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Measurement of fexofenadine concentration in micro-sample human plasma by a rapid and sensitive LC-MS/MS employing protein precipitation: application to a clinical pharmacokinetic study

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ABSTRACT: A simple, rapid and sensitive liquid chromatography/positive ion electro-spray tandem mass spectrometry method (LC-MS/MS) was developed and validated for the quantification of fexofenadine with 100 μL human plasma employing glipizide as internal standard (IS). Protein precipitation was used in the sample preparation procedure. Chromatographic separation was achieved on a reversed-phase C_{18} column (5 μm, 100 × 2.1 mm) with methanol : buffer (containing 10 mmol/L ammonium acetate and 0.1% formic acid; 70 : 30, v/v) as mobile phase. The total chromatographic runtime was approximately 3.0 min with retention time for fexofenadine and IS at approximately 1.9 and 2.1 min, respectively. Detection of fexofenadine and IS was achieved by LC-MS/MS in positive ion mode using 502.1 → 466.2 and 446.0 → 321.1 transitions, respectively. The method was proved to be accurate and precise at linearity range of 1–600 ng/mL with a correlation coefficient (*r*) of ≥0.9976. The validated method was applied to a pharmacokinetic study in human volunteers following oral administration of 60 or 120 mg fexofenadine formulations, successfully. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: fexofenadine; protein precipitation; LC-MS/MS; pharmacokinetic

Introduction

Fexofenadine {(±)-2-[4-[1-hydroxy-4-[4-(hydroxydiphenylmethyl) piperidino]butyl] phenyl]-2-methylpropanoic acid, Fig. 1(a)}, a second-generation, long-lasting H_1-receptor antagonist, is chronically administered for the relief of allergy symptoms. It is a non-sedating histamine H_1 antagonist which does not cause cardiotoxicity.

Several analytical methods for the pharmacokinetic study have been reported to determine fexofenadine in biological matrices. Some of these methods (Robbins *et al.*, 1998; Unoa *et al.*, 2004; Miura *et al.*, 2007; Pathak *et al.*, 2008), based on liquid chromatography (LC) using fluorescence or ultraviolet detection with the lower limit of quantification (LLOQ) at 1–25 ng/mL, have been published. Recently, many methods (Hofmann *et al.*, 2002; Nirogi *et al.*, 2007; Isleyen *et al.*, 2007; Bharathi *et al.*, 2008) using LC-MS with LLOQs at 0.5–3 ng/mL have also been reported, which are much more specific and sensitive analytical techniques. In these methods, solid-phase extraction (SPE) and liquid–liquid extraction techniques have been used for handling samples, which has been proven to be an effective technique. However, the technique is time-consuming and commercially available columns for SPE are relatively expensive, especially for pharmacokinetic studies, for which many samples must be treated.

The aim of our research work was to establish a simpler and more rapid protein precipitation method for determination of fexofenadine in humam plasma to carry out clinical pharmacokinetic studies efficiently in terms of small processing volume, low concentration and a short run time. To best of our knowledge, none of the previously published method of handing plasma samples for determination of fexofenadine in human plasma have employed such a simple technique. In our paper, the method of protein precipitation with methanol guaranteed a high absolute recovery (>82%) and good purification from matrix

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Abbreviations used: CID, collision-induced dissociation; ESI, electro-spray ionization; MRM, multiple-reaction monitoring mode.

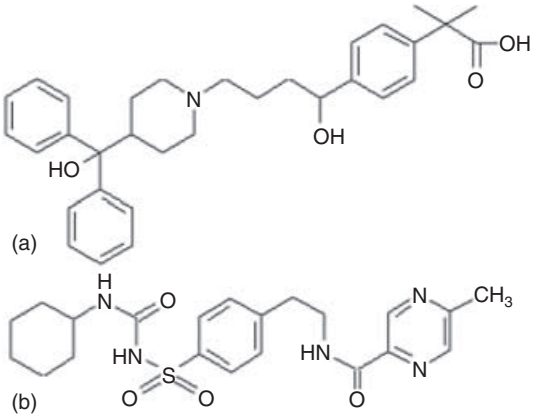


Figure 1. Chemical structures of fexofenadine (a) and glipizide (b).

interference. Meanwhile, we developed and validated a highly sensitive LC-MS/MS method (LLOQ 1.0 ng/mL) with a high throughput (runtime of approximately 3.0 min) for quantification of fexofenadine in human plasma and applied this method to derive pharmacokinetic parameters for fexofenadine in humans.

Experimental

Chemicals and Reagents

Fexofenadine hydrochloride (99.7% pure) was purchased from the National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China). Glipizide hydrochloride (IS, 99.8% pure) was obtained from Weihai Disha Pharmaceutical Co. Ltd (Shandong, China). The chemical structures of these compounds are shown in Fig. 1. Methanol was of HPLC grade (TEDIA, USA). Water was deionized and purified using a TTL-30 purification system (Tongtai Science and Technology Development Co. Ltd, Beijing, China) and was used to prepare all aqueous solutions. All other chemicals and solvents were of the highest analytical grade available. Drug-free plasma was from different batches of human blank plasma.

Instrument and Conditions

The LC-MS/MS equipment consisted of a Surveyor LC pump, a Surveyor auto-sampler and a tandem mass spectrometer equipped with an ESI ion source (TSQ Discovery Max, Thermo Electron Corporation, San Jose, CA, USA). Xcalibur software version 1.3 was used for data acquisition and analysis. LC separation was performed on an Agilent Zorbax SB-C_{18} column (5 μm, 100 × 2.1 mm) with a mobile phase of methanol : buffer (containing 10 mmol/L ammonium acetate and 0.1% formic acid; 70 : 30, v/v) at a flow rate of 0.20 mL/min. The column temperature was maintained at 30°C. An aliquot of 5 μL of the purified sample was injected automatically into the LC system for LC-MS/MS analysis. The analyses were conducted in positive ion detection mode. The ion spray voltage and capillary temperature were set at 4200 V and 350°C, respectively. The nitrogen sheath, ion sweep and auxiliary gasses were set at 18, 0 and 10 arbitrary units, respectively. The collision induced dissociation (CID) after the source was set at −2 V. The collision energy was selected as 25 eV for fexofenadine and 15 eV for glipizide. The transitions *m/z* 502.1 → 466.2 for fexofenadine and *m/z* 446.0 → 321.1 for glipizide (IS) were monitored using multiple-reaction monitoring (MRM) mode.

Preparation of Standard Stock and Plasma Samples

The stock solution (50 μg/mL) and working solutions (500, 50 and 5 ng/mL) of fexofenadine were prepared by dissolving an accurately weighed quantity of fexofenadine hydrochloride and serial dilution with methanol. The stock (50 μg/mL) and working solutions (1.0 μg/mL) of IS of glipizide were obtained in the same way. All the solutions were stored at −20°C and brought to room temperature (25 ± 1°C) before use. Drug-free plasma, i.e. control (blank) plasma, was withdrawn from the deep freezer and allowed to completely thaw before use. The calibration standards and quality control (QC) samples (LQC, low-quality control; MQC, medium-quality control; HQC, high-quality control) were prepared by spiking blank plasma with respective working solutions. Calibration standards were made at 1.0, 3.0, 10.0, 30.0, 100, 300 and 600 ng/mL for fexofenadine. Quality controls were prepared at 3.0 ng/mL (LQC), 50.0 ng/mL (MQC) and 400 ng/mL (HQC). The spiked plasma samples at all levels were stored at −65 ± 2°C for validation and analysis.

All frozen samples were allowed to thaw at room temperature and homogenized by vortexing. A 100 μL aliquot plasma sample was transferred to a 1.5 mL centrifuge tube together with 50 μL of IS working solution (1.0 μg/mL). The sample mixture was mixed with 0.45 mL of methanol and vortexed for approximate 3 min, and then allowed to stand for approximate 5 min to deproteinize and the precipitate was removed by centrifugation at 16,000 rpm for 3 min. The supernatant was pipetted into an injected vial and a 5 μL aliquot was injected into the LC-MS/MS system for analysis.

Assay Validation

A full validation according to the FDA guidelines (US DHHS, FDA, CDER, 2001) was performed for the assay in human plasma.

Specificity and selectivity. The specificity of the method was evaluated by analyzing human plasma samples from at least six different sources to investigate the potential interferences at the LC peak region for analyte and IS.

Matrix effect. The matrix effect (ME) was defined as the direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in the sample (Viswanathan *et al.*, 2007). The method of matrix effect was evaluated by comparing the peak area of analytes dissolved in the post-extracted blank plasma solution (A) with that dissolved in mobile phase (B) at equivalent concentrations (Hubert *et al.*, 1999; Dams *et al.*, 2003). The ME was calculated by using the formula: ME (%) = A/B × 100%. Three different concentration levels of fexofenadine at 3.0, 5.0 and 400 ng/mL were evaluated by analyzing five samples at each level. The blank plasma samples used in this study were from five different batches of healthy human blank plasma. If the ratio was <85% or >115%, a matrix effect was implied.

Calibration curve. The calibration curve was acquired by plotting the ratio of sum of peak area of fexofenadine to that of IS against the nominal concentration of calibration standards. The final concentrations of calibration standards obtained for plotting the calibration curve were 1.0, 3.0, 10.0, 30.0, 100, 300 and 600 ng/mL. The results were fitted to linear regression analysis using $1/x^2$ as the weighting factor. The calibration curve had to have a correlation coefficient (r) of 0.99 or better. The acceptance criteria for each back-calculated standard concentration were 15% deviation from the nominal value except at the LLOQ, which was set at 20% (US DHHS *et al.*, 2001).

Precision and accuracy. The intra-batch assay precision and accuracy were estimated by analyzing five replicates at three different QC levels, i.e. 3.0, 50.0 and 400 ng/mL. The inter-batch precision and accuracy were determined by analyzing the three levels QC samples on three different runs. The accuracy is the degree of closeness of the determined value to the nominal true value under prescribed conditions. Accuracy is defined as the relative deviation in the calculated value (E) of a standard from that of its true value (T) expressed as a percentage [RE(%)]. It was calculated using the formula: RE (%) = $(E - T)/T \times 100$. The criteria for acceptability of the data included accuracy within 15% standard deviation (SD) from the nominal values and a precision of within 15% relative standard

deviation (RSD), except for at the LLOQ, where it should not exceed 20% of accuracy as well as precision (US DHHS *et al.*, 2001).

Stability experiments. The stability of fexofenadine and IS in the injection solvent was determined periodically by injecting replicate preparations of processed samples up to 10 h (in the autosampler, 10 ± 1°C) after the initial injection. The concentration of the fexofenadine obtained at 0 h on day 1 was used as the reference to determine the relative stability of the analyte at subsequent points. In all stability studies three QC concentrations were used, LQC, MQC and HQC. The stability of fexofenadine in human plasma during 6 h exposure at bench-top (25 ± 1°C) was determined five replicates at each concentration. The freezer stability of fexofenadine in human plasma was assessed by analyzing the QC samples stored at −65 ± 2°C for 30 days. The freeze–thaw stability of fexofenadine in human plasma was assessed using QC samples following three cycles. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (± 15% SD) and precision (± 15% RSD).

Pharmacokinetic Study in Humans

The volunteers had the following clinical characteristics (expressed as mean ± SD [range]): age, 23.2 ± 2.3 [18–27]; height, 168.1 ± 7.4 cm [157–180]; and body weight, 60.5 ± 8.8 kg [49–75]. Twenty healthy volunteers including 10 males and 10 females were randomly divided into two groups, group A and group B. Each group was made up of five males and five females. They were selected after passing a clinical screening procedure including a physical examination and laboratory tests, which included hematology, blood biochemistry and urine analysis. No volunteers had a history or evidence of a renal, gastrointestinal, hepatic or hematologic abnormality or any acute or chronic disease, or an allergy to any drugs. This was done to ensure that the existing degree of variation would not be due to an influence of illness or other medications. All volunteers avoided using other drugs for at least 2 weeks prior to the study and until after its completion. This study was performed according to the revised Declaration of Helsinki for biomedical research involving human subjects and the rules of Good Clinical Practice (GCP). The protocol of this study was approved by the ethics committee of Nanjing First Hospital of Nanjing Medical University (Nanjing, China). All participants signed a written informed consent after they had been informed of the nature and details of the study. Volunteers were hospitalized at 9:00 p.m. one day before this study and fasted 10 h before each drug administration. At 8:00 a.m., groups A and B were administered a single dose of fexofenadine 60 and 120 mg with 250 mL water, respectively. Heparinized blood samples (5 mL) were collected from a suitable forearm vein using an indwelling catheter into heparin-containing tubes before (0 h) and 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 11, 15, 24, 36 and 48 h after dosing. The blood samples were centrifuged at 4500 rpm for 10 min, and plasma samples were separated and stored at −65 ± 2°C until required for analysis. An 100 µL thawed plasma sample was spiked with IS and processed as described in the Sample Preparation section. Along with study samples, QC samples at low, medium and high concentration were assayed in duplicate and were distributed among unknown samples in the analytical run. The criteria for acceptance of the analytical runs encompassed the following: (i) not more than 33% of the QC samples were greater than ±15% of the nominal concentration; (ii) not less than 50% at each QC concentration level met the acceptance criteria. Plasma concentration–time data of fexofenadine were analyzed using the Drug and Statistics software (version 2.0, Anhui, China).

Results and discussion

Method development

Sample preparation is usually required for the determination of pharmaceuticals in biological samples owing to complex matrices in order to remove possibly interfering matrix components and increase the selectivity and sensitivity. SPE is a widely adopted method and often achieves clean analytes from biological samples (Robbins *et al.*, 1998; Hofmann *et al.*, 2002; Unoa *et al.*, 2004; Isleyen *et al.*, 2007; Miura *et al.*, 2007; Nirogi *et al.*, 2007; Bharathi *et al.*, 2008; Pathak *et al.*, 2008; Pathak *et al.*, 2008), but the technique is time-consuming and the SPE columns are relatively expensive. In the present work, protein precipitation with methanol was used for the extraction of fexofenadine from human plasma, which produced a clean chromatogram for a drug-free plasma sample and offered satisfactory absolute recoveries for the analyte. An Agilent Zorbax SB-C_{18} column (5 µm, 100 × 2.1 mm) was used for the chromatographic separation. For mobile phase, a mix of methanol : buffer (containing 10 mmol/L ammonium acetate and 0.1% formic acid; 70 : 30, v/v) at a flow rate of 0.20 mL/min was found to be optimal for the study, which provided symmetric peak shapes of the analytes and the IS as well as a short run time (*ca* 3 min). For the selection of the IS, similar extracted recovery and retention times were the significant factors. Several compounds (pioglitazone, cetirizine and gliclazide) were tried and glipizide was finally used as the IS in this work. A preliminary series of experiments indicated that fexofenadine and IS were very responsive to LC-MS/MS with positive ion detection.

Mass Spectrometry

As fexofenadine is a basic compound, ESI in positive ion mode was adopted for the LC-MS/MS determination of fexofenadine. During ionization, fexofenadine formed protonated molecules in the mobile phase containing methanol : buffer (containing 10 mmol/L ammonium acetate and 0.1% formic acid; 70 : 30, v/v). The optimum CID of −2 V was selected after testing values between −1 and −10 V. We investigated the collision energies of fexofenadine and glipizide. Finally, we selected 25 eV as the collision energy for fexofenadine and 15 eV for glipizide. Under CID and collision energies, the parent ion of fexofenadine *m/z* 502.1 gave a product ion at *m/z* 466.2 and glipizide at *m/z* 446.0 → 321.1. The mass spectra of fexofenadine and glipizide are shown in Figs 2 and 3.

Selectivity, Calibration Curve and Lower Limits of Quantification

Under the present chromatographic conditions, the retention time was approximately 1.9 min for fexofenadine and 2.1 min for the IS. Representative chromatograms are shown in Fig. 4. All samples were found to have no interferences at the retention times of the analytes. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighting factors ($1/x$, $1/x^2$ and none). The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/x^2$ weighting factor, giving a mean linear regression equation for the calibration curve of fexofenadine: $y = 0.0043\ (\pm 0.0001)\ x - 0.0001\ (\pm 0.0005)$ where y was the peak area ratio of fexofenadine to the IS and x was the concentration of fexofenadine. The mean correlation coefficient of the weighted calibration curve generated during the validation for fexofenadine was 0.9976 ± 0.0015; Table 1 summarized the calibration curve results.

Matrix Effect

Three different concentration levels of fexofenadineat 3.0, 5.0 and 400 ng/mL were evaluated by analyzing five samples at each

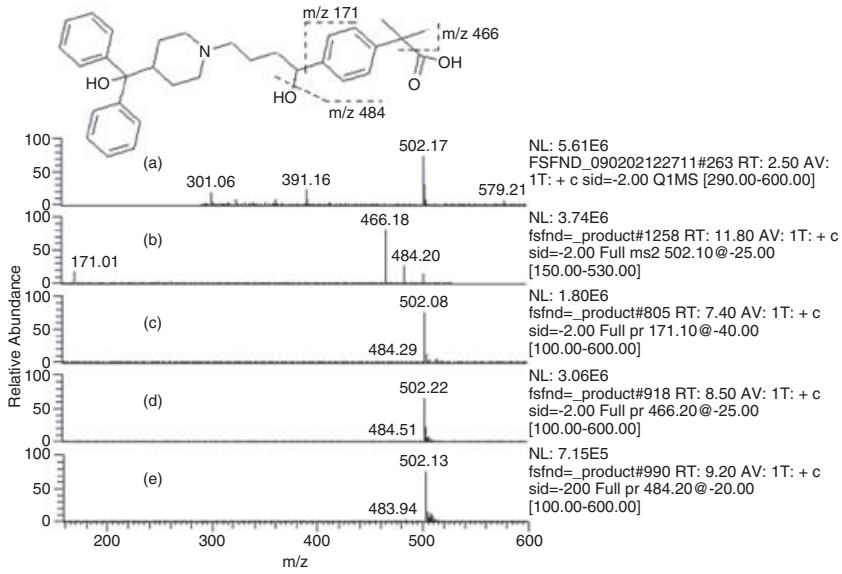


Figure 2. Fexofenadine mass spectrum of (a) full-scan mode; (b) product scan mode; (c–e) precursor scan mode.

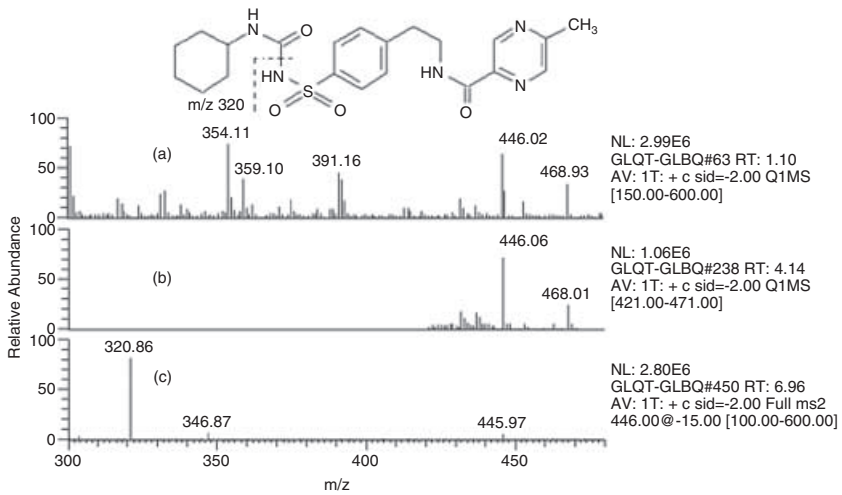


Figure 3. Glipizide (IS) mass spectrum of (a, b) full-scan mode; and (c) product scan mode.

level. The matrix effects of glipizide were evaluated by analyzing five samples at one level. The blank plasmas used in this study were from six individual of healthy human blank plasma. The ME data at three concentration levels of fexofenadine in five different batches of human plasma are presented in Table 2. The results showed that there was no matrix effect of the analytes observed from the matrix of plasma in this study.

Precision and Accuracy

The intra- and inter-batch precision and accuracy are summarized in Table 3. The results demonstrate that the precision and accuracy of this assay are within the acceptable range.

Absolute Recovery

Protein precipitation efficiency of methanol and acetonitrile was compared. The absolute recovery value of fexofenadine from human plasma with methanol was higher than acetonitrile. Methanol was chosen as the precipitant for its better deproteinization efficiency. The absolute recovery of fexofenadine was evaluated at three different concentration levels of 3.0, 50.0 and 400 ng/mL, respectively. The results of recovery studies are shown in Table 4.

Stability

The stability of fexofenadine was studied under a variety of storage and handling conditions. The results in Table 5 showed that no significant degradation of fexofenadine was observed under the tested conditions.

Application

The method was successfully applied to determine the plasma concentration of fexofenadine after groups A and B were administered respectively a single dose of 60 and 120 mg fexofenadine.

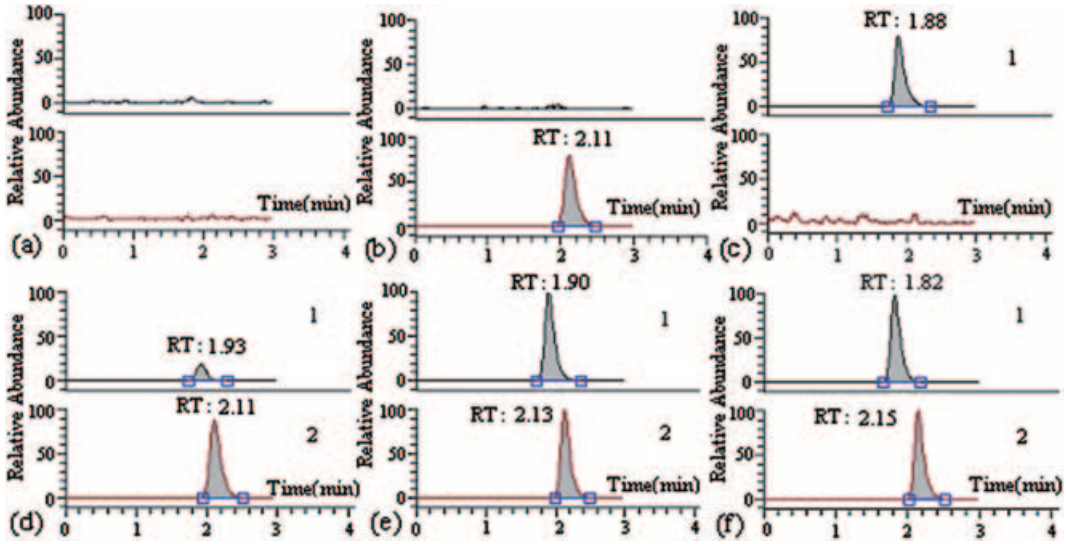


Figure 4. Typical LC-MS/MS chromatograms: (a) blank plasma; (b) IS references; (c) fexofenadine references; (d) LLOQ for fexofenadine (1.0 ng/mL); (e) plasma sipked with fexofenadine (100.0 ng/mL); and (f) plasma obtained from a volunteer at 3 h after oral administration 60 mg dose of fexofenadine (101.2 ng/mL) (1, fexofenadine; 2, IS).

Table 1. Precision and accuracy data of back-calculated concentrations of calibration samples for fexofenadine in human plasma ($n = 5$)

Nominal concentration (ng/mL)	Found concentration (mean ± SD) (ng/mL)	RE (%)	RSD (%)
1.0	1.02 ± 0.02	2.4	2.3
3.0	2.84 ± 0.21	–5.3	7.3
10	9.41 ± 0.16	–5.9	1.7
30	29.78 ± 0.27	–0.7	0.9
100	91.98 ± 1.8	–8.0	2.0
300	321.68 ± 5.4	7.2	1.7
600	644.28 ± 15.1	7.4	2.3

n, number of replicates; SD, standard deviation; RE, relative error; RSD, relative standard deviation.

Table 2. Matrix effects evaluation of fexofenadine and glipizide in human plasma ($n = 5$)

Samples	Nominal concentration (ng/mL)	ME (%)	RSD (%)
Fexofenadine	3.0	106.6	5.6
	50.0	105.8	4.9
	400	108.8	5.3
Glipizide	100	103.6	5.9

n, number of replicates; ME, matrix effect; RSD, relative standard deviation.

Table 3. Accuracy and precision for the analysis of fexofenadine in human plasma (three batches, five replicates per batch)

Analytes	Nominal concentration (ng/mL)	Found concentration (ng/mL)	RE (%)	Intra-batch RSD (%)	Inter-batch RSD (%)
Fexofenadine	3.0	3.04	1.4	8.5	8.2
	50.0	45.1	–9.8	3.4	4.9
	400	410.7	2.7	7.4	1.8

RE, relative error; RSD, relative standard deviation.

Table 4. Absolute recovery data of fexofenadine in human plasma ($n = 5$)

Analytes	Nominal concentration (ng/mL)	Absolute recovery (%) (mean ± SD)	RSD (%)
Fexofenadine	3.0	87.6 ± 5.4	4.8
	50.0	92.5 ± 3.2	3.0
	400	93.6 ± 2.6	2.4

n, number of replicates; SD, standard deviation; RSD, relative standard deviation.

Table 5. Stability of fexofenadine in human plasma under various conditions ($n = 3$)

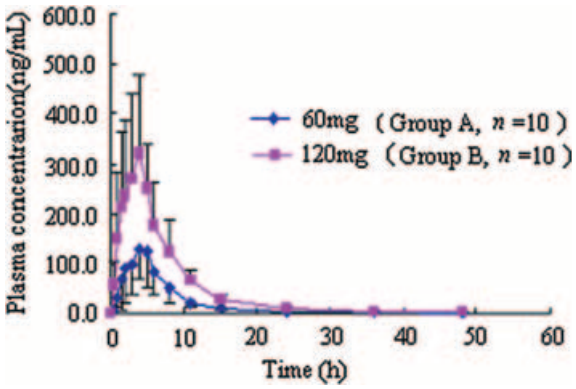
Nominal concentration (ng/mL)	Stability conditions	Found concentration (mean ± SD) (ng/mL)	RE (%)	RSD (%)
3.0	0 h (for all)	3.12 ± 0.21	4.0	6.6
	6 h at bench-top	3.14 ± 0.27	4.8	8.5
	10 h at autosampler	3.40 ± 0.03	13.5	0.8
	3 freeze–thaw cycles	3.39 ± 0.13	13.0	3.9
	30 days at –65 ± 2°C	3.05 ± 0.04	1.6	1.2
50.0	0 h (for all)	46.55 ± 0.38	–6.9	0.8
	6 h at bench-top	52.11 ± 1.13	4.2	2.2
	10 h at autosampler	49.53 ± 0.23	–0.9	0.5
	3 freeze–thaw cycles	46.90 ± 0.66	–6.2	1.4
	30 days at –65 ± 2°C	51.30 ± 0.37	2.6	0.7
400	0 h (for all)	407.87 ± 5.6	2.0	1.4
	6 h at bench-top	412.58 ± 4.6	3.1	1.1
	10 h at autosampler	442.48 ± 6.5	10.6	1.5
	3 freeze–thaw cycles	406.41 ± 16.1	1.6	4.0
	30 days at –65 ± 2°C	415.06 ± 9.5	3.8	2.3

n, number of replicates; SD, standard deviation; RE, relative error; RSD, relative standard deviation.

Table 6. Pharmacokinetic parameters of fexofenadine after the administration of an oral single dose of 60 or 120 mg in 10 healthy volunteers (groups A and B)

Pharmacokinetic parameters	Administration 60 mg (mean ± SD, $n = 10$)	Administration 120 mg (mean ± SD, $n = 10$)
$T_{1/2}$ (h)	11.14 ± 4.95	9.29 ± 3.61
T_{max} (h)	4.10 ± 0.88	3.58 ± 1.17
C_{max} (ng/mL)	152.62 ± 74.18	365.98 ± 168.02
$AUC_{(0-48)}$ (ng h/mL)	961.17 ± 409.97	2404.3 ± 885.5
$AUC_{(0-\infty)}$ (ng h/mL)	978.19 ± 411.06	2437.5 ± 885.9
$MRT_{(0-48)}$ (h)	8.24 ± 0.91	7.95 ± 1.01
$MRT_{(0-\infty)}$ (h)	9.42 ± 1.46	8.79 ± 1.84
CL (L/h)	72.24 ± 31.01	55.64 ± 21.32
V (L)	1189.86 ± 829.5	737.92 ± 376.49

n, number of healthy volunteers; SD, standard deviation; $T_{1/2}$, elimination half-life; T_{max}, time to C_{max}; C_{max}, peak concentration; AUC, area under plasma concentration–time curve; MRT, mean residence time; *CL*, clearance; *V*, volume of distribution.



Profiles of the mean plasma concentration–time are depicted in Fig. 5. Pharmacokinetic parameters of fexofenadine are shown in Table 6.

Conclusions

The current method has shown acceptable precision and adequate sensitivity for the quantification of fexofenadine in human

Figure 5. Plasma concentration–time profile of fexofenadine after the administration of an oral single dose of 60 or 120 mg in 10 healthy volunteers (groups A and B). Each point represents a mean + SD or –SD ($n = 10$).

plasma. The method consisted of sample preparation by protein precipitation, followed by chromatographic separation and ESI-MS/MS detection. The rapid extraction method and sample turnover rate of approximately 3 min make it an attractive procedure in high-throughput bioanalysis of fexofenadine. The method was successfully applied to quantify the concentration–time profiles of fexofenadine in a clinical pharmacokinetic study.

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