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Journal of Chromatography B, 852 (2007) 590–597
JOURNAL OF CHROMATOGRAPHY B
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Ultra sensitive determination of limaprost, a prostaglandin E_1 analogue, in human plasma using on-line two-dimensional reversed-phase liquid chromatography–tandem mass spectrometry

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Received 22 September 2006; accepted 16 February 2007
Available online 24 February 2007

Abstract

A highly sensitive and selective method has been developed and validated to determine limaprost, a prostaglandin (PG) E_1 analogue, in human plasma by on-line two-dimensional reversed-phase liquid chromatography–tandem mass spectrometry (2D-LC/MS/MS) due to the lack of efficient methods to determine very low levels of limaprost in plasma. Limaprost and its deuterium derivatives, used as internal standard, were extracted by protein precipitation and following three-step solid phase extractions. After extraction procedure, samples were analyzed by on-line 2D-LC/MS/MS with electrospray ionization in negative mode. The 2D-LC system consists of Phenyl column at first dimension and ODS at second dimension with a trapping column placed between the separation columns. The linear dynamic range of this method was 0.1–10 pg/ml with 3 ml of plasma ($r > 0.9987$). Acceptable precision and accuracy were obtained over the calibration curve ranges. The assay has been successfully used in analyses of human plasma samples to support clinical pharmacokinetics studies.
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Keywords: Limaprost; OP-1206; Prostaglandin; Two-dimensional

1. Introduction

Limaprost (OP-1206) is an orally active PGE_1 analogue that has strong vasodilatory and anti-platelet activities [1–3]. Limaprost alfadex (alpha-cyclodextrin clathrate) has been approved in Japan for the treatment of various ischemic symptoms, such as ulcers, pain, and cold sensations associated with thromboangitis obliterans (TAO) and treatment of subjective symptoms (pain and numbness of lower legs) and gait associated with acquired lumber spinal canal stenosis (LCS).

Since approval, limaprost alfadex has been prescribed to many TAO and LCS patients; however, the pharmacokinetics of limaprost, necessary to explain the pharmacological, physiological, and pathological effects of the drug, have not been evaluated. One reason for this is that limaprost is used in very small clinical doses, 5 μg, t.i.d. for LCS and 10 μg, t.i.d. for TAO, leading to extremely low plasma concentrations. Thus, in order to determine the pharmacokinetics of limaprost, it is necessary to develop a highly sensitive method to detect limaprost in human plasma.

Radioimmunoassays (RIA) [4,5] or enzyme linked immunosorbent assays (ELISA) [6,7] are typically used to determine the levels of natural PGs in biological samples. However, these methods have poor specificity. Gas chromatography/mass spectrometry (GC/MS)[8–11] has higher sensitivity and selectivity than immunoassays; however, difficult sample purification and complicated derivation procedures are necessary for GC/MS.

Recently, rapid progress has been made in improving liquid chromatography–tandem mass spectrometry (LC/MS/MS) instruments, and this technique has been used to determine natural PG levels [12-16]. Conventional or simple column-switching LC/MS/MS systems have achieved high sensitivity with low-pg/ml lower limits of quantification (LLOQ). However, limaprost has stronger activity than natural PGE_1 and the required LLOQ is lower than for natural PGs. Therefore,

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doi:10.1016/j.jchromb.2007.02.031

a sub-pg/ml LLOQ was set as a target sensitivity for measuring limaprost levels in human plasma; this level was not achieved in preliminary studies using conventional LC/MS/MS systems.

Two-dimensional liquid chromatography (2D-LC) and 2D-LC coupled with mass spectrometry (2D-LC/MS, 2D-LC/MS/MS) have become popular techniques in proteomic research [17–21]. These systems generate excellent resolution, enabling the comprehensive separation of complex biological mixtures. 2D-LC can be divided into two approaches, the on-line and off-line modes. The on-line approach narrows the choice of LC separation mode due to a requirement for mobile phase compatibility for direct transfer to the second dimension. However, this approach minimizes sample loss, which is an advantage for sensitivity compared to the off-line mode.

2D-LC systems have been applied to comprehensive analysis of small molecules [22–24]. However, to the best of our knowledge, on-line 2D-LC/MS/MS has not been used for routine quantitative analysis of specific small molecule drugs, except in cases employing solid phase extraction columns in the 1st dimension. Some reasons for this include (1) complexity of system optimisation; (2) long run time; and (3) in many cases; sufficient sensitivity and specificity of conventional one-dimensional LC/MS/MS systems have rendered 2D-LC unnecessary. However, superior resolving power was needed to separate limaprost, which has a chemical structure similar to that of natural PGs, from endogenous substances included in the biological matrix. Furthermore, multidimensional separation may increase the ESI–MS/MS sensitivity by removing interfering components that suppress limaprost ionization. For these reasons, on-line 2D-LC/MS/MS may be an ideal strategy for the quantitation of compounds that require extreme sensitivity and selectivity, such as limaprost.

In this study, we report the development and validation of a method to quantitate limaprost levels in human plasma using on-line 2D-LC/MS/MS. Utilizing this method, we have performed pharmacokinetic studies of limaprost alfadex at clinical dosages (5 μg and 10 μg).

2. Experimental

2.1. Chemicals and reagents

Limaprost (Fig. 1) and deuterated limaprost (used as an internal standard (IS)) were synthesized at Ono Pharmaceutical Company (Osaka, Japan). HPLC-grade acetonitrile, methanol, ethanol, ethyl acetate, hexane, water, special grade potassium acetate, and 1 mol/l hydrochloric acid were purchased from Kishida Chemical (Osaka, Japan). LC/MS grade acetonitrile was obtained from Kanto Chemical (Tokyo, Japan). Special grade acetic acid and ammonium acetate were purchased from Sigma–Aldrich Japan (Tokyo, Japan). PGE_1 was obtained form Cayman Chemical (Ann Arbor, MI, USA). Deionized water was generated in-house with a Barnstead Nanopure Infinity basic filtration system (Dubuque, IA USA). Bond Elut C_{18} (3CC, 500 mg) and Bond Elut DEA (3CC, 500 mg) were purchased from Varian (Lake Forest, CA, USA). OASIS HLB (1CC, 30 mg) was obtained from Waters (Milford, MA, USA).

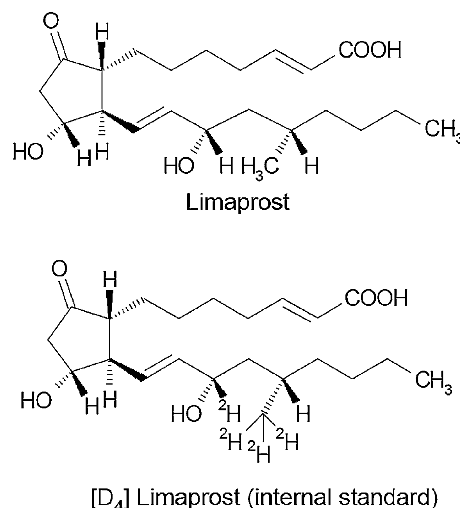


Fig. 1. Structures of limaprost and the internal standard.

Control plasma (sodium heparin anticoagulant) was obtained from human volunteers at Ono Pharmaceutical and was stored at −20 °C until use.

2.2. Stock solutions and working solutions

Limaprost was dissolved in ethanol to give 1 mg/ml stock solution. Separate stock solutions were made for the standards and QC samples. The stock solutions were serially diluted with ethanol to obtain working standard solutions of 10, 20, 50, 100, 200, 500, 800 and 1000 pg/ml. IS working solution containing 1000 pg/ml was generated in the same manner. All working solutions were stored at −20 °C until required.

2.3. Preparation of standards and QC samples

In a 10 ml glass tube, 3 ml of human plasma was added to 30 μl of each working standard solution and 30 μl of IS working solution to obtain standard samples ranging from 0.1 to 10 pg/ml and QC samples. For blank or double blank samples, 30 or 60 μl of ethanol was added, respectively, instead of standard or IS working solution. After the addition of human plasma, 100 μl of PGE_1 solution (100 ng/ml in ethanol) was added to each glass tube to block non-specific adsorption of the analyte to the surface of materials, e.g. glass tubes or SPE cartridges. Calibration standards and QC samples were freshly prepared except in the case of stability assessments.

2.4. Extraction procedure

To precipitate proteins, 6 ml of acetonitrile was added to the standard and QC samples and tubes were vortex-mixed for 10 s. Each tube was centrifuged for 5 min at 1500 × g and the supernatants were transferred into clean glass tubes. The residues were washed with 2 ml of acetonitrile and this solvent was added to the supernatant. The supernatant was concentrated to approximately 2 ml and 1 ml of water/acetic acid (99:1, v/v) was added. Each concentrated supernatant was applied

to Bond Elut C_{18} pre-conditioned with 2.5 ml each of ethyl acetate, methanol, and water/acetic acid (99:1, v/v). Following sample loading, the columns were consecutively washed with 5 ml of water, water/methanol (3:2, v/v), and hexane, and were then eluted with 4 ml of ethyl acetate. After concentration to approximately 2 ml, each fraction was loaded onto Bond Elut DEA pre-conditioned with 2.5 ml each of 1 mol/l hydrochloric acid, water, methanol/0.5 mol/l potassium acetate (pH 10) (3:2, v/v), and ethyl acetate. Following sample loading, the columns were consecutively washed with 2.5 ml of ethyl acetate and water/acetic acid (99:1, v/v), and then eluted with (water/methanol (3:2, v/v))/acetic acid (99:1, v/v). The elutes were concentrated to approximately 1 ml and applied to OASIS HLB, pre-conditioned with 1 ml each of ethyl acetate, methanol, and water/acetic acid (99:1, v/v). The columns were washed with water/acetic acid (99:1, v/v), eluted with 3 ml of ethyl acetate, and each elute was evaporated to dryness. The residues were reconstituted with 110 μl of mobile phase A (see Table 1). After centrifugation at 1500 × *g* for 1 min, samples were transferred to autosampler vials. Then, a 100 μl aliquot was injected for LC/MS/MS.

2.5. Liquid chromatography

The on-line 2D-LC system developed in this study consisted of an HP1100 binary pump, column oven, auto sampler (Agilent, Palo Alto, CA, USA), isocratic pump Nano Space SI-1 (Shiseido, Tokyo, Japan), capillary pump (GL Sciences, Tokyo, Japan), and column-switching valve (Valco, Houston, TX, USA). Capcell PAK Phenyl UG120, 5 μm, 2.0 mm × 150 mm (1st column) was purchased from Shiseido. Inertsil ODS-3, 5 μm, 1.0 mm × 10 mm (trapping column) and Inertsil ODS-3, 3 μm, 0.7 mm × 150 mm (2nd column) were obtained form GL Sciences.

2.6. Mass spectrometer

A Sciex API4000 (Applied Biosystems, Foster City, CA, USA) triple quadrapole mass spectrometer equipped with Turbo Ion Spray interface in negative ion mode was used. Quantification was performed in multiple reaction monitoring (MRM) mode. The probe temperature was 500 °C, the ionization voltage was −4500 V, the declustering voltage was −70 V, the collision gas was 6 psi, and the collision potential was −16 V for limaprost and −18 V for IS. The limaprost was monitored at a transition of *m/z* 379–233 and the IS at *m/z* 383–233. Data acquisition was performed with dwell times of 1500 ms for limaprost and 200 ms for IS. The Analyst 1.4 software (Applied Biosystems, CA, USA) was used to control the LC/MS/MS system and perform sample data analysis.

2.7. Validation parameters

Analytical method validation was performed according to the FDA guideline [25]. The validation parameters included specificity, linearity, intra- and inter-assay precision and accuracy, extraction recovery, and stability.

The specificity of the method was assessed by analyzing normal and limaprost-spiked plasma from six (three male and three female) individuals. Ion chromatograms were examined to determine the presence of any endogenous constituents which might potentially interfere with the analysis of limaprost and IS.

Extraction recoveries were carried out in triplicate at concentrations of 0.2, 1, and 8 pg/ml for limaprost and 10 pg/ml for the IS. Matrix effect on ionization was evaluated by comparing the absolute peak areas of limaprost standard added to the extract of plasma with those of neat standards. Linearity, intra- and inter-assay precision (expressed as percentage coefficient of variation, %CV) and accuracy (expressed as relative error from nominal concentration, %RE) were determined from the analysis of three separate analytical batches including standards and QC samples. Each batch contained standards consisting of seven points (0.1, 0.2, 0.5, 1, 2, 5, and 10 pg/ml) and two blanks (blank and double blank), which were not included in the calculations, and four concentrations (0.1, 0.2, 1, and 8 pg/ml) of QC samples in five replicates for each concentration. Calibration curves were established by linear least-squares regression ($1/x$ weighting) from peak area ratios (limaprost/IS) versus nominal concentrations. Intra- and inter-assay experiments had ≤15% CV and RE except for the lower limit of quantification (LLOQ) where ≤20% was acceptable for both parameters.

The short-term stability at room temperature for 4 h, the stability following three freeze–thaw cycles (−80 °C to room temperature), and the long-term stability (7 days at −20 °C or 73 days at −80 °C) were examined for spiked plasma samples. Stability of processed samples was also assessed. In each case, triplicate QC samples at 0.2 and 8 pg/ml were analyzed. In addition, short-term stability (for 4 h at 4 °C) in human whole blood was determined.

2.8. Clinical study

A single dose study of limaprost alfadex was conducted at Osaka Pharmacology Research Clinic (Osaka, Japan). Study protocols were approved by the Institutional Review Board of Osaka Pharmacology Research Clinic, and written informed consent was obtained from each study subject. Study subjects were healthy male Japanese volunteers, aged between 20 and 35 years old, with body mass index between 18.5 and 25.0. Twenty-four subjects were enrolled in the study and were assigned to two dose groups. Each subject of Group 1 was given 5 μg of limaprost orally as one tablet of limaprost alfadex (OPALMON®, Ono Pharmaceutical Co., Ltd), and each subject of Group 2 was given 10 μg as two tablets. The drug was administered to both groups following breakfast.

Blood samples (24 ml per point) were collected into tubes containing sodium heparin at the following time points: predosing, 0.25, 0.5, 1, 1.5, 2, 2.5, 3, and 4 h post-dosing. Samples were kept in ice until centrifugation. Within 1 h of blood collection, the tubes were centrifuged at 1500 × *g* for 10 min at 4 °C. The plasma was placed into polypropylene tubes and stored at −80 °C until assayed.

3. Results and Discussion

3.1. Development of the extraction procedure and on-line 2D-LC/MS/MS system

The aim of this study was to find a procedure with sufficient sensitivity and specificity to evaluate the pharmacokinetics of limaprost. Therefore, we prioritized increased sensitivity and specificity rather than time and labor in developing the method.

Sample extraction procedures were important because a large amount (3 ml) of plasma was needed to achieve the target LLOQ. An effective four-step extraction method was developed by modifying in-house method of natural PGs for ELISA: (1) protein precipitation with acetonitrile; (2) Bond Elut C_{18}; (3) Bond Elut DEA; (4) OASIS HLB, details are given in Section 2.4.

MRM condition was optimized with infusion of limaprost and IS, followed by flow injection analysis using the automatic tuning tool of the Analyst software. The negative ion full-scan mass spectra (Q1) of limaprost and the IS indicated that the deprotonated molecular ion $[M-\mathrm{H}]^-$ was the predominant ion for each compound, with *m/z* 379 and 383 for limaprost and IS, respectively. The product ion mass spectra were obtained by choosing each deprotonated molecule as precursor ions (Fig. 2). *m/z* 343, corresponding to the loss of two water molecules from limaprost, showed the highest abundance; however, *m/z* 233 was selected for MRM because of the poor specificity of *m/z* 343 in analysis of plasma samples.

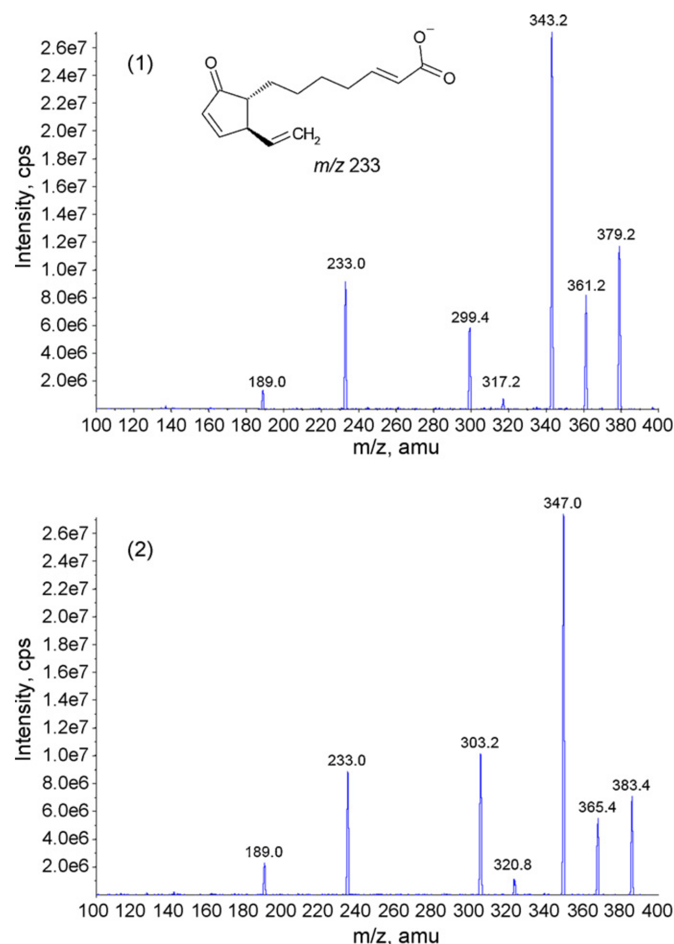


Fig. 2. Negative ion product scan mass spectra for (1) limaprost and (2) $[D_4]$ limaprost.

Before set up 2D-LC/MS/MS systems, conventional 1D-LC/MS/MS systems were evaluated. Fig. 3 shows the chromatograms obtained from 1D-LC/MS/MS systems equipped with or without column-switching. Both systems could not detect distinct peak of limaprost spiked into plasma at the concentration of target LLOQ because of endogenous interferences, though the peak was detected when standard solution at corresponding concentration were analyzed by each system. These results indicated that sensitivity and selectivity of 1D-LC/MS/MS systems were inadequate. Thus, in order to reach the target LLOQ, we employed and optimized on-line 2D-LC/MS/MS systems. In developing 2D-LC systems, choice of column combination was the most important issue. Current 2D-LC approaches for proteomics usually combine 1st dimension ion-exchange (IE) mode and 2nd dimension reversed-phase (RP) mode. However, in our early studies, some IE columns lacked retention time reproducibility, an unacceptable defect for the 1st dimension in routine quantitative analysis. The best combination for the determination of limaprost turned out to be RP (Phenyl)-RP (C_{18}), because it had not only sufficient sensitivity and specificity but also good robustness and ruggedness. Schematic diagrams of the on-line 2D-LC/MS/MS system used in this study and the time program of the system are shown in Fig. 4 and Table 1, respectively. First, the sample was injected by autosampler and the analyte underwent primary separation from endogenous interfering substances on the 1st column (Phenyl). The fraction including limaprost and the IS was diluted with ammonium acetate buffer to reduce the ratio of organic solvent, and was then concentrated on the head of the trapping column. The analyte was next separated further on the 2nd column prior to MS/MS. As shown in Fig. 3A and A′, about two times higher peak response of limaprost standard was obtained using 0.7 mm I.D. column with flow a rate of 20 μl/min comparing to a 2.1 mm I.D. column with 200 μl/min. Since linear velocity of each condition was approximately equal, it was supposed that more rapid evaporation of mobile phase following efficient ion production resulted in improved sensitivity at low flow rate. Therefore, a small bore (0.7 mm I.D.) column with low flow rate (20 μl/min) was employed for 2nd dimension in our system.

The run time of this system was relatively long (50 min); however, it was difficult to shorten run time without reducing specificity.

3.2. Validation

3.2.1. Specificity

Typical chromatograms of normal human plasma, human plasma spiked with the IS, and human plasma spiked with 0.1 pg/ml of limaprost and the IS are shown in Fig. 5. No significant interfering peaks, which would affect the accuracy or precision of the method, were observed, although a broad signal of endogenous substances was appeared around the retention time of limaprost.

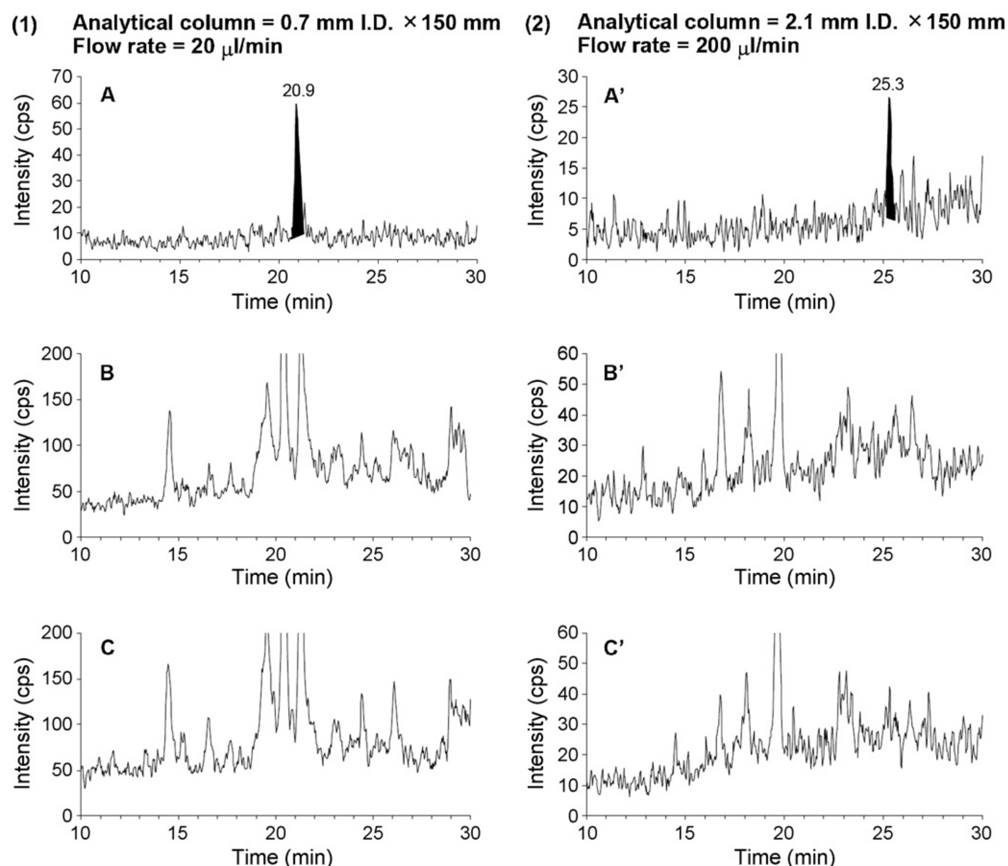
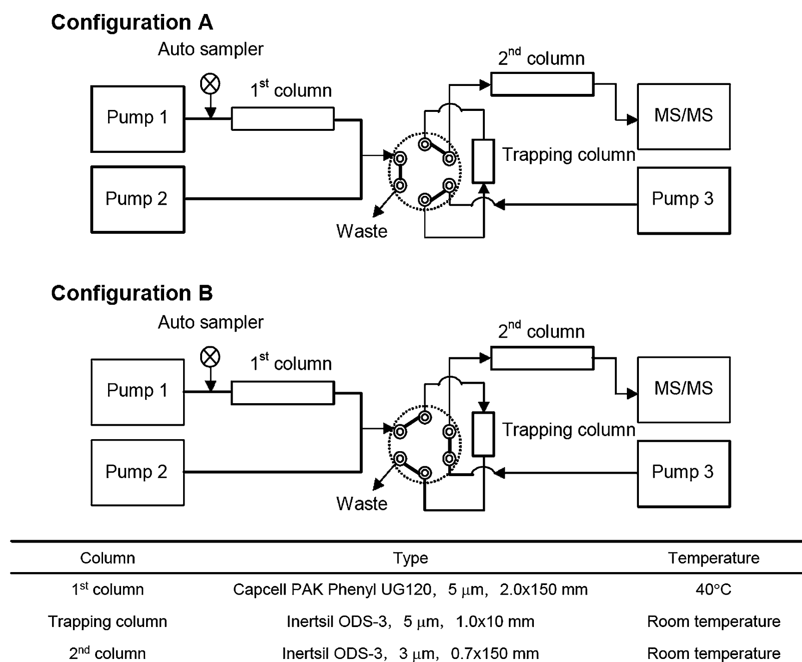


Fig. 3. Representative mass chromatograms of limaprost using 1D-LC/MS/MS (1) with or (2) without column-switching. (A, A′) Limaprost standard corresponding concentration of target LLOQ (0.1 pg/ml). (B, B′) Blank human plasma. (C, C′) Human plasma containing 0.1 pg/ml limaprost. For both systems, gradient conditions in separation step were corresponded with that of 2nd dimension of the 2D-LC/MS/MS system described in Table 1 (pump 3) except flow rate.



Column	Type	Temperature
1st column	Capcell PAK Phenyl UG120, 5 μm, 2.0x150 mm	40°C
Trapping column	Inertsil ODS-3, 5 μm, 1.0x10 mm	Room temperature
2nd column	Inertsil ODS-3, 3 μm, 0.7x150 mm	Room temperature

Fig. 4. A schematic representation of the 2D-LC/MS/MS system.

Table 1
Time program for the switching valve and pumps

Time (min)	Configuration	Analyte status
0–17.5	A	Primary separation on the 1st column
17.5–21.5	B	Concentration on the trapping column
21.5–50.0	A	Separation on the 2nd column and application to MS/MS

Pump 1			Pump 2		Pump 3		
Flow rate: 200 μl/min			Flow rate: 400 μl/min		Flow rate: 20 μl/min		
Time	Mobile phase		Time	Mobile phase	Time	Mobile phase	
(min)	A	B	(min)	C	(min)	D	E
0.0	100	0	0.0	100	0.0	5	95
22.0	100	0	50.0	100	5.0	5	95
22.1	10	90			5.1	84	16
28.0	10	90			20.0	84	16
28.1	100	0			50.0	46	54
50.0	100	0					

Mobile phase A: 5 mmol/l ammonium acetate (pH 4.5)/acetonitrile (7:3), B: acetonitrile, C: 5 mmol/l ammonium acetate (pH 4.5), D: 1 mmol/l ammonium acetate (pH 4.5)/acetonitrile (8:2), E: 1 mmol/l ammonium acetate (pH4.5)/acetonitrile (2:8).

3.2.2. Linearity

Good linearity was observed in the concentration range of 0.1–10 pg/ml in human plasma. The relative errors (%RE) were between 0.2% and 16.1% for LLOQ (0.1 pg/mL) and between −7.4% and 7.9% for other concentrations, and the correlation coefficients were greater than 0.9987.

3.2.3. Intra- and inter-day precision and accuracy

Intra-day precision and accuracy were within acceptable limits, as shown in Table 2. Precision (%CV) values were between 1.7% and 9.7% and accuracy (%RE) was between 8.9 and 17.6 for LLOQ, between −9.2 and 12.3 for other concentrations.

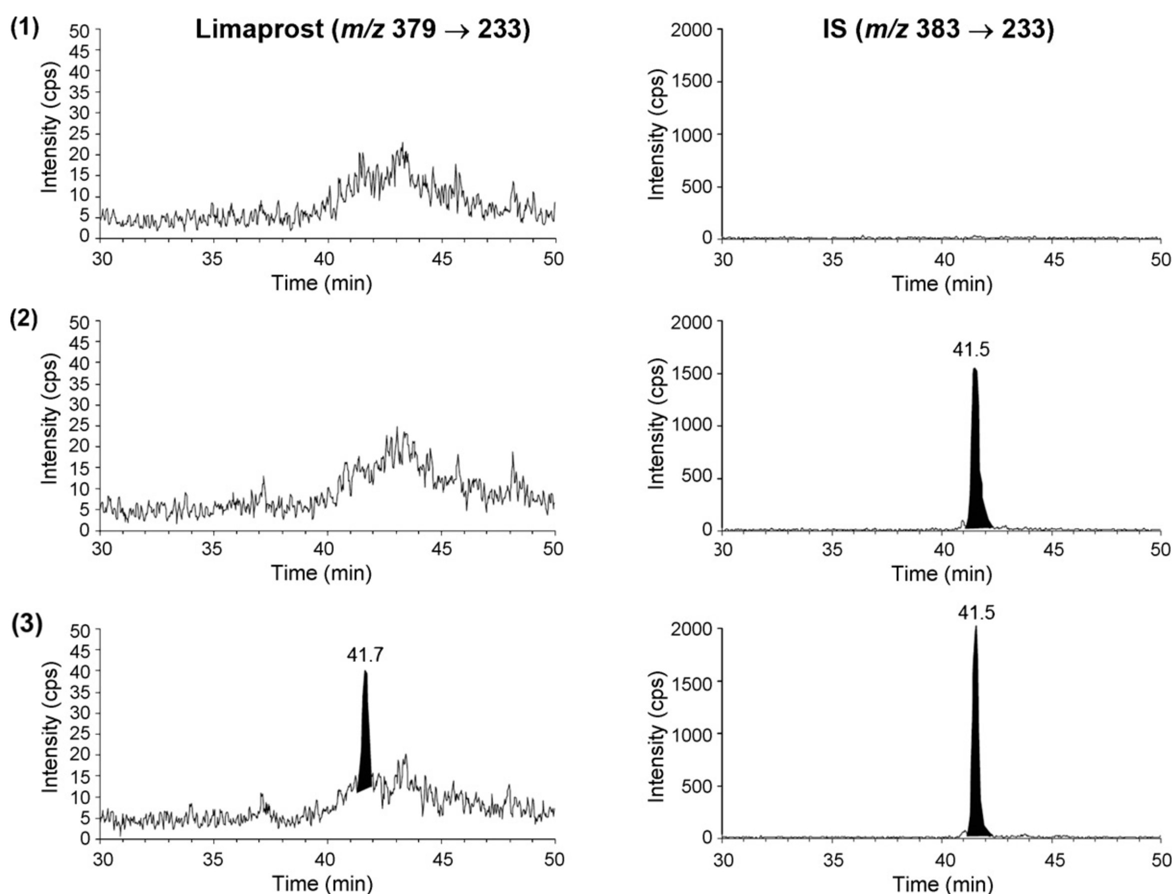


Fig. 5. Representative mass chromatograms of (1) blank human plasma; (2) human plasma spiked with the IS (10 pg/ml); and (3) human plasma spiked with limaprost (0.1 pg/ml) and the IS (10 pg/ml) using the 2D-LC/MS/MS system.

Table 2
Intra-day precision and accuracy for detection of limaprost in human plasma

Nominal concentration (pg/ml)	Batch	n	Measured concentration (pg/ml)		CV (%)	RE (%)
			Mean	SD		
0.100	1	5	0.114	0.007	13.5	6.0
	2	5	0.118	0.011	17.6	9.7
	3	5	0.109	0.006	8.9	5.6
0.200	1	5	0.208	0.019	3.8	9.0
	2	5	0.224	0.011	12.0	5.1
	3	5	0.190	0.015	−4.9	7.9
1.00	1	5	0.967	0.072	−3.3	7.5
	2	5	1.05	0.04	4.7	3.5
	3	5	0.908	0.035	−9.2	3.9
8.00	1	5	8.53	0.15	6.6	1.7
	2	5	8.98	0.35	12.3	3.9
	3	5	8.50	0.18	6.3	2.2

Table 3
Inter-day precision and accuracy for detection of limaprost in human plasma

Nominal concentration (pg/ml)	n	Measured concentration (pg/ml)		CV (%)	RE (%)
		Mean	SD		
0.100	15	0.113	0.009	13.3	7.6
0.200	15	0.207	0.020	3.6	9.7
1.00	15	0.974	0.076	−2.6	7.8
8.00	15	8.67	0.32	8.4	3.7

The results of inter-day precision and accuracy are summarized in Table 3. The %CV values were between 3.7% and 9.7%, and the %RE values were between −2.6% and 13.3%. Each result satisfied the acceptance criteria.

3.2.4. *Recovery and matrix effect*

Extraction recovery was determined by comparing the peak area ratio obtained for control plasma that was spiked with a known concentration of limaprost before extraction with control plasma that was spiked after the extraction procedure. Mean absolute recoveries at concentrations of 0.2, 1, and 8 pg/ml were 74.5, 64.9, and 67.4%, respectively. The effect of ion suppression on the peak of limaprost was examined at the concentration of 1 pg/ml ($n = 3$), and was 19.8%.

3.2.5. *Stability*

The results of stability tests are summarized in Table 4. The stability experiments were carried out using QC samples at low and high levels. The mean value of the stability QC samples at each level was compared to the nominal value except the stability in whole blood, which was compared to the initial measured concentrations. The analyte was considered to be stable if the %RE was no greater than ±15%. Using these criteria, limaprost was determined to be stable in human plasma for up to 73 days of storage at −80 °C, three freeze–thaw cycles, up to 4 h at room temperature, in human whole blood for 4 h at 4 °C, and in processed samples for 96 h at 4 °C.

3.3. *Pharmacokinetics of limaprost in humans*

The validated determination method was applied to determine the pharmacokinetics of limaprost in 24 healthy subjects for 4 h following a single 5 or 10 μg oral dose. The plasma concentrations versus time profiles are shown in Fig. 6. The mean maximum plasma concentrations (C_{max}) at 5 and 10 μg oral dose were 1.02 and 1.93 pg/ml, respectively, occurring at 1.0 h

Table 4
Stability of limaprost in human plasma, whole blood, and processed samples

Nominal concentration (pg/ml)	Difference (%, $n = 3$)[a]					
	Plasma				Whole blood	Processed sample
	Room temperature for 4 h	−20 °C for 7 days	−80 °C for 73 days	Three freeze/thaw cycles	4 °C for 4 h	4 °C for 96 h
0.200	−3.2	−1.2	4.3	6.0	−4.0	4.3
8.00	−13.5	12.0	2.3	−11.3	−9.6	−8.5

[a] Difference from nominal concentration except for whole blood, which was compared to the initial measured concentrations.

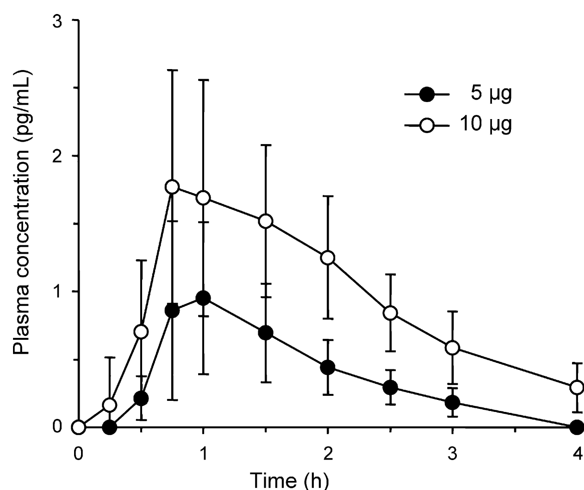


Fig. 6. Mean plasma concentration–time profiles of limaprost following a 5 μg or 10 μg oral dose in healthy male subjects. Each value represents the mean ± SD of 12 subjects.

post dose. The mean areas under the plasma concentration–time curves (AUC) were 1.71 and 4.06 pg h/ml, respectively, and the apparent plasma half lives of limaprost were 0.82 and 0.92 h, respectively. These data indicate that the plasma concentration of limaprost increased in a dose-dependent manner at two clinical doses.

4. Conclusions

An ultra sensitive and selective on-line 2D-LC/MS/MS method for the quantification of limaprost in human plasma was developed and validated. This method allowed quantitation of limaprost in the sub-pg/ml range and was able to assess the pharmacokinetics of limaprost for 2.5 h following a single 5 or 10 μg oral dose to healthy subjects. Since limaprost is a PGE_1 analogue and has a chemical structure similar to those of natural PGs, minimizing the influence of endogenous interfering substances was crucial. The four-step extraction procedure and 2D-LC/MS/MS system developed in this study showed excellent performance in specificity. In particular, employment of a 2D-LC system with low flow gradient achieved significant improvement in sensitivity and specificity. This system can be applied to the measurement of natural PGs and other PG analogues in biological matrices.

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