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Microdose clinical trial: Quantitative determination of fexofenadine in human plasma using liquid chromatography/electrospray ionization tandem mass spectrometry

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Abstract

A sample treatment procedure and high-sensitive liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) method for quantitative determination of fexofenadine in human plasma was developed for a microdose clinical trial with a *cold drug*, i.e., a non-radioisotope-labeled drug. Fexofenadine and terfenadine, as internal standard, were extracted from plasma samples using a 96-well solid-phase extraction plate (Oasis HLB). Quantitation was performed on an ACQUITY UPLC system and an API 5000 mass spectrometer by multiple reaction monitoring. Chromatographic separation was achieved on an XBridge C18 column (100 mm × 2.1 mm i.d., particle size 3.5 μm) using acetonitrile/2 mM ammonium acetate (91:9, v/v) as the mobile phase at a flow rate of 0.6 ml/min. The analytical method was validated in accordance with the FDA guideline for validation of bioanalytical methods. The calibration curve was linear in the range of 10–1000 pg/ml using 200 μl of plasma. Analytical method validation for the clinical dose, for which the calibration curve was linear in the range of 1–500 ng/ml using 20 μl of plasma, was also conducted. Each method was successfully applied for making determinations in plasma using LC/ESI-MS/MS after administration of a microdose (100 μg solution) and a clinical dose (60 mg dose) in eight healthy volunteers.

Keywords: Microdosing; Fexofenadine; Electrospray ionization; Tandem mass spectrometry; Validation

1. Introduction

The development of new drugs has been stagnated in the last decade, the cause of which has been neither prohibitive costs nor lack of industriousness. Research and development budgets in pharmaceutical companies are increasing beyond their cost-cutting efforts. A major problem is how misleading extrapolation of absorption, distribution, metabolism and excretion (ADME) data from animals to dosing in humans can be. In fact, of the new drugs under development, up to 40% are dropped at Phase I even though promising ADME data were obtained in animals [1]. Pharmacokinetic (PK) data in humans prior to a traditional first-in-human study (Phase I study) is highly advantageous in selecting candidate drugs. These factors gave rise to the concept of a microdosing study. The aim of the microdosing study is to obtain human PK data for a single or multiple drug candidates prior to Phase I studies with the intention of reducing the dropout rates in Phase I development. A position paper released in 2003 by the European Agency for the Evaluation of Medicinal Products defined the amounts for microdosing in human to be less than 1/100th of the therapeutic dose predicted from animal and *in vitro* models, while also not exceeding 100 μg [2]. Thus, analysis of drug concentration in human plasma requires the use of ultra-sensitive instruments such as that used in accelerator mass spectrometry (AMS) [3]. Among the merits of AMS is its ability to obtain sensitivity in the range of 10^{-21} to 10^{-18} mol

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[4], thus enabling the instrument to directly detect ^{14}C atoms which are 10^{-12} less existent on earth than ^{12}C. Subsequently, 0.5–100 μg amounts are sufficient for dosing in human and the volume of blood collection can be reduced from the beginning. On the other hand, there are several disadvantages. The dosing of ^{14}C-labeled drugs, or "*hot drugs*", is absolutely essential and the syntheses involved are both costly and time consuming. It puts financial burden on the pharmaceutical company and can cause temporary suspension at development stages. Additionally, the process for sample treatment takes extra man-hours and a spacious facility is needed to install AMS equipment. The AMS equipment itself is expensive and its analysis is also costly compared with that of analysis using liquid chromatography–tandem mass spectrometry (LC/MS/MS) [4].

In contrast to this is LC/electrospray ionization (ESI)-MS/MS, which has been used most frequently in the analysis of drugs in human matrices. ESI makes it possible to ionize almost all polar compounds [5]. The most remarkable aspect is that determination of concentration in plasma can be done after administration of a non-labeled drug (*cold drug*). We have already reported on actual examples of determination of drugs at a picogram per milliliter level in human plasma using LC/ESI-MS/MS [6].

In this report, we show that we have developed a sample treatment procedure and analytical methods for a microdose clinical trial using LC/ESI-MS/MS after administration of a *cold drug*, having been fexofenadine in this case, and studied how the determination of drug concentration using LC/ESI-MS/MS is useful and effective in a microdose study. As a subject of comparison, a clinical dose study was also conducted.

2. Experimental

2.1. Materials and reagents

Fexofenadine hydrochloride and terfenadine hydrochloride were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). HPLC grade acetonitrile and methanol, and analytical grade ammonium acetate and acetic acid were purchased from Wako Pure Chemical Industries, Ltd. (Kyoto, Japan). The solid-phase extraction (SPE) plate, Oasis HLB (10 mg/well) was supplied by Waters Corporation (Milford, MA, USA).

2.2. Preparation of stock and standard solutions

Fexofenadine stock solution (100 μg/ml) and terfenadine stock solution (100 μg/ml) for the internal standard (IS) were prepared by dissolving with acetonitrile/water (7:3, v/v). The fexofenadine stock solution and IS stock solution were serially diluted with acetonitrile/water (7:3, v/v) as follows. For validation of an analytical method for microdosing, we prepared standard solutions for the calibration curve at concentrations of 0.2, 0.4, 1, 2, 4, 10 and 20 ng/ml, standard solutions for quality control (QC) samples at concentrations of 0.2, 0.4, 2 and 16 ng/ml, and an IS solution at a concentration of 0.5 ng/ml. These solutions were treated as the solutions for microdosing. For validation of an analytical method for clinical dosing, we prepared standard solutions at concentrations of 20, 40, 100, 200, 400, 1000, 2000, 4000 and 10,000 ng/ml, standard solutions for QC samples at concentrations of 4, 20, 40, 400, 800 and 8000 ng/ml, and an IS solution at a concentration of 10 ng/ml. These solutions were treated as the solutions for clinical dosing. These stock and standard solutions were stored in a refrigerator at 5 °C.

2.3. Calibration standards, zero sample and QC samples

Calibration standards for microdosing at concentrations of 10, 20, 50, 100, 200, 500 and 1000 pg/ml were prepared by spiking 200 μl of the blank human plasma with 10 μl of the standard solutions (0.2, 0.4, 1, 2, 4, 10 and 20 ng/ml). Calibration standards for clinical dosing at concentrations of 1, 2, 5, 10, 20, 50, 100, 200 and 500 ng/ml were prepared by spiking 190 μl of the blank human plasma with 10 μl of the standard solutions (20, 40, 100, 200, 400, 1000, 2000, 4000 and 10,000 ng/ml). A 20 μl aliquot of the mixture was used. The zero sample for microdosing was prepared by spiking 200 μl of the blank human plasma with 10 μl of acetonitrile/water (7:3, v/v). The zero sample for clinical dosing was prepared by using 20 μl of the blank human plasma. The QC samples for accuracy and precision were prepared in human plasma at concentrations of 10, 20, 100 and 800 pg/ml for microdosing, and at concentrations of 1, 2, 20 and 400 ng/ml for clinical dosing. The QC samples for stability studies were prepared in human plasma at concentrations of 20, 100 and 800 pg/ml for microdosing, and at concentrations of 2, 20 and 400 ng/ml for clinical dosing. The samples for dilution reproducibility were prepared in human plasma at concentrations of 2000 pg/ml for microdosing and 1000 ng/ml for clinical dosing.

2.4. LC/ESI-MS/MS analysis

The samples for both microdosing and clinical dosing were analyzed according to the same analytical methods. The concentrations of fexofenadine in human plasma were determined using LC/MS/MS. The LC system was in the form of an ACQUITY UPLC system (Waters Corporation). The mass spectrometer was an API 5000 system (Applied Biosystems/MDS Sciex, Foster City, CA, USA) equipped with a turbo electrospray ionization source. The analytical column used was an XBridge C18 (100 mm × 2.1 mm i.d., particle size 3.5 μm; Waters Corporation). The column was heated to 30 °C. The mobile phase, consisting of acetonitrile/2 mM ammonium acetate (91:9, v/v), was pumped at a flow rate of 0.6 ml/min. The injection volume was 1 μl. The Turbo ion spray interface was operated in the positive ion mode at an ionization voltage of 4500 V with a turbo gas heater at 600 °C. Nebulizer gas, turbo gas, curtain gas and collision gas were at 30 psi (207 kPa), 60 psi (414 kPa), 10 psi (69 kPa) and 7, respectively. Quantitation was performed by multiple reaction monitoring (MRM). The mass transition was from *m/z* 502 to 466 for fexofenadine and from *m/z* 472 to 436 for the IS. The collision energy and dwell time for fexofenadine and the IS were 30 V and 500 ms, respectively. The

analytical data were processed with Analyst software, version 1.4.1 (Applied Biosystems/MDS Sciex).

2.5. Sample preparation

A 200 μl aliquot of modeled microdose plasma samples was transferred into a micro tube and 20 μl of IS solution (0.5 ng/ml) was added. After 400 μl of 10 mmol/l ammonium acetate–acetic acid buffer (pH 4.0) was added, the mixed solution was loaded into an SPE plate, OASIS HLB, which had been conditioned with 1 ml of methanol, 1 ml of water and 1 ml of 10 mmol/l ammonium acetate–acetic acid buffer (pH 4.0). The SPE plate was washed with 1 ml of water and 1 ml of acetonitrile/water (1:9, v/v). A 200 μl aliquot of acetonitrile/water (7:3, v/v) was added to elute fexofenadine and the IS into the 96-well 350 μl plate.

A 20 μl aliquot of modeled clinical dose plasma samples was transferred into a micro tube and 20 μl of IS solution (10 ng/ml) was added. After 60 μl of 10 mmol/l ammonium acetate–acetic acid buffer (pH 4.0) was added, the mixed solution was loaded into an SPE plate, OASIS HLB, which had been conditioned with 1 ml of methanol, 1 ml of water and 1 ml of 10 mmol/l ammonium acetate–acetic acid buffer (pH 4.0). The SPE plate was washed with 300 μl of water and 300 μl of acetonitrile/water (1:9, v/v). A 200 μl aliquot of acetonitrile/water (7:3, v/v) was added to elute fexofenadine and the IS. A 20 μl aliquot of the eluted solution was mixed with 180 μl of acetonitrile/water (7:3, v/v) prior to analysis.

2.6. Analytical validation

The analytical method was validated for selectivity, accuracy and precision in accordance with the FDA guideline for validation of bioanalytical methods [7]. Analytical methods for both microdosing and clinical dosing were conducted using the same validation parameters.

2.6.1. Selectivity

Selectivity was assessed by extracting from six different sources of plasma. No interfering peaks from endogenous substances were confirmed at the eluting positions of fexofenadine and the IS.

2.6.2. Calibration curve

Calibration standards for microdosing and clinical dosing consisted of a zero sample and seven non-zero samples (10–1000 pg/ml), and a zero sample and nine non-zero samples (1–500 ng/ml), respectively. Linearity was assessed by a weighted ($1/y^2$) least regression analysis. The relative error (RE) of the back-calculated value for each calibration curve should be within ±15% except for the lower limit of quantification (LLOQ), where it should be within ±20%. At least 75% of non-zero standards should meet the above criteria, including the LLOQ and the calibration standard at the highest concentration. The correlation coefficient (r) of standards was 0.99 or greater.

2.6.3. Lower limit of quantification

The LLOQ was defined as the lowest concentration of the calibration curve where the RE should be within ±20% and the coefficient of variation (CV) should not exceed 20%. When there is an interfering peak at the eluting position of fexofenadine, the response at the LLOQ should be at least five times that in comparison with the blank response.

2.6.4. Precision and accuracy

The intra-day and inter-day precision and accuracy were determined by analyzing five sets of QC samples at four concentration levels on three different days. The CV should not exceed 15% at each concentration except for the lower limit of quality control (LLQC), where the CV should not exceed 20%. The RE of the mean value should be within ±15% at each concentration except for the LLQC, where the RE should be within ±20%.

2.6.5. Recovery

Recovery was evaluated using the samples at three concentration levels (20, 100 and 800 pg/ml for microdosing, and 2, 20 and 400 ng/ml for clinical dosing), and carried out in triplicate. The peak areas of the extracted samples were compared with those of the corresponding blank plasma samples which were spiked with a known amount of standard solution after extraction to calculate the recovery of fexofenadine. Also, the peak areas of the extracted samples at one concentration level (50 pg/ml for microdosing and 10 ng/ml for clinical dosing) in triplicate were compared with those of the corresponding blank plasma samples which were spiked with a known amount of IS solution after extraction to calculate recovery of the IS.

2.6.6. Stability studies

The stability of fexofenadine in human plasma or processed samples was determined by analyzing three sets of QC samples at three concentration levels. The QC samples analyzed immediately after preparation were used as the samples for initial values. Stability was evaluated according to the difference from the mean of initial values, and expressed as RE. Stability of fexofenadine was assured when the RE of the mean value was within ±15%.

The QC samples for freeze and thaw cycles were kept frozen for more than 24 h at −70 °C or below in a freezer, and thawed at room temperature. The second and third freeze periods were between 12 and 24 h. The QC samples were analyzed after 1, 2 and 3 freeze and thaw cycles.

The QC samples for short-term stability were analyzed after being kept frozen for more than 24 h in a freezer at −70 °C or below, thawed at room temperature, and then kept in storage for 4 h.

The QC samples for long-term stability were analyzed after being kept frozen at −20 ± 5 °C, and at −70 °C or below for 30 days.

The processed samples of QC samples for post-preparative sample stability were analyzed after 24 and 48 h storage in the autosampler (set at 5 °C).

2.6.7. Matrix effects

The matrix effects were investigated using six independent sources of plasma. The peak areas of extracted blank plasma samples which were spiked with standard solution at one concentration level (0.4 ng/ml for microdosing and 4 ng/ml for clinical dosing) were compared with peak areas of the same concentration of standard solution diluted in acetonitrile/water (7:3, v/v). Also, the peak areas of extracted blank plasma samples which were spiked with IS solution at one concentration level (0.5 ng/ml for microdosing and 10 ng/ml for clinical dosing) were compared with peak areas of the same concentration of IS solution diluted in acetonitrile/water (7:3, v/v). The matrix effects were evaluated according to the ratios of peak areas of fexofenadine or the IS in spiked plasma post-extraction to those in acetonitrile/water (7:3, v/v), and expressed as ME.

2.6.8. Dilution reproducibility

QC samples at a concentration level of 2000 pg/ml for microdosing and 1000 ng/ml for clinical dosing were analyzed after 10-fold dilution by blank human plasma. Precision and accuracy were calculated using the mean of the measured concentration. It was established that the CV should not exceed 15% and the RE of the mean value should be within ±15%.

2.6.9. Stock and standard solutions stability

The stability of fexofenadine stock solution (100 μg/ml), IS stock solution (100 μg/ml), standard solutions (0.2, 20 and 10,000 ng/ml) and IS solutions (0.5 and 10 ng/ml) was determined by analyzing three sets of each solution after 6 h storage at room temperature and after 30 days storage at 5 °C. Stability was evaluated according to the difference from the mean of the initial values, and expressed as RE. The stability of fexofenadine and the IS was assured when the RE of the mean value was within ±15%.

2.7. Clinical trial

This clinical trial was conducted at the Clinical Investigation Center of Kitazato University East Hospital after approval by the Institutional Review Board of the hospital. Eight healthy male volunteers who provided written informed consent participated in the trial. The design of the trial was a randomized, two-period crossover study. A single group consisted of four subjects. All subjects were given a single oral dose of fexofenadine hydrochloride under fasting conditions with a 2-week washout between administrations. The dose levels were a clinical dose (60 mg Allegra tablet, Sanofi-Aventis Pharmaceuticals Inc.) and a microdose (100 μg solution prepared with Allegra tablet). The time points for blood collection were at predosing, and 0.5, 1, 2, 3, 4, 6, 8 and 12 h post-dosing.

3. Results and discussion

3.1. LC/ESI-MS/MS analysis

We selected an ACQUITY UPLC system and an XBridge C18 analytical column with the aim of greater high-throughput analysis because these have been designed to tolerate high pressure [8]. We attempted to obtain a sharper peak and shorter retention time for fexofenadine on MRM chromatograms. This was accomplished by increasing the organic solvent content and flow rate of the mobile phase by use of a column which is packed with particles synthesized by ethylene-bridged hybrids technology [9]. This made it clear that usage of the system and column with a mobile phase consisting of acetonitrile/2 mM ammonium acetate (91:9, v/v) at a flow rate of 0.6 ml/min had been proven to be very effective. The retention times were 1.0 min for fexofenadine and 2.2 min for the IS, with a total run time of 2.4 min. This retention time for fexofenadine was the shortest ever reported [10].

Terfenadine which is a prodrug of fexofenadine was used as the IS. Although a stable isotope-labeled form of fexofenadine has been marketed commercially, it is not suitable for use in the analysis of drug in plasma in a microdosing study. It is common knowledge that using a stable isotope-labeled form as the IS gives high precision and accuracy for quantitative determination. The objection of the microdosing study is to select a promising drug from among multiple candidates. If it is assumed that synthesis of stable isotope-labeled forms of these candidate drugs would be carried out, then it would also mean a necessarily large use of time and money. We thought that quantitative determination should not be part of the rate-determining process at the microdosing study stage. Therefore, we selected the chemically related substance fexofenadine. The structures of fexofenadine and the IS are shown in Fig. 1.

Mass spectral and tandem mass spectral measurements of fexofenadine and the IS were performed with these standard solutions by infusion. Protonated ions $[M+H]^+$ at *m/z* 502 for fexofenadine and *m/z* 472 for the IS, which showed up as base peaks in mass spectra, were selected as the precursor ions. The selection of product ions was accomplished by utilizing the "Quantitative Optimization" function of "Analyst" software. The product ions at *m/z* 466 for fexofenadine and *m/z* 436 for the

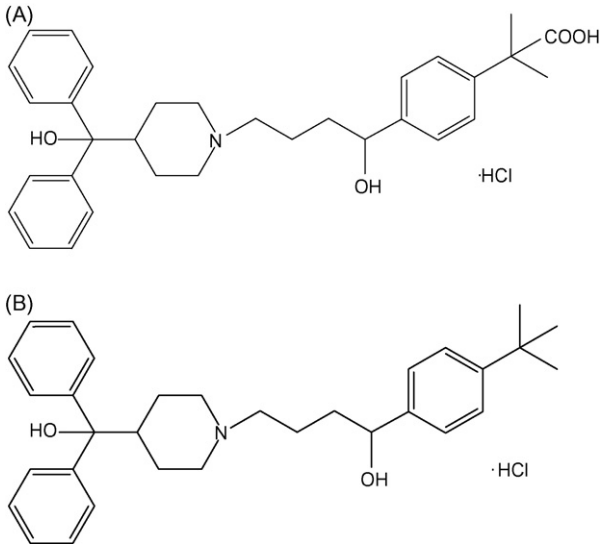

Fig. 1. Structural formulas of fexofenadine hydrochloride (A) and terfenadine hydrochloride (B).

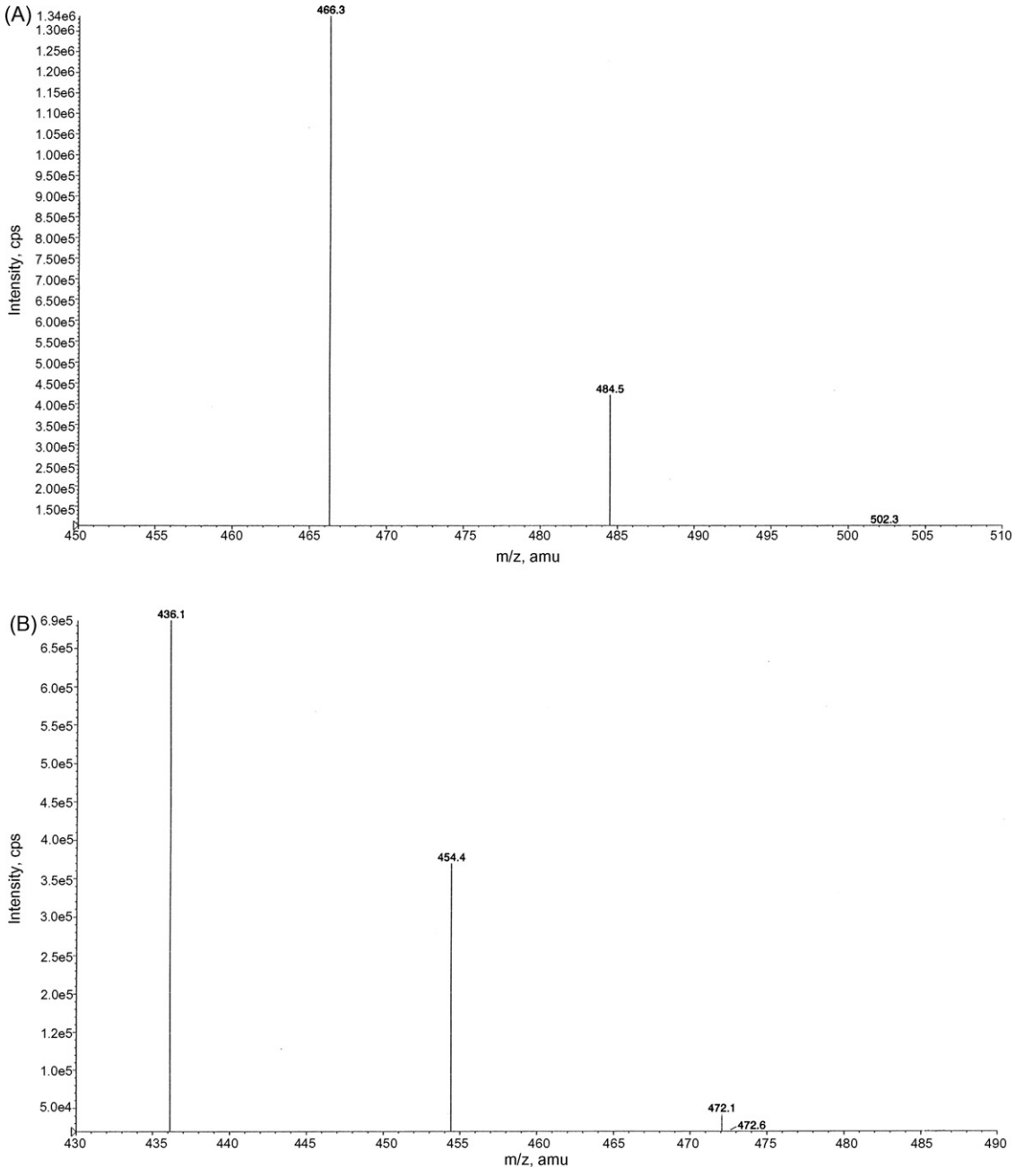

Fig. 2. Product ion spectra of fexofenadine (A) and terfenadine (B).

IS were automatically selected at the highest peak intensity in tandem mass spectra (Fig. 2). Each ion $[M-2H_2O+H]^+$ corresponded with the loss of two molecules of water from the precursor ions. Subsequently, optimization in the ion source was performed by flow injection analysis. This was carried out using the same function as in "Analyst" software, automatically searching out optimal conditions for contained nebulizer gas, turbo gas, curtain gas, collision gas, ion spray voltage and temperature. Determination of the analytical conditions for MS using the software was very simple. Under such LC/MS/MS conditions it was possible to determine absolute amounts as low as 10 fg fexofenadine.

3.2. Sample treatment

Solid-phase extraction using a 96-well plate is suitable for high-through-put processing and for its simplicity of use. We selected the Oasis HLB because the sorbents are divinylbenzene – which prevents loss of recovery due to dryness – and an *N*-vinylpyrrolidone polymer that provides hydrophobic retention [11]. The sorbents, which are without residual silanol-groups, are also expected to produce high recovery of the base drug. Sample treatment for fexofenadine using Oasis HLB has reported that wash solvent was comprised of water, and elution solvent was comprised of acetonitrile/10 mM ammonium acetate/formic

acid (90:10:0.1, v/v/v); the latter also having been used as mobile phase [10]. After the samples spiked fexofenadine was adjusted to pH 4.0, the samples were loaded into the SPE plate. The method of loading samples was the same for both the example in reference [10] and our sample treatment. We investigated the profiles for wash and elution solvents being added to the SPE plate at different ratios of acetonitrile/water. For the wash solvent we decided on acetonitrile/water (1:9, v/v), which does not elute fexofenadine or the IS because eluting of fexofenadine began at the SPE plate with acetonitrile/water (2:8, v/v). We tried to elute only fexofenadine and the IS by lowering the acetonitrile content in the elution solvent as much as possible and thereby selected acetonitrile/water (7:3, v/v). In addition, when the ratio of organic solvent for processed solution, acetonitrile/water (7:3, v/v), was lower than that of the mobile phase, acetonitrile/2 mM ammonium acetate (91:9, v/v), it was effective in producing sharp peaks for fexofenadine and the IS. The concentrations of the samples for clinical dosing were 100 times higher than those of the samples for microdosing. Therefore, plasma was decreased to 20 μl (1/10 of sample volume for microdosing), and the solutions eluted from the SPE plate were diluted 10-fold with acetonitrile/water (7:3, v/v) prior to analysis.

3.3. Analytical validation

3.3.1. Selectivity

Typical MRM chromatogram of blank plasma samples for microdosing and clinical dosing are shown in Figs. 3A and 4A, respectively. There were no interfering peaks in elution positions of fexofenadine and the IS. The other five blank plasma samples were also similar. In typical MRM chromatograms of the zero sample for microdosing and clinical dosing shown in Figs. 3B and 4B, no interfering peaks were observed at the eluting positions of fexofenadine.

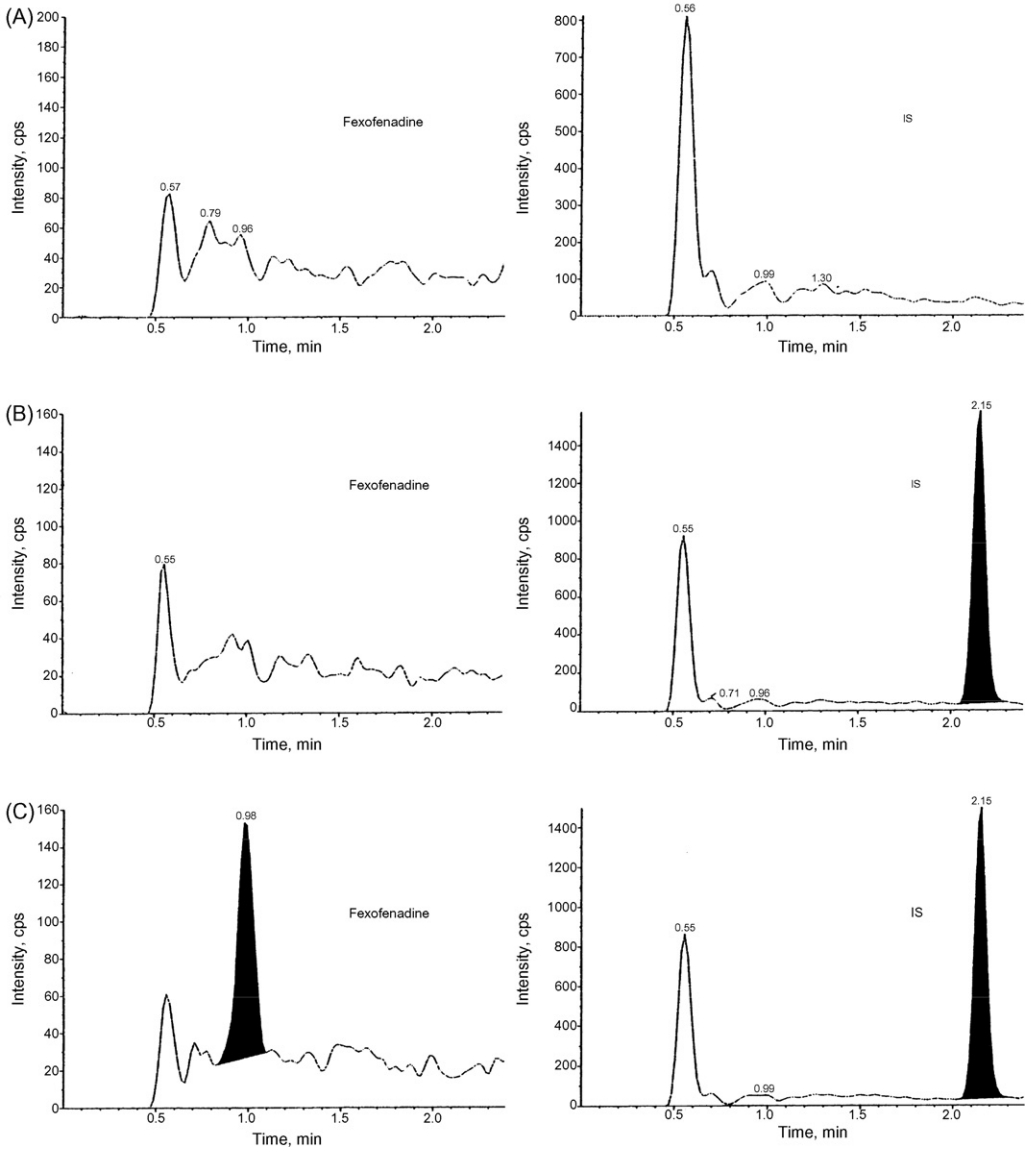

Fig. 3. Typical MRM chromatograms of calibration curve for the microdose: blank sample (A), zero sample (B) and standards at LLOQ (10 pg/ml) (C). IS: terfenadine.

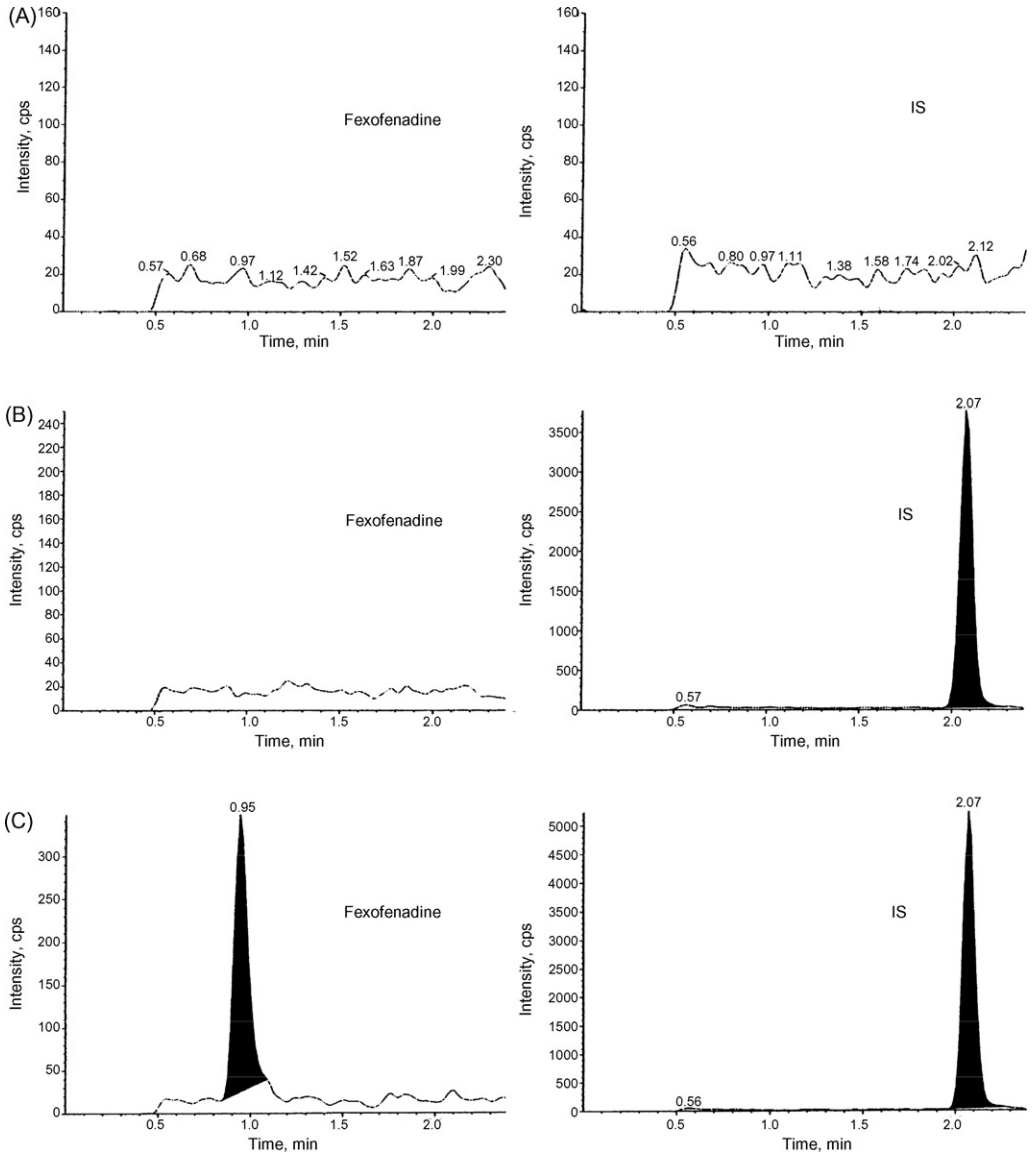

Fig. 4. Typical MRM chromatograms of calibration curve for the clinical dose: blank sample (A), zero sample (B) and standards at LLOQ (1 ng/ml) (C). IS: terfenadine.

3.3.2. Calibration curve

The calibration curve was linear over a concentration range of 10–1000 pg/ml for microdosing and 1–500 ng/ml for clinical dosing using a weighted ($1/y^2$) least squares linear regression. The main equations of the calibration curve for microdosing and clinical dosing were $y = 0.00934 (\pm 0.00149)x + 0.0186(\pm 0.0166)$ and $y = 0.0661(\pm 0.0104)x - 0.00586(\pm 0.00360)$, respectively. The equation for the calibration curve, $y = ax + b$, was obtained from the relationship between the ratio (y) of peak area of fexofenadine to the IS peak area and nominal concentrations (x) of fexofenadine. The correlation coefficient (r) for both calibration curves was >0.992. Typical calibration curves for microdosing and clinical dosing are shown in Fig. 5. The mean, CV and RE for back-calculated concentrations in each concentration of the calibration curves for microdosing and clinical dosing are summarized in Tables 1 and 2, respectively. The precision and accuracy of the calibration curve for microdosing were less than 8.5% for CV and within ±7.5% for RE. The precision and accuracy of the calibration curve for clinical dosing were less than 10.5% for CV and within ±9.1% for RE.

Table 1
Precision and accuracy of calibration standards for the microdose ($n = 3$)

Nominal concentration (pg/ml)	Back-calculated concentration		
	Mean (pg/ml)	CV (%)	RE (%)
10.0	10.4	5.6	4.3
20.0	19.0	5.5	−5.0
50.0	48.4	6.1	−3.3
100	105	8.4	4.5
200	192	8.0	−4.0
500	538	8.5	7.5
1000	1037	8.1	3.7

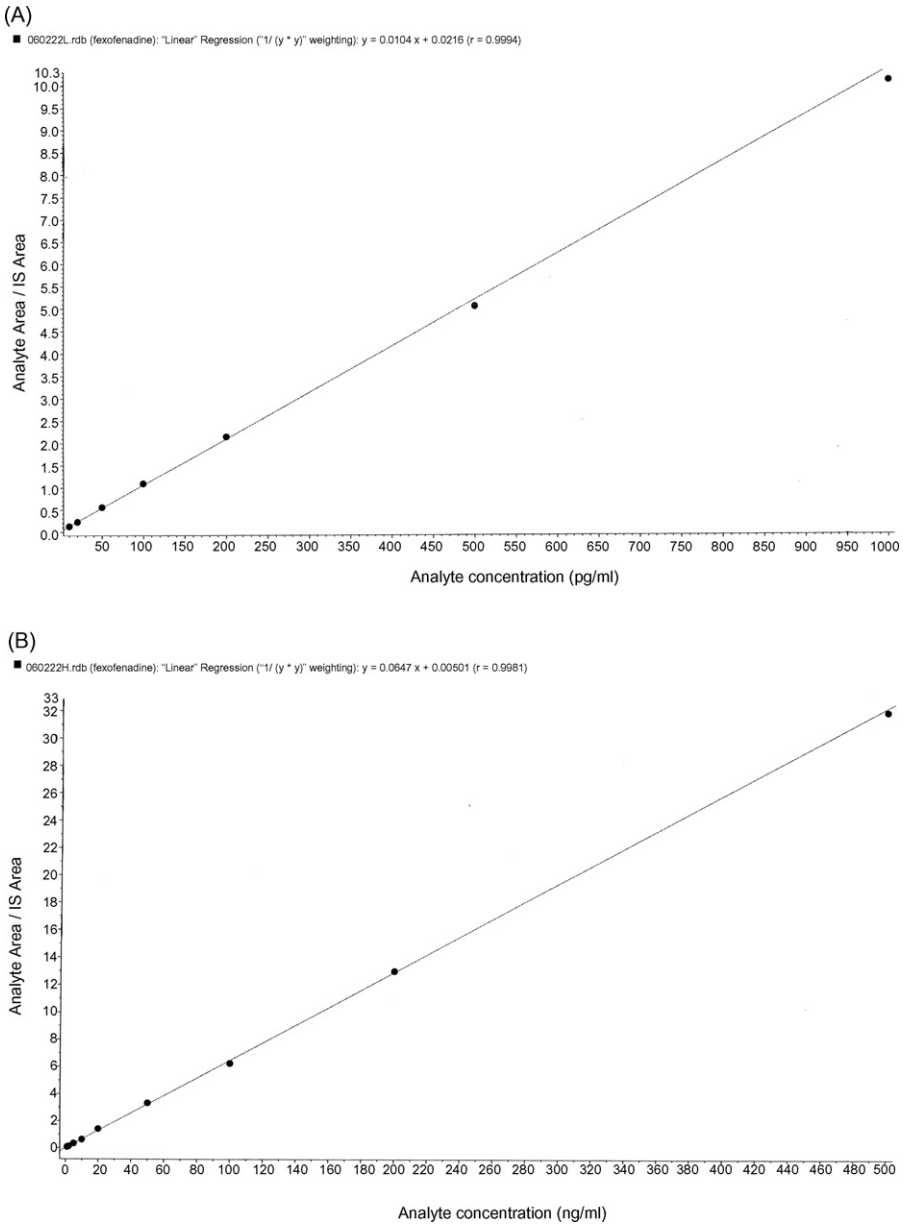

Fig. 5. Typical calibration curves: for microdosing (A) and for clinical dosing (B).

Table 2
Precision and accuracy of calibration standards for the clinical dose ($n = 3$)

Nominal concentration (ng/ml)	Back-calculated concentration		
	Mean (ng/ml)	CV (%)	RE (%)
1.00	1.02	3.0	1.7
2.00	1.92	5.2	−4.2
5.00	5.26	1.2	5.2
10.0	10.1	4.2	1.2
20.0	19.5	10.5	−2.3
50.0	54.6	5.2	9.1
100	93.9	2.5	−6.1
200	198	1.8	−1.0
500	515	3.5	2.9

3.3.3. Lower limit of quantification

The LLOQ was defined as the lowest concentration of the calibration curve with acceptable precision and accuracy, which were confirmed to be 10 pg/ml on the calibration curve for microdosing and 1 ng/ml on the calibration curve for clinical dosing. In typical MRM chromatograms of LLOQ for microdosing and clinical dosing shown in Figs. 3C and 4C, both peaks of fexofenadine were observed with good peak shapes.

3.3.4. Precision and accuracy

The intra-day and inter-day precision and accuracy were determined by analyzing five sets of QC samples at four concentration levels on three different days (Table 3). The intra-day precision and accuracy of QC samples for microdosing were less than 12.4% for CV and within ±3.5% for RE. The inter-

Table 3
Intra-day and inter-day precision and accuracy of QC samples for the microdose and clinical dose

QC samples	Intra-day				Inter-day		
	Nominal concentration (pg/ml)	Measured concentration			Measured concentration		
		Mean (pg/ml)	CV (%)	RE (%)	Mean (pg/ml)	CV (%)	RE (%)
For the microdose	10.0	10.4	10.2	3.5	10.1	9.4	1.4
	20.0	20.5	8.3	2.6	19.6	9.2	−2.2
	100	100	12.4	0.0	101	8.4	1.1
	800	779	7.8	−2.7	827	6.4	3.3
QC samples	Intra-day				Inter-day		
	Nominal concentration (ng/ml)	Measured concentration			Measured concentration		
		Mean (ng/ml)	CV (%)	RE (%)	Mean (ng/ml)	CV (%)	RE (%)
For the clinical dose	1.00	1.00	9.0	0.4	1.00	10.3	0.0
	2.00	1.98	5.4	−1.0	1.91	5.7	−4.5
	20.0	21.8	3.4	8.9	20.4	6.3	2.1
	400	428	3.7	7.1	408	8.6	2.0

day precision and accuracy of QC samples for microdosing were less than 9.4% for CV and within ±3.3% for RE. The intra-day precision and accuracy of QC samples for clinical dosing were less than 9.0% for CV and within ±8.9% for RE. The inter-day precision and accuracy of QC samples for clinical dosing were less than 10.3% for CV and within ±4.5% for RE. These results satisfied the criteria.

3.3.5. Recovery

Recovery was evaluated by peak areas of the extracted samples at three concentration levels for fexofenadine and at one concentration level for the IS compared with those of post-extracted blank plasma samples which were spiked with a known amount of standard solution. Average recoveries of the samples for recovery for microdosing were 92.5 ± 6.5% for fexofenadine (CV = 7.1%) and 73.8 ± 5.4% for the IS (CV = 7.3%). Average recoveries of the samples for recovery for clinical dosing were 84.8 ± 7.5% for fexofenadine (CV = 8.8%) and 78.1 ± 7.4% for the IS (CV = 9.4%). The recovery of IS for both microdosing and clinical dosing was lower than that of fexofenadine. However, CV of recovery for IS was less than 9.4%. Therefore, we judged that this matter presented no problems to quantitative determination.

3.3.6. Stability studies

The stability data of QC samples for microdosing and clinical dosing are shown in Tables 4 and 5, respectively. In the freeze (−70 °C or below) and thaw stability, short-term stability at room temperature and post-preparative stability (in the autosampler set at 5 °C) studies, fexofenadine was stable for 3 cycles, 4 h and 48 h, respectively. Fexofenadine was stable in human plasma for 30 days storage at −20 ± 5 °C, and −70 °C or below.

3.3.7. Matrix effects

The ME values for microdosing were 97.4 ± 6.1% for fexofenadine (CV = 6.3%) and 96.3 ± 4.8% for the IS (CV = 5.0%). The ME values for clinical dosing were 101.9 ± 9.2% for fexofe-

Table 4
Stability studies of QC samples for the microdose ($n = 3$)

Parameters for stability studies	Concentrations of fexofenadine in human plasma (pg/ml)								
	20.0			100			800		
	Mean (pg/ml)	CV (%)	RE (%)	Mean (pg/ml)	CV (%)	RE (%)	Mean (pg/ml)	CV (%)	RE (%)
Stability in matrix during freeze and thaw cycles									
Cycle 1	18.4	6.3	−8.0	97.7	6.7	−2.3	847	3.2	5.9
Cycle 2	20.9	7.1	4.5	98.4	5.6	−1.6	839	7.3	4.9
Cycle 3	19.1	4.7	−4.5	110	4.1	10.0	866	1.7	8.3
Long-term stability in matrix for 30 days at −20 ± 5 °C	20.5	6.6	2.5	92.1	3.8	−7.9	752	6.0	−6.0
Long-term stability in matrix for 30 days at −70 °C or below	20.0	11.9	0.0	96.5	8.8	−3.5	782	7.2	−2.3
Short-term stability in matrix for 4 h at room temperature	19.2	7.4	−4.0	99.8	4.9	−0.2	860	4.2	7.5
Post-preparative stability in processed sample in autosampler set at 5 °C									
For 24 h	18.1	4.4	−9.5	93.2	3.6	−6.8	795	9.5	−0.6
For 48 h	19.9	2.0	0.5	102	12.9	4.0	817	4.3	2.1

Table 5
Stability studies of QC samples for the clinical dose ($n = 3$)

Parameters for stability studies	Concentrations of fexofenadine in human plasma (ng/ml)								
	2.00			20			400		
	Mean (ng/ml)	CV (%)	RE (%)	Mean (ng/ml)	CV (%)	RE (%)	Mean (ng/ml)	CV (%)	RE (%)
Stability in matrix during freeze and thaw cycles									
Cycle 1	1.80	0.6	−10.0	17.3	0.5	−2.3	363	1.9	−9.3
Cycle 2	1.86	7.2	7.0	19.2	13.5	−1.6	365	0.5	−8.8
Cycle 3	2.03	3.4	1.5	20.0	4.0	10.0	391	5.0	−2.3
Long-term stability in matrix for 30 days at −20 ± 5 °C	2.00	2.8	0.0	17.6	3.3	−12.0	411	4.2	2.8
Long-term stability in matrix for 30 days at −70 °C or below	1.92	8.8	−4.0	18.2	2.9	−9.0	384	5.0	−4.0
Short-term stability in matrix for 4 h at room temperature	1.93	1.6	−3.5	20.3	2.2	1.5	436	2.6	9.0
Post-preparative stability in processed sample in autosampler set at 5 °C									
For 24 h	2.01	8.7	0.5	20.3	3.1	1.5	410	3.7	2.5
For 48 h	1.80	2.2	−10.0	18.7	2.7	−6.5	369	2.5	−7.8

nadine (CV = 9.0%) and 108.0 ± 11.0% for the IS (CV = 11.2%). ME values less than 100% and those more than 100% express ionization suppression and ionization enhancement, respectively. The results showed that the analysis of fexofenadine and IS both for microdosing and clinical dosing were not interfered with by endogenous substances in plasma.

3.3.8. Dilution reproducibility

QC samples at a concentration level of 2000 pg/ml for microdosing and 1000 ng/ml for clinical dosing were analyzed after 10-fold dilution by blank human plasma. The precision and accuracy were less than 11.2% for CV and within ±9.3% for RE, which matched with their original nominal concentrations.

3.3.9. Stock and standard solutions stability

The RE for fexofenadine and the IS was within ±13.1% and within ±14.4%, respectively. Fexofenadine stock solution (100 μg/ml), IS stock solution (100 μg/ml), standard solutions (0.2, 20 and 10,000 ng/ml) and IS solutions (0.5 and 10 ng/ml) were stable for 6 h at room temperature and for 30 days at 5 °C.

3.4. Clinical trial

The validated methods were applied to the analysis of fexofenadine in human plasma after microdosing and clinical dosing. The mean plasma concentration–time profiles of fexofenadine when having received 100 μg solution of fexofenadine or 60 mg oral dose of fexofenadine are shown in Fig. 6A and B, respectively. The concentrations of plasma samples exceeded the upper limit of the calibration curve when they were diluted with blank human plasma. The quality control of the analysis was done by analyzing the calibration curve and the QC samples with each run. In analysis using LC/ESI-MS/MS, it was possible to obtain the concentrations at all time points after microdosing. We selected fexofenadine hydrochloride, a non-sedating H_1 antihistamine drug, as the drug for administration because it is a substrate of organic anion transporting polypeptide-A and P-glycoprotein [12]. It was thought that the linear PK profiles between clinical dosing and microdosing were obtained. If the linear PK profiles work out, concentrations in human plasma after an oral dose of 100 μg must be determinable using LC/ESI-MS/MS. The concentrations were from 39.1 to 1082 pg/ml and the expected range of the calibration curve was within appropriate limits. The MRM chromatograms at 12 h after microdosing and 0.5 h after clinical dosing are shown in Fig. 7. The PK profiles were enough for producing statistics on PK parameters. Concentrations at all time points after clinical dosing were also obtained satisfactorily and the mean of C_{max} value was 275 ng/ml at 2.0 h which was similar to previous reported data [13].

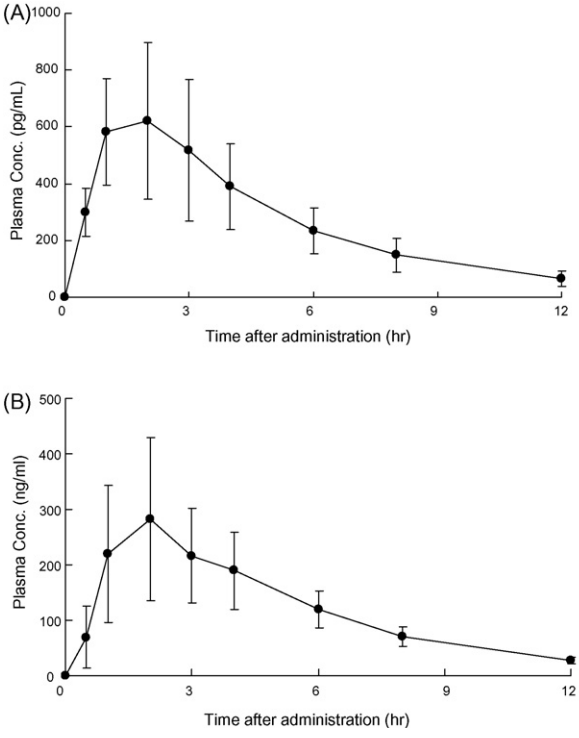

Fig. 6. Mean plasma concentration–time profiles of fexofenadine when having received 100 μg solution of fexofenadine (A) and 60 mg oral dose of fexofenadine (B).

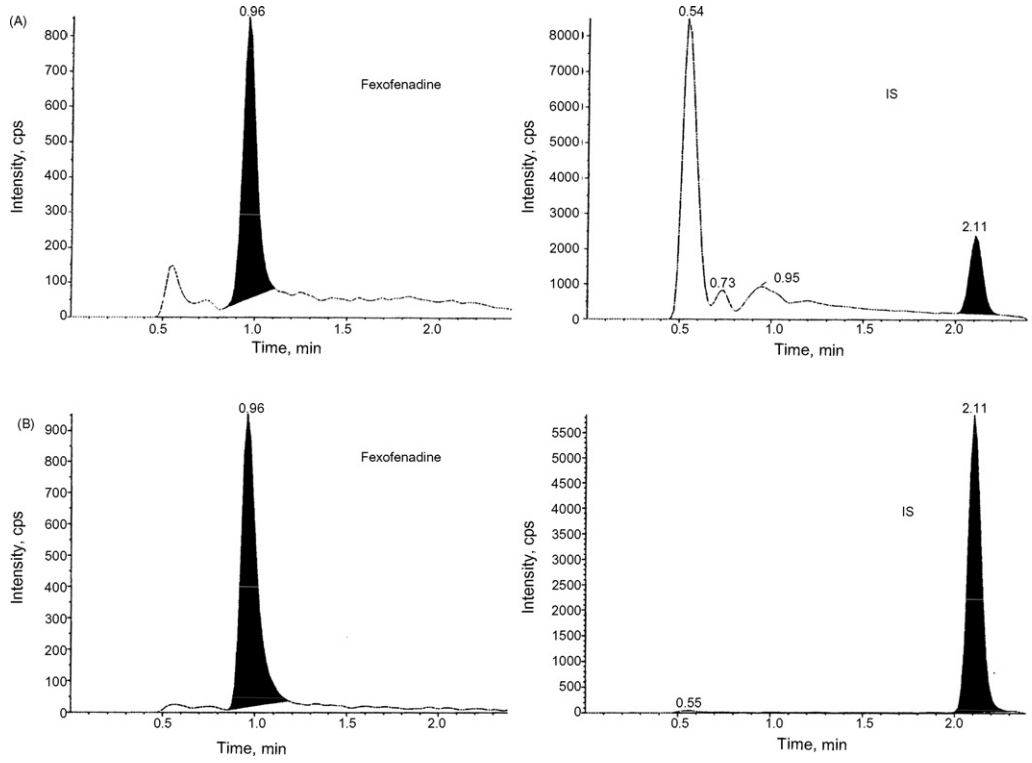

Fig. 7. MRM chromatograms: at 12 h after microdosing (39.1 pg/ml) (A) and at 0.5 h after clinical dosing (3.34 ng/ml) (B).

4. Conclusions

The sample treatment procedure and high-sensitive LC/ESI-MS/MS method we developed for quantitative determination of fexofenadine in human plasma were applied to the analysis of fexofenadine in human plasma for a microdose clinical study. We concluded that the quantitative determination of drug at a picogram per milliliter order in human plasma using LC/ESI-MS/MS was useful and effective for a microdosing study. There is the potential that analysis using LC/ESI-MS/MS could be advanced to being utilized for a microdose clinical trial with a *cold drug* (non-radioisotope-labeled drug).

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