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ORIGINAL RESEARCH

Quantification of fexofenadine in human plasma by liquid chromatography coupled to electrospray tandem mass spectrometry using mosapride as internal standard

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ABSTRACT: A rapid high-performance liquid chromatography/positive ion electrospray tandem mass spectrometry method was developed and validated for the quantification of fexofenadine in human plasma using mosapride as internal standard. Following solid-phase extraction, the analytes were separated using an isocratic mobile phase on a reverse-phase column and analyzed by MS/MS in the multiple reaction monitoring mode using the respective $[M+H]^+$ ions, *m/z* 502/466 for fexofenadine and *m/z* 422/198 for the IS. The method exhibited a linear dynamic range of 1–500 ng/mL for fexofenadine in human plasma. The lower limit of quantification was 1 ng/mL with a relative standard deviation of less than 5% for fexofenadine. Acceptable precision and accuracy were obtained for concentrations over the standard curve range. The total chromatographic run time of 2 min for each sample made it possible to analyze more than 400 human plasma samples per day. The validated method has been successfully used to analyze human plasma samples for application in pharmacokinetic, bioavailability or bioequivalence studies. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: fexofenadine; electrospray ionization; tandem mass spectrometry; human plasma; pharmacokinetic study

INTRODUCTION

The recently introduced H-1 receptor antagonist fexofenadine (Fig. 1) belongs to the second-generation antihistamines. It is approved for treatment of seasonal allergic rhinitis and urticaria. The probability that cardiotoxic side effects occur in connection with fexofenadine is assessed as being extremely low (Simpson and Jarvis, 2000). Fexofenadine is a non-impairing and non-sedating even when administered at doses up to twice that recommended (Hindmarch, 2002). *In vitro* results have shown that fexofenadine is transported by various mechanisms across biological membranes, including transcellular passive diffusion and, to some extent, P-glycoprotein (ABCB1) and organic anion transporting polypeptides (OATPs) (Cvetkovic *et al.*, 1999; Dresser *et al.*, 2002; Kobayashi *et al.*, 2003; Nozawa *et al.*, 2004; Perloff *et al.*, 2002; Petri *et al.*, 2004). Several clinical studies have reported drug–drug and drug–diet interactions between fexofenadine and substrates or inhibitors of both these transporters (Dresser *et al.*, 2002; Davit *et al.*, 1999; Hamman *et al.*, 2001; Tannergren *et al.*, 2003; Wang *et al.*, 2002).

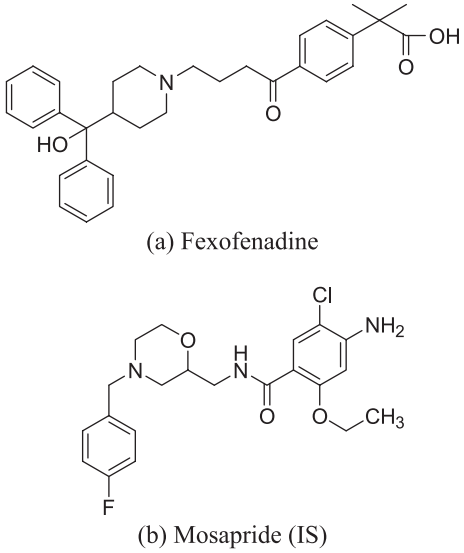

Figure 1. Chemical structures for fexofenadine and IS (mosapride).

The bioanalytical component of a pharmacokinetic study requires a drug assay with simplicity, selectivity,

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Abbreviations used: APCI, atmospheric pressure chemical ionization; OATPs, organic anion transporting polypeptides.

sensitivity, small volume requirements and rapid turnaround time. Several methods for the quantification of fexofenadine in biological fluids have been described. Most of these methods have been based on high-performance liquid chromatography (HPLC) using fluorescence detection (Coutant *et al.*, 1991; Surapaneni and Khalil, 1994; Uno *et al.*, 2004; Robbins *et al.*, 1998). In these HPLC methods, the chromatographic run time was long and/or the sensitivity was inadequate for pharmacokinetic studies. A liquid chromatography–mass spectrometry (LC-MS) method with an improved lower limit of quantification (LLOQ) of 0.5 ng/mL was reported (Hofmann *et al.*, 2002). However, this method required chromatography under gradient conditions. In addition, the run time of this method was 10 min, which is relatively long for an HPLC-MS procedure. Recently, a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method utilizing atmospheric pressure chemical ionization (APCI) has been described (Fu *et al.*, 2004); this method was applied to determine fexofenadine in human plasma after solid-phase extraction of 500 µL samples, achieving linearity over a 1–200 ng/mL concentration range with an LLOQ of 1 ng/mL. However, most laboratories are not equipped with APCI, and deuterated internal standard is rarely commercially available. The total runtime was also long (4 min).

The present paper describes the development and validation of an LC-electrospray ionization (ESI)-MS/MS method using a commercially available compound mosapride as internal standard, and the sample turnaround time of 2 min makes it an attractive procedure in high-throughput bioanalysis of fexofenadine.

EXPERIMENTAL

Chemicals. Fexofenadine hydrochloride drug substance was obtained from Wockhardt Research Center (Aurangabad, India) and mosapride citrate dihydrate (internal standard, IS) was obtained from Torrent Research Ltd (Ahmedabad, India). Chemical structures are presented in Fig. 1. HPLC-grade LiChrosolv methanol and LiChrosolv acetonitrile were purchased from Merck (Darmstadt, Germany). Formic acid was purchased from Merck (Worli, Mumbai, India). HPLC-grade water from a Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were analytical grade.

LC/MS/MS instrument and conditions. The HPLC SIL HTC system (Shimadzu Corporation, Kyoto, Japan) was equipped with an LC-AD VP binary pump, a DGU20A5 degasser and an SIL-HTC auto sampler equipped with a CTO-10AS VP thermostated column oven. The chromatography was performed using Waters symmetry® C_{18} (5 µm, 150 × 4.6 mm i.d.) at 30°C temperature. The isocratic mobile phase composition was a mixture of 0.03% formic acid–acetonitrile (40:60, v/v), which was pumped at a flow-rate of 1.0 mL/min with a split ratio of load to waste of 10:90.

Mass spectrometric detection was performed on an API 3000 triple quadrupole instrument (MDS-SCIEX, Concord, Ontario, Canada) using multiple-reaction monitoring (MRM). A turboionspray interface operating in positive ionization mode was used. Typically, setting source conditions were as follows: the turbo-gas temperature was set at 250°C and the ion spray needle voltage was adjusted at 5000 V. The common parameters, viz., nebulizer gas, curtain gas and collision gas, were set at 9, 6 and 3, respectively. The mass spectrometer was operated at unit resolution for both Q1 and Q3 in the MRM mode, with a dwell time of 200 ms per MRM channel. The precursor/product ion pairs monitored were *m/z* 502.2/466.1 for fexofenadine and *m/z* 422.3/198.4 for IS. The collision energy was set at 40 and 30 V for fexofenadine and IS, respectively. Data acquisition was performed with Analyst software (Version 1.4.1).

Sample preparation. Standard stock solutions of fexofenadine (1 mg/mL) and the IS (1 mg/mL) were separately prepared in methanol. Working solutions for calibration and controls were prepared by appropriate dilution in water–methanol (50:50, v/v; diluent). The IS working solution (10 µg/mL) was prepared by diluting its stock solution with diluent. Working solutions (0.2 mL) were added to drug-free human plasma (9.8 mL) as a bulk, to obtain fexofenadine concentration levels of 1, 2, 5, 10, 20, 50, 100, 200 and 500 ng/mL as a single batch at each concentration. Quality control (QC) samples were also prepared as a bulk on an independent weighing of standard drug, at concentrations of 1 (LLOQ), 3 (low), 200 (medium) and 400 ng/mL (high) as a single batch at each concentration. The calibration and control bulk samples were divided into aliquots in micro centrifuge tubes (Tarson, 2 mL) and stored in the freezer at below –50°C until analysis.

A plasma sample (0.5 mL) was pipetted into a microcentrifuge tube and then 25 µL of IS working solution (10 µg/mL) were added. The mixture was vortex-mixed for 10 s and loaded onto an OASIS™ HLB SPE cartridge (30 mg, Waters Co., Milford, USA), which had been conditioned by washing with methanol (1 mL) followed by water (1 mL). The cartridge was rinsed in RapidTrace (Zymark, Hopkinton, MA, USA) with water (1 mL) followed by 10% methanol in water (1 mL), and then eluted with 0.1% formic acid in methanol (0.5 mL). The elutant was evaporated to dryness using an evaporator at 40°C under a stream of nitrogen, the dried extract was reconstituted in mobile phase (250 µL) and a 10 µL aliquot was injected into the chromatographic system.

Bioanalytical method validation. A calibration curve was constructed from a blank sample (a plasma sample processed without the IS), a zero sample (a plasma processed with the IS) and nine non-zero samples covering the total range 1–500 ng/mL for fexofenadine, including the LLOQ. The calibration curves were generated using the analyte to IS peak area ratios by weighted ($1/x$) least-squares linear regression on consecutive days. The acceptance criterion for a calibration curve was a correlation coefficient (r) of 0.99 or better, and each back-calculated standard concentration within 15% deviation from the nominal value except at the LLOQ, for which the maximum acceptable deviation was set at 20%. At least 67% of non-zero standards were required to meet the

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above criteria, including acceptable LLOQ and upper limit of quantification.

The within-batch precision and accuracy were determined by analyzing four sets of QC samples (LLOQ, low, medium and high concentrations) each comprising five replicates in a batch. The between-batch precision and accuracy were determined by analyzing three different such batches. The acceptance criteria for within- and between-batch precision were 20% or better for LLOQ and 15% or better for the other concentrations, and the accuracy was 100 ± 20% or better for LLOQ and 100 ± 15% or better for the other concentrations (Shah *et al.*, 1991).

Recovery of analyte from the extraction procedure was determined by a comparison of the peak area of analyte in spiked plasma samples (five each of low, medium and high QCs) with the peak area of analyte in samples prepared by spiking extracted drug-free plasma samples with the same amounts of analyte at the step immediately prior to chromatography. Similarly, recovery of IS was determined by comparing the mean peak areas of extracted QC samples ($n = 5$) to mean peak areas of IS in samples prepared by spiking extracted drug-free plasma samples with the same amounts of IS at the step immediately prior to chromatography.

The stability of the analyte and IS in human plasma under different temperature and timing conditions, as well as their stability in the stock solutions, was evaluated. QC samples were subjected to short-term room temperature conditions, to long-term storage conditions (–50°C), and to freeze–thaw stability studies. All the stability studies were conducted at two concentration levels (3 and 400 ng/mL as low and high QC values) with five replicates for each.

RESULTS AND DISCUSSION

Mass spectrometry

Pharmacokinetic applications require highly selective assays with high sample throughput capacity. Quantification of drugs in biological matrices by LC-MS/MS is becoming more common due to the improved sensitivity and selectivity of this technique. The product ion mass spectra of fexofenadine and the IS are shown in Fig. 2. $[M + H]^+$ was the predominant ion in the Q1 spectrum and was used as the precursor ion to obtain product ion spectra. The product ion mass spectrum of fexofenadine shows the formation of characteristic product ions at *m/z* 131, 171, 189, 233, 262, 466 and 484. The two most prominent ions (*m/z* 484 and 466) resulted from the loss of either one or two fragments with *m/z* 18, most likely attributable to the loss of one or two molecules of water from the parent ion. The product ion mass spectrum of IS shows the formation of characteristic product ions at *m/z* 170 and 198. The most sensitive mass transition was from *m/z* 502 to *m/z* 466 for fexofenadine and from *m/z* 422 to *m/z* 198 for the IS.

Method development. Choosing the appropriate internal standard is important to achieve acceptable method performance, especially with LC-MS/MS, where matrix effects can lead to poor analytical results. Ideally, isotopically labeled internal standards for all analytes should be used, but these are not commercially available. Several compounds were investigated to find a suitable IS, and finally mosapride was found to be suitable for the present purpose (Table 1). Clean chromatograms were obtained and no significant direct interferences in the MRM channels at the relevant retention times were observed. However, in ESI, signal suppression or enhancement may occur due to co-eluting endogenous components of the sample matrix. The importance of including the evaluation of matrix effect in any LC-MS/MS method is outlined in an excellent publication by Matuszewski *et al.* (2003). Their data strongly emphasize the need to use a blank matrix from (at least five) different sources/individuals instead of using one blank matrix pool to determine method precision and accuracy. Therefore, all validation experiments in this assay were performed with matrixes obtained from different individuals. In addition, validation experiments were performed using hemolytic and strongly lipemic matrixes. As all data falls within the guidelines, we conclude that the degree of matrix effect was sufficiently low to produce acceptable data, and the method can be considered as valid.

The chromatographic conditions, especially the composition of mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes for the analyte and IS, as well as a short run time. It was found that a mixture of 0.03% formic acid–acetonitrile (40:60, v/v) could achieve this purpose and was finally adopted as the mobile phase. The

Table 1. Matrix effect data for fexofenadine in human plasma

Concentration (ng/mL) $n = 5$	Peak area of fexofenadine		Peak area of IS		Peak area ratio of fexofenadine/IS		Matrix effect (%)
	Set 1	Set 2	Set 1	Set 2	Set 1	Set 2	
3	1.4	1.3	34.2	34.5	0.09	0.09	92.1
400	182	191	34.5	36.1	2.14	2.17	100.2

Set 1: Fexofenadine standard in water-methanol (1:1); set 2: fexofenadine spiked into post-extracted blank plasma; matrix effect expressed as the ratio of the mean peak area of fexofenadine spiked post-extraction (set 2) to the mean peak area of the fexofenadine standard (set 1) multiplied by 100.

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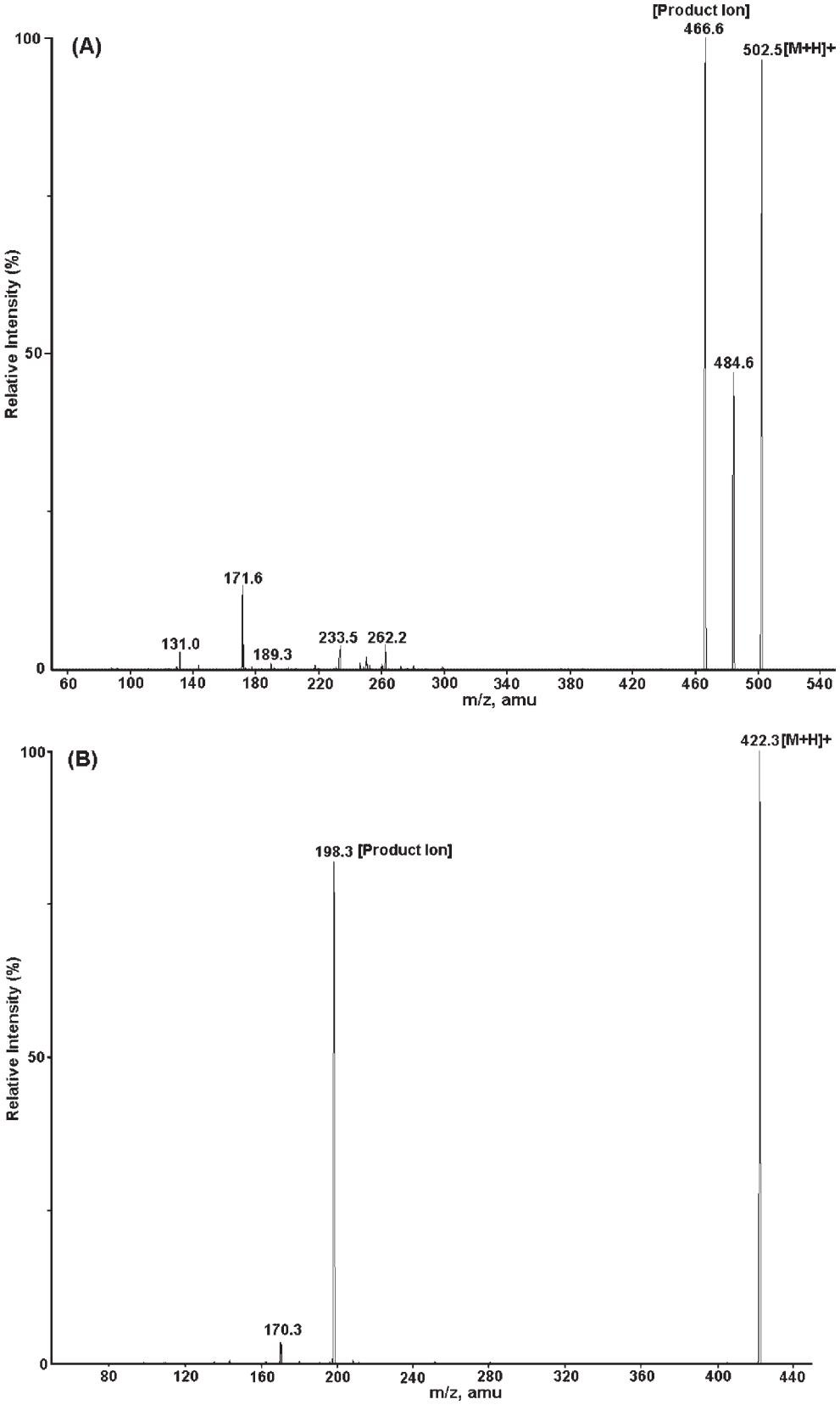

Figure 2. Full scan positive ion turboIonspray product ion mass spectra of (a) fexofenadine and (b) mosapride (internal standard).

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formic acid was found to be necessary in order to lower the pH to protonate the analytes and thus deliver good peak shape. The percentage of formic acid was optimized to maintain this peak shape while being consistent with good ionization and fragmentation in the MS. The high proportion of organic solvent eluted the fexofenadine and the IS at retention times of 1.06 and 1.08 min. A flow rate of 1.0 mL/min produced good peak shapes and permitted a run time of 2.0 min.

The solid-phase extraction procedure described here offers a rapid way to isolate the analyte from the plasma matrix. The technique provides clean injection extracts and was shown here to be robust. Several solid-phase packing materials from various manufacturers were tested for the present application, and the OASIS™ HLB cartridge was found to meet the criteria of clean injection extracts and high recovery. The average absolute recovery of fexofenadine from spiked plasma samples was 58.2 ± 1.5%. The recovery of the IS was 57.0 ± 2.4% at the concentration used in the assay (10 µg/mL). Recoveries of the analyte and IS were good, and it was consistent, precise and reproducible. Therefore, the assay has proved to be robust in high throughput bioanalysis.

Assay performance and validation. The nine-point calibration curve was linear over the concentration range 1–500 ng/mL for fexofenadine. 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 $1/x$ weighting factor, giving a mean linear regression equation for the calibration curve of fexofenadine:

$$y = 0.0024(\pm 0.0002)x + 0.0004(\pm 0.0005)$$

where y was the peak area ratio of the analyte to the IS and x was the concentration of the analyte. The mean correlation coefficient of the weighted calibration curve generated during the validation for fexofenadine was 0.9996 ± 0.0001; Table 2 summarizes the calibration curve results.

The selectivity of the method was examined by analyzing ($n = 5$) blank human plasma extract [Fig. 3(A)] and an extract spiked only with the IS [Fig. 3(B)]. As shown in Fig. 3(A), no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free human plasma at the retention time of the analyte. Similarly, Fig. 3(B) shows the absence of direct interference from the IS to the MRM channel of the analyte. Figure 3(C) depicts a representative ion-chromatogram for the LLOQ (1 ng/mL). Excellent sensitivity was observed for a 10 µL injection volume; the LLOQ corresponds to ca. 10 on-column.

The LLOQ was defined as the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision, and was found to be 1 ng/mL in human plasma. The mean response for the analyte peak at the assay sensitivity limit (1 ng/mL) was ~17-fold greater than the mean response for the peak in six blank human plasma samples at the retention time of the analyte. The between-batch CV at the LLOQ was 3.5%, and the between-batch accuracy was 99.6% (Table 3). The within-batch CV was 4.7% and the accuracy was 98.2%.

Table 2. Precision and accuracy data of back-calculated concentrations of calibration samples for fexofenadine in human plasma

Concentration added (ng/mL)	Concentration found (mean ± SD $n = 5$) (ng/mL)	Precision (%)	Accuracy (%)
1	1.04 ± 0.05	4.6	104.5
2	1.97 ± 0.06	3.0	98.5
5	4.9 ± 0.1	1.9	98.4
10	10.2 ± 0.3	2.7	102.5
20	20.4 ± 0.8	3.8	102.0
50	50.5 ± 1.8	3.6	101.1
100	100.4 ± 2.6	2.6	100.4
200	197.6 ± 5.5	2.8	98.8
500	504.4 ± 7.8	1.6	100.9

Table 3. Precision and accuracy of the method for determining fexofenadine concentrations in plasma samples

	Within-batch ($n = 5$)			Between-batch ($n = 3$)		
Concentration added (ng/mL)	Concentration found (mean ± SD) (ng/mL)	Precision (%)	Accuracy (%)	Concentration found (mean ± SD) (ng/mL)	Precision (%)	Accuracy (%)
1	0.99 ± 0.05	4.7	98.2	1.00 ± 0.03	3.5	99.6
3	3.1 ± 0.2	6.9	103.1	3.0 ± 0.1	4.3	99.7
200	212.0 ± 7.2	3.4	106.0	205.8 ± 8.9	4.3	102.9
400	433.3 ± 20.4	4.7	108.3	409.4 ± 28.0	6.8	102.3

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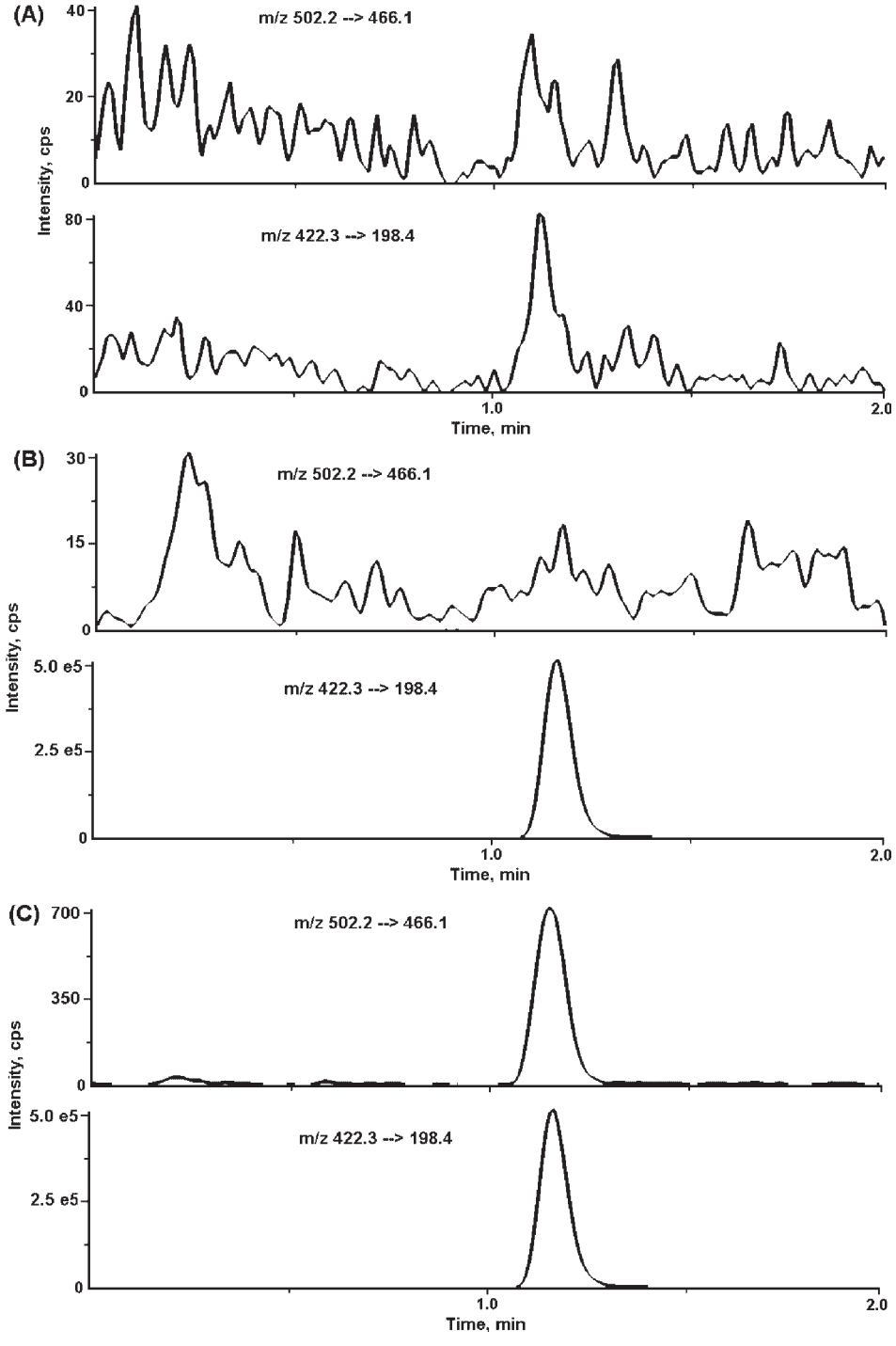

Figure 3. MRM chromatograms for fexofenadine and the IS resulting from analysis of: (a) blank (drug and IS free) human plasma; (b) zero sample (drug-free spiked with IS) human plasma; (c) 1 ng/mL (LLOQ) spiked with the IS.

The middle and upper quantification levels of fexofenadine ranged from 1 to 400 ng/mL in human plasma. For the between-batch experiments, the CV ranged from 4.3 to 6.8% and the accuracy from 99.7 to 102.9% (Table 3). For the within-batch experiments, the CV and accuracy for the analyte met the acceptance criteria (<±15%).

Stability studies. For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeding that expected to be encountered during routine sample preparation (around 24 h). Samples were extracted and analyzed as described above and the results indicate reliable stability behavior under the experimental

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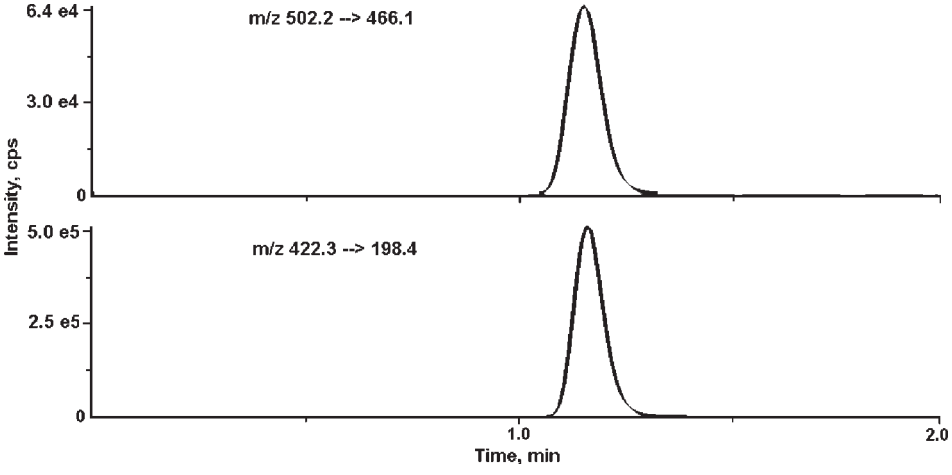

Figure 4. MRM chromatograms resulting from the analysis of a subject plasma sample after the administration of fexofenadine 60 mg single oral dose. The sample concentration was determined to be 78 ng/mL.

conditions of the regular analytical procedure. The stability of QC samples kept in the autosampler for 25 h was also assessed. The results indicate that solutions of the analyte and the IS can remain in the autosampler for at least 25 h without showing significant loss in the quantified values, indicating that samples should be processed within this period of time.

The stability data of the analyte in plasma over three freeze–thaw cycles indicate that the analyte is stable in human plasma for three freeze–thaw cycles, when stored at below –50°C and thawed to room temperature.

The long-term stability data of the analyte in human plasma stored for a period of 21 days at below –50°C showed reliable stability behavior, as the mean of the results of the tested samples were within the acceptance criteria of ±15% of the initial values of the controls. These findings indicate that storage of the analytes in plasma samples at below –50°C is adequate, and no stability-related problems would be expected during routine analyses for pharmacokinetic, bioavailability or bioequivalence studies.

The stability of the stock solutions was tested and established at room temperature for 4 and 21 h, and under refrigeration (~4°C) for 21 days (data not shown). The results revealed optimum stability for the prepared stock solutions throughout the period intended for their daily use.

Application. The present method allowed for the quantification of fexofenadine in human plasma in pharmacokinetic studies. The MRM chromatograms obtained for an extracted plasma sample of a healthy subject who participated in a pharmacokinetic study conducted on 24 subjects are depicted in Fig. 4. Fexofenadine was quantified as 78 ng/mL. Figure 5 shows the mean plasma concentration–time profile of fexofenadine after an oral dose of 60 mg in six healthy subjects.

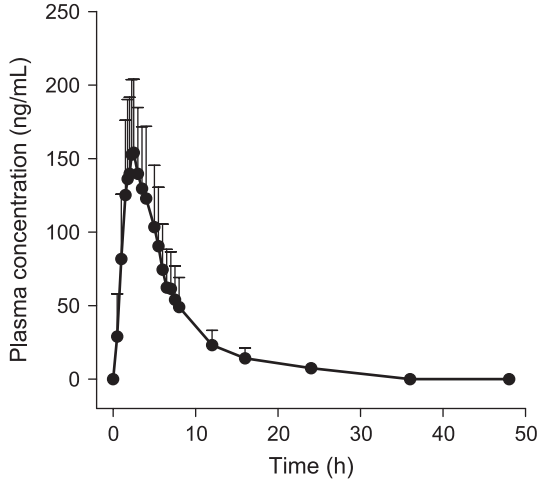

Figure 5. Representative data showing mean plasma concentration-time profile of fexofenadine after the administration of an oral single dose of 60 mg in six healthy subjects.

CONCLUSIONS

In summary, a method is described for the quantification of fexofenadine in human plasma by LC-MS/MS in positive electrospray ionization mode using a commercially available compound mosapride as internal standard and fully validated according to commonly accepted criteria (Shah *et al.*, 1991). The current method has shown acceptable precision and adequate sensitivity for the quantification of fexofenadine in human plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies. The rapid extraction method

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and sample turnover rate of 2 min per sample 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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