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Short communication

Determination of fexofenadine enantiomers in human plasma with high-performance liquid chromatography

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Abstract

A simple and sensitive high-performance liquid chromatography (HPLC) method was developed as an assay for fexofenadine enantiomers in human plasma. Fexofenadine enantiomers were separated using a mobile phase of 0.5% KH_2PO_4–acetonitrile (65:35, v/v) on a Chiral CD-Ph column at a flow rate of 0.5 ml/min and measurement at 220 nm. Analysis required 400 μl of plasma and involved solid-phase extraction with an Oasis HLB cartridge, which gave recoveries for both enantiomers from 67.4 to 71.8%. The lower limit of quantification was 25 ng/ml for (*R*)- and (*S*)-fexofenadine. The linear range of this assay was between 25 and 625 ng/ml (regression line $r^2 > 0.993$). Inter- and intra-day coefficients of variation were less than 13.6% and accuracies were within 8.8% over the linear range for both analytes. This method can be applied effectively to measure fexofenadine enantiomer concentrations in clinical samples.
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1. Introduction

Fexofenadine ((±)-2-[4-[1-hydroxy-4-[4-(hydroxydiphenyl-methyl)piperidino]butyl] phenyl]-2-methylpropanoic acid, Fig. 1) is a non-sedating histamine H_1 antagonist. This drug is a P-glycoprotein substrate that has been used as a probe drug to assess P-glycoprotein activity in vivo because it is not metabolized in humans [1,2]. However, this drug contains an asymmetric carbon in its chemical structure and is administered clinically or is used as a P-glycoprotein probe as a racemic mixture of *R*- and *S*-enantiomers (Fig. 1). To our knowledge, there is only one published clinical study of the pharmacokinetics of fexofenadine enantiomers [3]. The plasma concentration of (*R*)-fexofenadine in humans was reported to be higher than that of the corresponding (*S*)-enantiomer, but the analysis method for fexofenadine enantiomers in human plasma was not described in this report [3]. There also has been no HPLC-based method for determination of fexofenadine enantiomers in human plasma published to date. The lack of a reliable analysis method has caused delays in the study of chiral fexofenadine. This is the first report on the chiral separation of fexofenadine in human plasma with an HPLC method. The method is rapid and simple and consists of a solid-phase extraction followed by selective HPLC. To validate this method, the pharmacokinetics of fexofenadine enantiomers in humans was investigated.

2. Experimental

2.1. Chemicals and reagents

Fexofenadine and diphenhydramine were donated by Sanofi Aventis (Tokyo, Japan) and Tanabe Pharmaceutical Company Ltd. (Osaka, Japan), respectively. Fexofenadine enantiomers were fractionated by HPLC using a chiral column, and then the elution order of the enantiomers in the HPLC chromatogram was determined from the reported (*R*)-(+) and (*S*)-(−) rotations [3]. An Oasis HLB extraction cartridge was purchased from Waters (Milford, MA, USA). All other reagents were purchased from Nacalai Tesque (Kyoto, Japan). All solvents were of HPLC grade.

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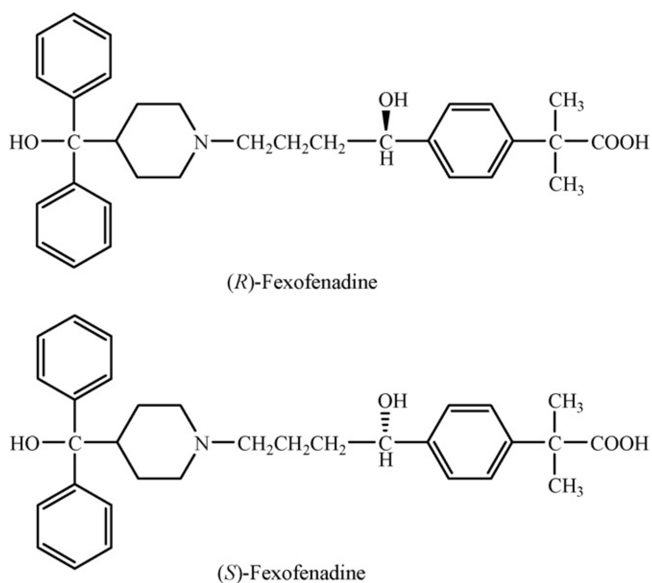

Fig. 1. Chemical structures of (*R*)-fexofenadine and (*S*)-fexofenadine.

Stock solutions for generating standard curves of fexofenadine and diphenhydramine were prepared by dissolving the dry reagents in methanol to yield concentrations of 1.0 mg/ml. Working standard solutions of fexofenadine (50, 125, 250, 500 and 1250 ng/ml) were prepared by serial dilution with methanol. Stock solutions are stable at 4 °C for at least 3 months according to the FDA [4].

2.2. Chromatographic conditions

A Model 510 chromatography pump (Waters) equipped with a Waters 486 ultraviolet detector was used. The HPLC column was a Chiral CD-Ph (250 mm × 4.6 mm i.d., Shiseido, Tokyo, Japan). The mobile phase was 0.5% KH_2PO_4 (pH 3.5)–acetonitrile (65:35, v/v), which was degassed in an ultrasonic bath prior to use. Before mixing with acetonitrile, the pH of the 0.5% KH_2PO_4 was adjusted with 50% phosphoric acid. The flow rate was 0.5 ml/min at ambient temperature and sample detection was carried out at 220 nm.

2.3. Extraction method

Following the addition of diphenhydramine (50 ng) in methanol (10 μl) to a 400 μl plasma sample as an internal standard, the plasma sample was diluted with 600 μl water and vortexed for 30 s. This mixture was applied to an Oasis HLB extraction cartridge that had been activated previously with methanol and water (1.0 ml each). The cartridge was then washed with 1.0 ml water and 1.0 ml 40% methanol in water, and eluted with 1.0 ml 100% methanol. Eluates were evaporated to dryness in a vacuum at 40 °C using a rotary evaporator (Iwaki, Tokyo, Japan). The residues were then dissolved in 50 μl methanol and vortex-mixed for 30 s; 50 μl mobile phase was added to each sample and samples were vortex-mixed for another 30 s. An aliquot of 50 μl of each sample was then processed on the HPLC apparatus.

2.4. Calibration line

The calibration line was obtained from spiked blank plasma samples in the concentration range of 25–625 ng/ml for each enantiomer of fexofenadine. Blank plasma samples were treated as described above. Calibration graphs were constructed from the peak-height ratio of each enantiomer of fexofenadine to the diphenhydramine internal standard from the HPLC chromatograms and the nominal concentration of each analyte.

2.5. Recovery

Recovery following the extraction procedure was determined by comparing the peak areas of blank plasma samples extracted according to the above procedure with those of non-extracted control samples. Control samples were prepared by mixing solutions containing the same amount of compound that was added to the plasma blank; but this compound was obtained by evaporating to dryness directly, rather than by extraction and was then reconstituted in methanol.

2.6. Assay validation

Inter-day precision and accuracy were determined from the analyses of control samples done on five different days, whereas intra-day precision and accuracy were determined by analyzing spiked controls that were run in random order five times over the course of one day. The precision of the method at each concentration was determined by comparing the coefficient of variation (CV), obtained by calculating the standard deviation (S.D.) as a percentage of the calculated mean concentration, with the accuracy estimated for each spiked control obtained by comparing the nominal concentration with the assayed concentration. The limits of quantification (LOQ) was determined as the lowest non-zero concentration measured with an intra-day CV of less than 20% and an accuracy of less than ±20% [5], and the limit of detection (LOD) was determined as the lowest concentration with a signal to noise ratio of 3.

2.7. Application to pharmacokinetics studies

The method was used to measure the plasma concentrations of fexofenadine enantiomers in human subjects. This study was approved by the Ethics Committee of Hirosaki University Hospital, and all patients gave written informed consent. Healthy subjects were given 60 mg of the Allegra® brand of fexofenadine (Sanofi Aventis). Blood samples were collected by venipuncture before and 0.5 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 h after oral administration of racemic fexofenadine. Plasma was isolated by centrifugation at 1900 × *g* for 15 min and stored at −30 °C until analysis. Plasma samples (400 μl) were then extracted as described above and 50 μl of extract was injected into the HPLC system. Pharmacokinetic analysis of fexofenadine enantiomers was done according to a standard non-compartmental method using WinNonlin software (Pharsight Co., Mountain View, CA, Version 3.1). Total area under the observed plasma

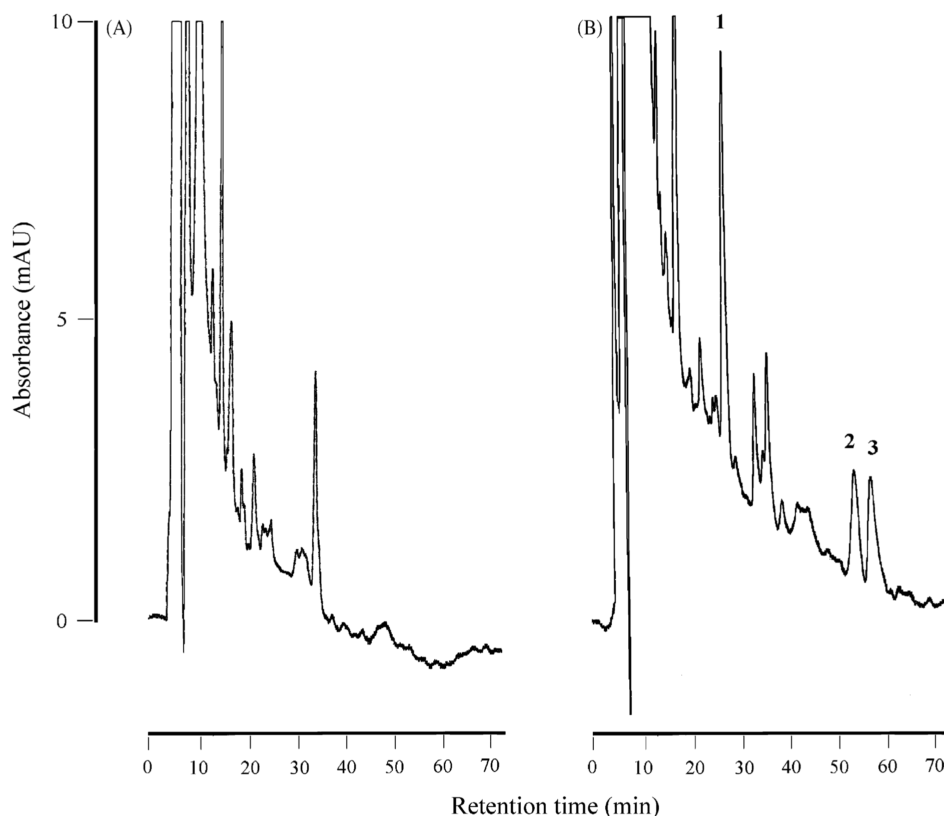

Fig. 2. Typical chromatograms of (A) plasma blank and (B) 400 μl plasma spiked with racemic fexofenadine (100 ng) and diphenhydramine (50 ng). Peaks: 1, diphenhydramine (IS); 2, (*S*)-fexofenadine; 3, (*R*)-fexofenadine.

concentration–time curve (AUC) was calculated using the linear trapezoidal rule. Values for the maximum plasma level (C_{max}) and time to reach the peak (t_{max}) were obtained directly from the profile.

3. Results and discussion

3.1. Chromatograms

Typical chromatograms obtained for blank plasma and for plasma samples spiked with racemic fexofenadine are shown in Fig. 2. Although the total HPLC run time was relatively long (about 60 min), the separation of fexofenadine enantiomers was satisfactory and free of interfering peaks in the biological matrix. Initial conditions using phosphate buffer at different pH values (ranging from 2.5 to 8) in mobile phase preparations on a reversed-phase Chiralcel OD-RH and Chiralpak AS-RH column (150 mm × 4.6 mm i.d., Daicel Chemical Ind., Tokyo, Japan) did not give a good resolution of (*R*)- and (*S*)-enantiomers from racemic fexofenadine. Consequently, fexofenadine enantiomer peaks were clearly separated using a mobile phase of 0.5% KH_2PO_4 (pH 3.5)–acetonitrile (65:35, v/v) on a Chiral CD-Ph column at a flow rate of 0.5 ml/min.

The Chiral CD-Ph analytical column is recommended washing in 70% methanol in water to ensure maximum column life according to the fact sheet from manufacturer. The chiral column was washed for 1 h after measuring about 10 plasma samples. More than 600 samples were injected onto the same column without loss in resolution.

3.2. Calibration line

Calibration lines for fexofenadine enantiomers in plasma were linear over the concentration range of 25–625 ng/ml. Typical calibration lines (obtained using the least-squares method) could be expressed for (*R*)-fexofenadine as $Y = 0.0022X–0.0305$ ($r^2 = 0.994$) and for (*S*)-fexofenadine as $Y = 0.0021X–0.0097$ ($r^2 = 0.993$), where Y is the peak height ratio and X is the concentration in ng/ml.

3.3. Recovery

The results of recovery studies from human plasma are shown in Table 1, with the recoveries of (*R*)- and (*S*)-fexofenadine determined by adding five known racemic rabeprazole concentrations (50, 125, 250, 500 and 1250 ng/ml) to drug-free plasma. The extraction recovery values for (*R*)- and (*S*)-fexofenadine were 67.4–71.8, and 67.2–71.6%, respectively (Table 1).

Fexofenadine can be extracted from plasma samples by liquid–liquid extraction [6] or solid-phase extraction on C_{18} cartridges [7,8] with similar extraction recoveries of 81–93 or 78–88 and 73–77%, respectively. Although the extraction recoveries by Oasis HLB cartridge were slightly lower than those by

Table 1
Accuracy and precision of HPLC assay for the determination of fexofenadine enantiomer concentrations in human plasma ($n = 5$)

Added (ng/ml)	Intra-day			Inter-day			Recovery (%)
	Found (mean ± S.D.)	CV (%)	Accuracy (%)	Found (mean ± S.D.)	CV (%)	Accuracy (%)	
(*R*)-Fexofenadine							
25.0	23.4 ± 2.6	11.1	−6.4	22.8 ± 3.1	13.6	−8.8	67.4
62.5	60.3 ± 3.7	6.1	−3.5	59.8 ± 4.2	7.0	−4.3	68.6
125	120 ± 7.6	6.3	−4.2	122 ± 8.1	6.6	−2.4	68.5
250	261 ± 12	4.6	4.4	258 ± 9.8	3.8	3.2	70.4
625	619 ± 16	2.6	−1.0	621 ± 18	2.9	−0.6	71.8
(*S*)-Fexofenadine							
25.0	23.1 ± 2.8	12.1	−7.6	23.0 ± 3.0	13.0	−8.0	67.2
62.5	60.1 ± 4.1	6.8	−3.8	59.6 ± 3.9	6.5	−4.6	68.3
125	119 ± 7.8	6.6	−4.8	121 ± 7.9	6.5	−3.2	68.4
250	263 ± 14	5.3	5.2	256 ± 12	4.7	2.4	70.2
625	619 ± 11	1.8	−1.0	620 ± 16	2.6	−0.8	71.6

C_{18} cartridge, the present extraction method was the simpler, faster and shorter run time compared to the previous reported methods.

3.4. Precision and accuracy

The CV and accuracy for intra- and inter-day assays were determined at concentrations of 25–625 ng/ml for (*R*)- and (*S*)-fexofenadine. CVs for intra- and inter-day assays were less than 13.6% for (*R*)-fexofenadine and were 13.0% for (*S*)-fexofenadine (Table 1). Accuracies for intra- and inter-day assays were within 8.8% for (*R*)-fexofenadine and 8.0% for (*S*)-fexofenadine (Table 1).

3.5. Sensitivity

The value of the LOQ was 25 ng/ml and of the LOD was 12.5 ng/ml for each enantiomer of fexofenadine.

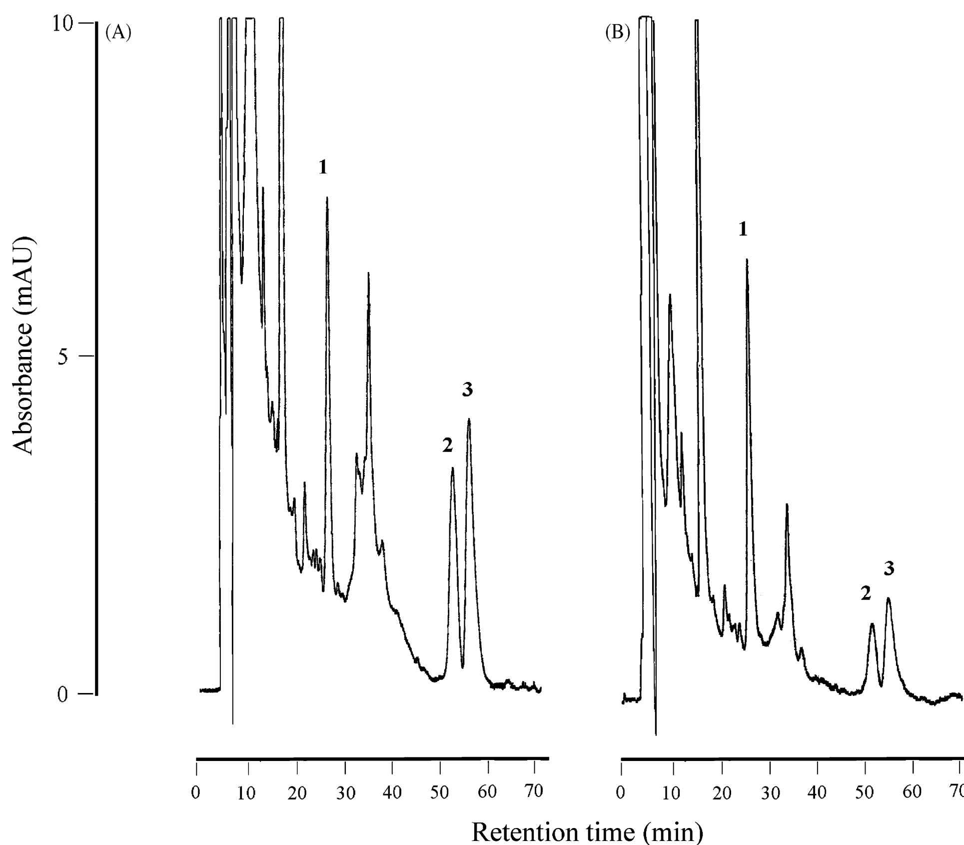

Fig. 3. Typical chromatogram of a plasma samples taken (A) 1 h and (B) 6 h after oral administration of 60 mg racemic fexofenadine. Calculated concentrations from (A) are 256 ng/ml of (*S*)-fexofenadine and 320 ng/ml of (*R*)-fexofenadine and concentrations from B) are 87 ng/ml of (*S*)-fexofenadine and 125 ng/ml of (*R*)-fexofenadine. Peaks: 1, diphenhydramine (IS); 2, (*S*)-fexofenadine; 3, (*R*)-fexofenadine.

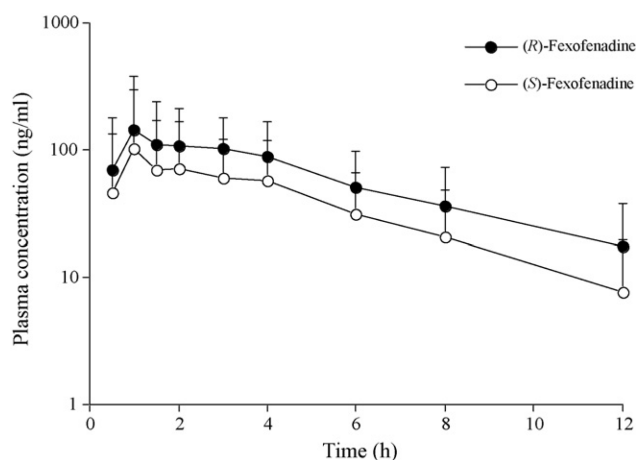

Fig. 4. Plasma concentration–time profiles of (*R*)-fexofenadine (solid circles) and (*S*)-fexofenadine (open circles) in three healthy subjects who received 60 mg racemic fexofenadine.

Table 2
Pharmacokinetic parameters of fexofenadine enantiomers in three healthy subjects

Parameter	(*R*)-Fexofenadine	(*S*)-Fexofenadine
C_{max} (ng/ml)	178 ± 220	121 ± 185
Elimination half-life (h)	5.3 ± 2.0	4.0 ± 1.8
$AUC_{0-\infty}$ (ng h/ml)	979 ± 720	564 ± 555

Values are the mean ± S.D. of three subjects.
C_{max}, maximum plasma concentration; $AUC_{0-\infty}$, area under the plasma concentration–time curve from 0 to ∞.

3.6. Stability

No significant degradation of spiked fexofenadine in blank plasma samples during 6 months at −80 °C was detected. Plasma samples for the pharmacokinetic study were stored at −80 °C and analyzed within 3 months after sampling, and then were stable at −80 °C for 6 months.

3.7. Application

Typical chromatograms of a plasma sample collected from a subject 1 and 6 h after administration of a 60 mg oral dose of racemic fexofenadine are shown in Fig. 3. The peaks corresponding to (*R*)- and (*S*)-fexofenadine were separated, with no interfering peaks detected at the retention of each analyte.

Time courses of the mean plasma concentrations of (*R*)- and (*S*)-fexofenadine in three healthy subjects receiving 60 mg fexofenadine are shown in Fig. 4, and the pharmacokinetic parameters for each enantiomer of fexofenadine are given in Table 2. Similar to the report by Robbins et al. [3], the plasma concentrations of (*R*)-fexofenadine were higher than those of the corresponding (*S*)-enantiomer. The mean C_{max} for (*R*)- and (*S*)-fexofenadine in the 60-mg dose (clinical dose) study were 178 and 121 ng/ml, respectively. The elimination half-life ($t_{1/2}$) values for (*R*)- and (*S*)-fexofenadine were 5.3 and 4.0 h, respectively; and the mean $AUC_{0-\infty}$ values were 979 and 564 ng h/ml, respectively. The *R*/*S* ratio for the AUC of fexofenadine after a 60-mg racemic fexofenadine dose was 2.1 ± 0.5. Such difference in the AUC between (*R*)- and (*S*)-fexofenadine was due to their stereoselective absorption. Therefore, P-glycoprotein may have ability for chiral discrimination. The pharmacokinetics of each fexofenadine enantiomer will be elucidated in future by using this HPLC assay.

4. Conclusion

The present HPLC method is the first analytical method described for the chiral separation of fexofenadine in human plasma. The precision and accuracy of the HPLC assay is suitable for pharmacokinetic studies. This method can be applied effectively to measure fexofenadine enantiomer concentrations in clinical samples.

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