plasma (0.2 ml) as an internal standard. The sample was diluted with 2 ml of distilled water and acidified to pH 4 by addition of 50 μl of 1 N HCl. The acidified plasma sample was applied to a Sep-pak C18 column which had been preconditioned with 4 ml of methanol and 4 ml of distilled water, and the column was washed with 4 ml of distilled water and 2 ml of methanol-distilled water (10:90, v/v). LTB_4 and d_4-LTB_4 were then eluted with 2 ml of methanol from the column and the eluate was evaporated to dryness.

The derivatization procedure was as follows. The residue was dissolved in 30 μl of acetonitrile, 10 μl of PFBB–acetonitrile (30:70, v/v) and 10 μl of *N*,*N*-diisopropylethylamine–acetonitrile (10:90, v/v). The mixture was then reacted at 40°C for 20 min for the derivatization of LTB_4 and d_4-LTB_4 into their PFB esters. The reacted mixture was evaporated to dryness and the residue was dissolved in 50 μl of BSTFA. The mixture was then reacted at 70°C for 60 min for the derivatization of PFB-LTB_4 and PFB-d_4-LTB_4 into their TMS ethers. Thus, LTB_4 and d_4-LTB_4 were finally derivatized into PFB-TMS-LTB_4 and PFB-TMS-d_4-LTB_4, respectively.

An aliquot (1 μl) of the reacted mixture including PFB-TMS-LTB_4 and PFB-TMS-d_4-LTB_4 derivatives was injected into the NICI/GC/MS or NICI/GC/MS/MS system.

2.4. GC/MS and GC/MS/MS conditions

Instrumental analysis was performed on a TSQ-700 triple-stage quadrupole mass spec-

trometer (Finnigan MAT, San Jose, CA, USA) equipped with a Varian 3400 gas chromatograph (Varian, Walnut Creek, CA, USA).

Gas chromatography conditions were as follows. Fused silica capillary column DB-1 (J&W Scientific, Folsom, CA, USA), 12.5 m × 0.25 mm i.d., with a film thickness of 0.25 μm was used. The initial temperature of the column was maintained at 80°C for 1 min, increased to 300°C at 28°C min^{-1} in a linear gradient mode, and then maintained at 300°C for 4 min. The injector and transferline temperatures were 280°C and 300°C, respectively. Helium gas was used as the carrier gas at a pressure of 10 psi.

Mass spectrometry conditions were as follows. Analysis was carried out in the NICI mode and methane gas was used as CI reagent gas at a pressure of 1.0×10^{-5} mTorr. The emission current was 200 μA, the ionization voltage was 70 V, and the multiplier voltage was 1700 V. The ion source temperature was 150°C and the manifold temperature was 70°C. In MS/MS mode, argon gas was used as the collision gas at a pressrue of 1 mTorr and the collision energy was 10 eV.

2.5. Validation procedure

Calibration curve for LTB_4

Standard solutions of LTB_4 at six concentrations (0.2, 0.5, 1.0, 2.0, 5.0, 10.0 pg μl^{-1}) in ethanol and a standard solution of d_4-LTB_4 (100 pg μl^{-1}) in ethanol were prepared. 10 μl of each standard solution of LTB_4 and d_4-LTB_4 were taken into the same test tube; each LTB_4 at six concentrations (2, 5, 10, 20, 50, 100 pg per tube) and d_4-LTB_4 at a constant concentration (1 ng per tube) were obtained. These ethanol solutions were evaporated to dryness under reduced pressure, and LTB_4 and d_4-LTB_4 were derivatized and quantified as previously described in Sections 2.3 and 2.4. Each peak area of PFB-TMS-LTB_4 and PFB-TMS-d_4-LTB_4 derivatives was obtained by monitoring at m/z 299 and m/z 303 of [M-PFB-2TMSOH]$^-$ in the SRM technique, respectively. A calibration curve for LTB_4 was constructed in the range of 2–100 pg (corresponding to injection of 0.04–2 pg onto the GC system), by plotting the peak area ratio of PFB-TMS-LTB_4 to PFB-TMS-d_4-LTB_4 derivative versus the concentration of LTB_4. In both intra-assay (within-day) and inter-assay (between-day), standard solutions were prepared and the calibration curves for LTB_4 were constructed six times.

Regression curve of spiked human plasma samples with authentic LTB_4

Spiked human plasma samples (0.2 ml) containing each authentic LTB_4 at seven concentrations (0, 2, 5, 10, 20, 50, 100 pg per tube) and d_4-LTB_4 at a constant concentration (1 ng per tube) were prepared. These samples were extracted and derivatized as described previously in Section 2.3 and quantified as described in Section 2.4. Each peak area of PFB-TMS-LTB_4 and PFB-TMS-d_4-LTB_4 derivatives was obtained as described above, and the concentrations of LTB_4 in the spiked human plasma samples were calculated by calibration curve for LTB_4 in the SRM technique. A regression curve of spiked human plasma samples with authentic LTB_4 was constructed in the range of 0–500 pg ml^{-1}, by plotting the detected concentrations of LTB_4 versus the spiked concentrations of LTB_4 in human plasma samples. In both intra-assay (within-day) and inter-assay (between-day), spiked human plasma samples were prepard and the regression curves were constructed six times in order to examine the linearity, precision and analytical recovery in the present method.

3. Results and discussion

3.1. Comparison of NICI/GC/MS/MS analysis with NICI/GC/MS analysis

Typical NICI/MS and NICI/MS/MS spectra of PFB-TMS-LTB_4 derivative are shown in Figs. 2(a) and 2(b), respectively. These spectra were obtained by the injection of the same derivatized authentic LTB_4 (1000 pg) sample into the NICI/GC/MS and NICI/GC/MS/MS systems. In the NICI/MS spectrum (Fig. 2(a)), the quasi-molecular ion was hardly observed but the fragment ion [M-PFB]$^-$ (m/z 479), formed by loss of the PFB group from the quasi-molecular ion, predominated. In contrast, the main product ion in the NICI/MS/MS spectrum (Fig. 2(b)), formed by loss of the two TMS groups from the parent ion [M-PFB]$^-$, was [M-PFB-2TMSOH]$^-$ (m/z 299).

Based on these spectral data, LTB_4 was quantified by both the SIM and SRM techniques. This was accomplished by monitoring at m/z 479 of [M-PFB]$^-$ and at m/z 299 of [M-PFB-2TMSOH]$^-$, respectively. Typical SIM and SRM chromatograms of the spiked human plasma sample (0.2 ml) with authentic

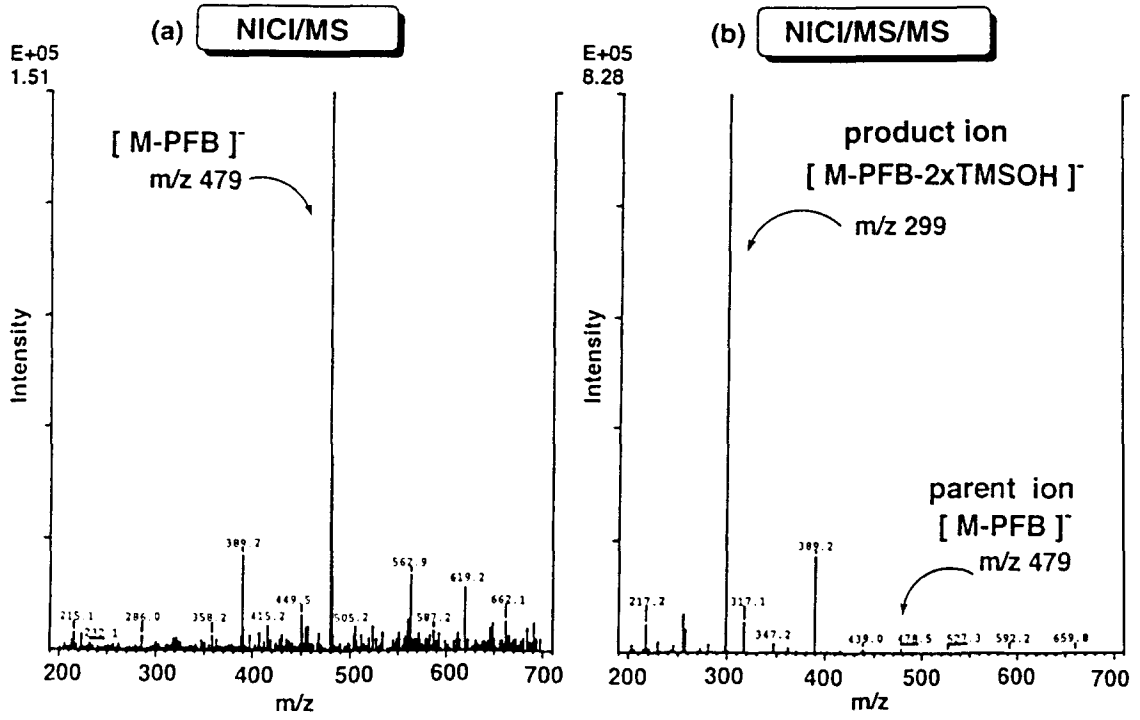

Fig. 2. Typical NICI MS and NICI MS/MS spectra of PFB-TMS-LTB$_4$ derivative: (a) NICI MS spectrum; (b) NICI MS/MS spectrum.

LTB$_4$ (100 pg) are shown in Figs. 3(a) and 3(b), respectively. These chromatograms were obtained by injection of the same spiked human plasma sample after extraction and derivatization into the NICI/GC/MS and NICI/GC/MS/MS systems. The peaks of PFB-TMS-LTB$_4$ derivative had a retention time of 10.2 min on both mass chromatograms. On the SIM chromatogram, there was serious interference from the reagents, which were added in large excess for the derivatization, and also other endogenous substances in human plasma. On the SRM chromatogram, such interference was significantly decreased. The signal to noise ratio (S/N) and the peak shape of PFB-TMS-LTB$_4$ derivative on the SRM chromatogram were superior to those on the SIM chromatogram.

Typical SRM chromatograms of PFB-TMS derivatives of authentic LTB$_4$ (100 pg) and d_4-LTB$_4$ (1 ng) are shown in Figs. 4(a) and 4(b), respectively.

These results suggested that NICI/GC/MS/MS analysis for the determination of LTB$_4$ in human plasma offers much greater sensitivity and selectivity than NICI/GC/MS analysis. Therefore, NICI/GC/MS/MS analysis was selected for the determination of LTB$_4$ in human plasma, and the validation was checked in this method as follows.

3.2. *Validation (the linearity, precision and analytical recovery)*

Calibration curves for LTB$_4$ in the range of 2–100 pg per tube in intra-assay ($n = 6$) and inter-assay ($n = 6$) are shown in Figs. 5(a) and 5(b), and Table 1. These showed good linearity, with a correlation coefficient of greater than 0.999; the y-intercepts were negligible. These calibration curves showed good reproducibility in the given range, with the relative standard deviation (RSD) of the slope being 8.3% in intra-assay and 9.0% in inter-assay. S/N on the chromatogram at the concentration of 2 pg per tube of LTB$_4$ (corresponding to injection of 0.04 pg onto the GC system) was greater than 10.

Regression curves of spiked human plasma samples with authentic LTB$_4$ in the range of 0–500 pg ml^{-1} in intra-assay ($n = 6$) and inter-assay ($n = 6$) are shown in Figs. 6(a) and 6(b), and Table 2. Regression curves of spiked human plasma samples with authentic LTB$_4$ showed good linearity in this range, with a correlation coefficient of greater than 0.999; the slopes were almost 1.0. The precisions of the assay for LTB$_4$ in human plasma at the seven spiked concentrations (0, 10, 25, 50, 100, 250, 500 pg ml^{-1}) were less than 12.0% in intra-as-

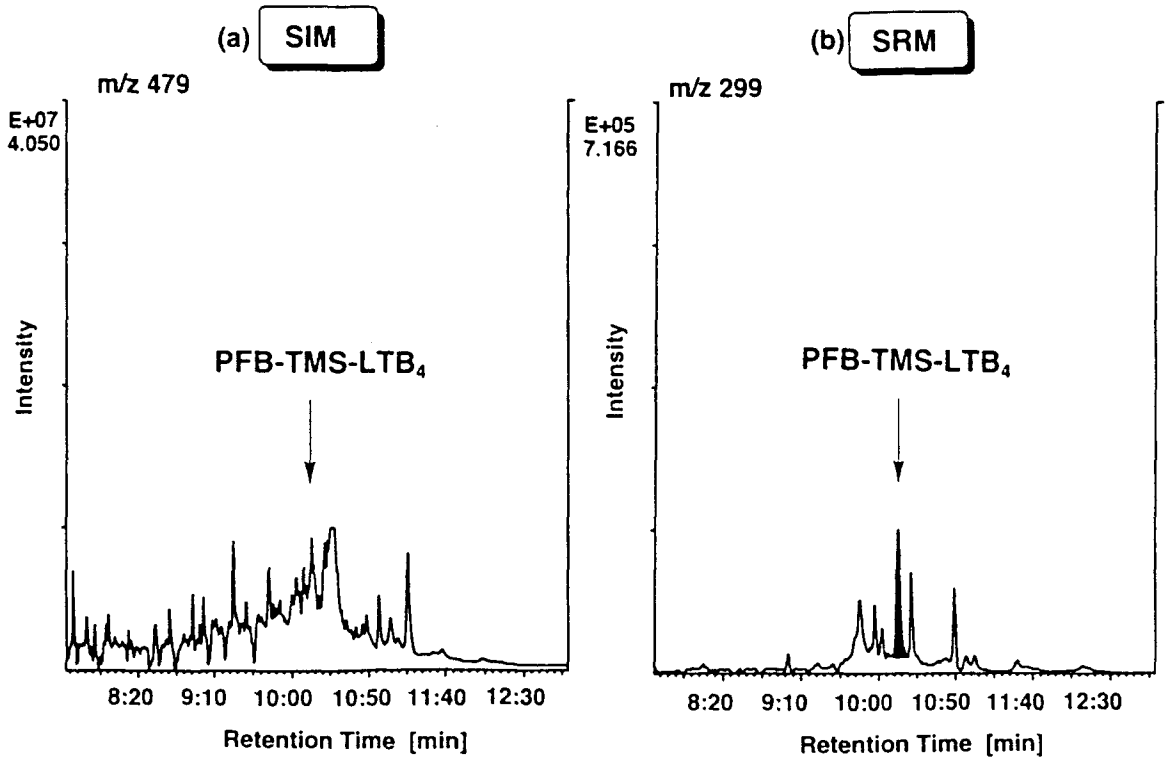

Fig. 3. Typical SIM and SRM chromatograms of the same spiked human plasma sample (0.2 ml) with authentic LTB_4 (100 pg) after extraction and derivatization into the NICI GC/MS and NICI GC/MS/MS systems: (a) SIM chromatogram; (b) SRM chromatogram.

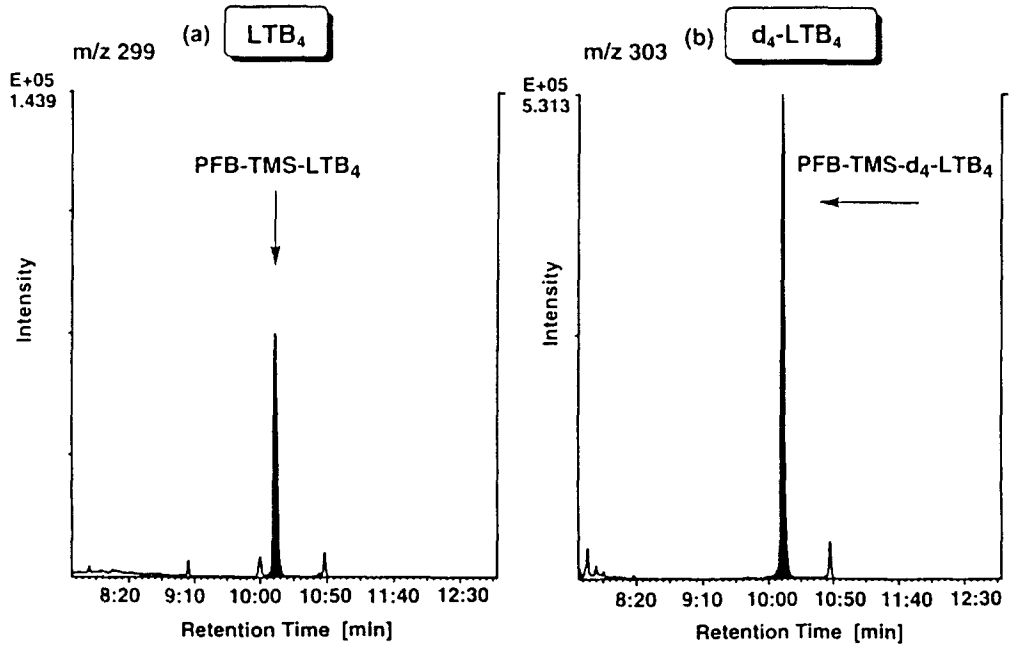

Fig. 4. Typical SRM chromatograms of PFB-TMS derivatives of authentic LTB_4 (100 pg) and d_4-LTB_4 (1 ng): (a) PFB-TMS-LTB_4; (b) PFB-TMS-d_4-LTB_4.

say and 14.2% in inter-assay. The analytical recoveries of LTB_4 in human plasma at the six spiked concentrations (10, 25, 50, 100, 250, 500 pg ml^{-1}), calculated by the equation shown in Table 2, were 96.5–111.8% in intra-assay and 87.0–109.1% in inter-assay.

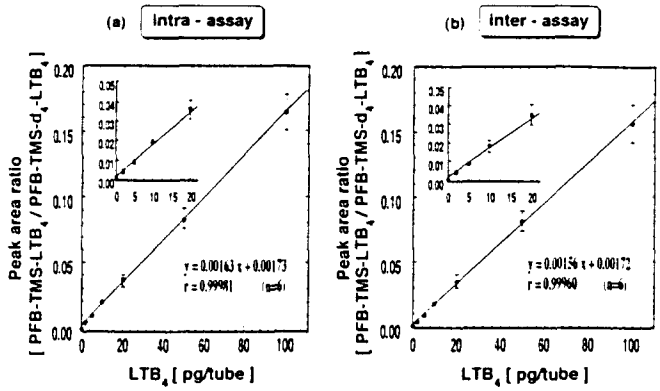

Fig. 5. Calibration curves for LTB_4 in the range of 2–100 pg per tube: (a) intra-assay ($n = 6$); (b) inter-assay ($n = 6$).

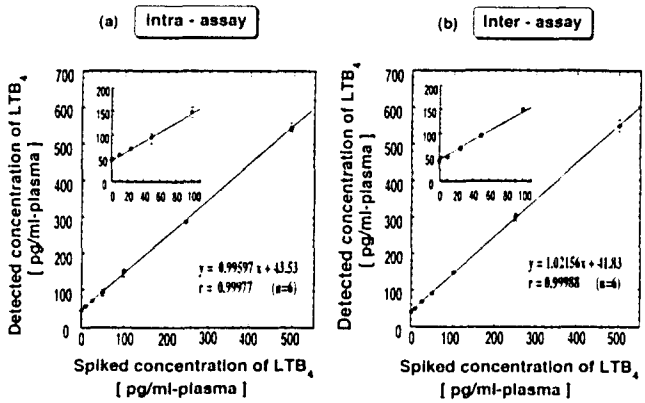

Fig. 6. Regression curves of spiked human plasma samples (0.2 ml) with authentic LTB_4 in the range of 0–500 pg ml^{-1}: (a) intra-assay ($n = 6$); (b) inter-assay ($n = 6$).

Thus, the present method showed good linearity, precision and analytical recovery. Based upon these results, the detection limit for the quantification of LTB_4 in human plasma was 10 pg ml^{-1} in the present method.

3.3. *The determination of LTB_4 in the plasma of healthy male volunteers*

The detected levels of LTB_4 in the plasma of healthy male volunteers were 33.85 ± 33.91 pg ml^{-1} (mean $\pm$ S.D. of six volunteers) in the present method. These levels were lower than those previously reported [3,5,6,9,14]. This result suggested that the present method by the use of NICI/GC/MS/MS analysis was more selective than previously reported methods.

4. Conclusions

The developed method for the determination of LTB_4 in human plasma by the use of NICI/GC/MSMS analysis showed high sensitivity, high selectivity and good reproducibility with a simple and rapid extraction procedure by only a Sep-pak C18 column. The present method was sufficiently sensitive to determine concentrations of endogenous LTB_4 in human plasma with high reliability, and would be useful to examine the relationship between various disease states and the levels of LTB_4 in biological fluids.

Table 1
Calibration curves for LTB_4 in the range of 2–100 pg per tube
Intra-assay

Concentration of LTB_4 (pg per tube)	Peak area ratio (PFB-TMS-LTB_4 / PFB-TMS-d_4-LTB_4) Mean ± S.D.
100	0.16452 ± 0.01353
50	0.08320 ± 0.00681
20	0.03608 ± 0.00483
10	0.01961 ± 0.00242
5	0.00906 ± 0.00145
2	0.00419 ± 0.00063
0	0.00057 ± 0.00051

Parameter	Mean ± S.D.	RSD (%)
Slope	0.00163 ± 0.00013	
Intercept	0.00173 ± 0.00135	8.3
Correlation coefficient	0.99981 [a]	

Inter-assay

Concentration of LTB_4 (pg per tube)	Peak area ratio (PFB-TMS-LTB_4 / PFB-TMS-d_4-LTB_4) Mean ± S.D.
100	0.15624 ± 0.01442
50	0.08156 ± 0.00750
20	0.03513 ± 0.00528
10	0.01821 ± 0.00291
5	0.00842 ± 0.00165
2	0.00375 ± 0.00054
0	0.00029 ± 0.00019

Parameter	Mean ± S.D.	RSD (%)
Slope	0.00156 ± 0.00014	9.0
Intercept	0.00172 ± 0.00112	
Correlation coefficient	0.99960 [a]	

The data represent mean ± S.D. of six experiments, except for correlation coefficient.
[a] Correlation coefficient was calculated from the mean values of peak area ratio.

Table 2
Precision of the assay for LTB_4 and analytical recovery of LTB_4 in human plasma
Intra-assay

Spiked concentration of LTB_4 (pg ml^{-1})	Detected concentration of LTB_4 (pg ml^{-1}) mean ± S.D.	Precision(%)	Analytical recovery (%) [a]
500	544.72 ± 12.93	2.4	100.3
250	284.66 ± 8.07	2.8	96.5
100	146.86 ± 11.47	7.8	103.6
50	92.22 ± 11.02	12.0	97.8
25	69.67 ± 7.44	10.7	105.5
10	54.48 ± 4.31	7.9	111.8
0 (non-spiked)	43.31 ± 2.98	6.9	–

Linear regression

Slope	0.99597
Intercept	43.53
Correlation coefficient	0.99977

Inter-assay

Spiked concentration of LTB_4 (pg ml^{-1})	Detected concentration of LTB_4 (pg ml^{-1}) mean ± S.D.	Precision(%)	Analytical recovery (%) [a]
500	550.84 ± 17.22	3.1	102.1
250	298.89 ± 12.79	4.3	103.4
100	149.52 ± 8.00	5.3	109.1
50	91.75 ± 4.38	4.8	102.7
25	69.49 ± 5.96	8.8	108.4
10	49.09 ± 5.12	10.4	87.0
0 (non-spiked)	40.39 ± 5.73	14.2	–

Linear regression

Slope	1.02156
Intercept	41.83
Correlation coefficient	0.99988

[a] Analytical recovery (%)

$$= \frac{\text{detected concentration} - \text{non-spiked concentration}}{\text{spiked concentration}} \times 100\ (\%).$$

The data of analytical recovery (%) represent the average of six experiments.

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