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Determination of fexofenadine in human plasma using 96-well solid phase extraction and HPLC with tandem mass spectrometric detection

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Abstract

A fast and sensitive HPLC–MS/MS method, utilizing atmospheric pressure chemical ionization, for the determination of fexofenadine in human plasma is described. A deuterated analog, d_6-fexofenadine is used as the internal standard (IS). Plasma samples are prepared using 96-well solid phase extraction with plates containing Waters Oasis HLB sorbent. The analytes are chromatographed on a Restek Ultra IBD column (3.2 mm × 50 mm, 3 μm) using a mobile phase consisting of a mixture of 90% acetonitrile and 10% 10 mM ammonium acetate buffer and 0.1% formic acid. Quantitation of the analyte is based on the response from the multiple reaction monitoring of the precursor to product ion pairs for fexofenadine (m/z 502 → 466) and d_6-fexofenadine (m/z 508 → 472). The assay has been validated over the concentration range of 1–200 ng/ml based on the analysis of 0.5 ml aliquots of plasma. Within-day assay accuracy was between 97 and 102% of nominal, while within-day precision was better than 3.5% CV at all points on the standard curve. Analyte extraction recovery was better than 70% over the range of the standard curve. The method was found to be suitable for the analysis of human plasma samples obtained 24 h following the administration of a single 60 mg dose of fexofenadine.

Keywords: Fexofenadine; P-glycoprotein; Antihistamine; HPLC; Mass spectrometry; Plasma

1. Introduction

P-glycoprotein (P-gp), a transmembrane protein, is an ATP-dependent efflux transporter that is increasingly recognized for its involvement in the disposition of many drugs [1,2]. Intestinal P-gp, which is believed to function in a protective role in the body, pumps xenobiotics back to the gut lumen, thus reducing drug absorption from the gastrointestinal tract. The presence of P-gp in the blood–brain barrier prevents substrate drugs from entering the central nervous system.

Modification of P-gp activity, by induction or inhibition, has been reported to be the mechanism responsible for several drug–drug interactions [3,4].

Fexofenadine (Fig. 1), a non-sedating antagonist of the histamine H1-receptor, is known to be a sub-

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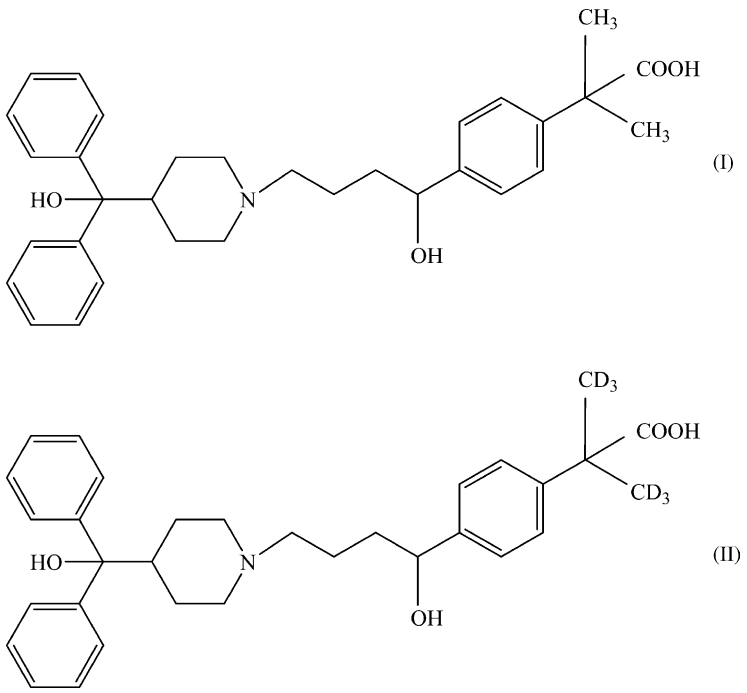

Fig. 1. Chemical structures of fexofenadine (I) and d_6-fexofenadine (II).

strate of P-gp [5,6]. Fexofenadine undergoes minimal metabolism in vivo [5], thus making it a potential probe for investigating the effect of co-administered drug candidates on intestinal P-gp. For example, the increased plasma levels of fexofenadine that resulted following co-administration with erythromycin or ketoconazole [7,8] were attributed to the inhibition of intestinal P-gp by the co-administered compounds.

To support pharmacokinetic studies in which a single dose of fexofenadine is administered as a probe of P-gp activity, a sensitive, rapid and reliable method for quantitating fexofenadine in human plasma is required. Published methods using fluorescence detection [9,10] have limits of quantitation (LOQ) at about or above 5 ng/ml which may not be sufficiently sensitive to support single dose studies. An LC–MS assay with an improved LOQ of 0.5 ng/ml was recently reported [11]. However, this method required chromatography under gradient conditions. In addition, the run-time of this assay was 10 min, which is relatively long for an HPLC–MS procedure. Finally, the sample preparation utilized discrete C_{18} SPE cartridges, a procedure that is not generally considered high-throughput or amenable to automation.

Presented here is a simple and rapid HPLC–MS/MS assay for the determination of fexofenadine in human plasma. Half milliliter aliquots of plasma are prepared using solid phase extraction in the 96-well format, a technique that is much more efficient than the use of individual extraction cartridges. Use of this methodology permits the extraction of 96 samples in under 1 h. Extracted samples are analyzed under isocratic HPLC conditions. The run time is 4 min and the LOQ of the assay is 1.0 ng/ml.

2. Experimental

2.1. Materials

Fexofenadine and d_6-fexofenadine were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Acetonitrile, methanol (Omnisolv HPLC grade) and ammonium hydroxide (GR) were from EM science (Gibbstown, NJ). Glacial acetic

acid (A.C.S. reagent) was obtained from Fisher Scientific (Pittsburgh, PA). Formic acid (A.C.S. reagent) and ammonium acetate (99.9%) were purchased from Aldrich (Milwaukee, WI). Drug-free heparinized plasma was from SeraCare (Oceanside, CA). Waters Oasis HLB (10 mg) solid phase extraction 96-well plates were supplied by Waters (Milford, MA).

2.2. Instrumentation

The HPLC–MS/MS system consisted of a Perkin-Elmer (Norwalk, CT) 200 series LC pump, a Varian (Walnut Creek, CA) Prostar 430 96-well autosampler, and an API 3000 triple quadrupole tandem mass spectrometer equipped with a heated nebulizer interface (PE Sciex, Thornhill, Canada).

2.3. Chromatographic conditions

The mobile phase consisted of acetonitrile (ACN)–ammonium acetate (10 mM)–formic acid (90:10:0.1, v/v/v) and was filtered through a nylon membrane (0.20 μm) prior to use. An Ultra IBD (3.2 mm × 50 mm, 3 μm) column from Restek (Bellefonte, PA) was used with a flow rate of 0.5 ml/min. The run time was 4 min.

2.4. Mass spectrometric conditions

The mass spectrometer was connected to the HPLC system via a heated nebulizer interface. Positive ions were generated by atmospheric pressure chemical ionization (APCI). Nebulizer (nitrogen) pressure was set at 60 psi and the temperature of the probe was set at 450 °C. Operational parameters for the MS/MS detection were optimized using the automated procedures incorporated into the instrument control software. The MS was operated under unit mass resolution. The detection of the analytes was based on the multiple reaction monitoring (MRM) of the protonated molecules $(M+H)^+$ and their major collision-induced fragments (m/z 502 → 466 for fexofenadine and m/z 508 → 472 for d_6-fexofenadine). The dwell time for both channels was 450 ms.

2.5. Preparation of standards

Stock solutions of fexofenadine and d_6-fexofenadine (10 μg/ml) were prepared in ACN–water (50:50, v/v). A series of dilutions with ACN–water (50:50, v/v) was made from the stock solution of fexofenadine to give working standards at the following concentrations of fexofenadine: 0.02, 0.04, 0.1, 0.2, 0.4, 1.0, 2.0, and 4.0 μg/ml. The stock solution of d_6-fexofenadine was diluted with ACN–water (50:50, v/v) to make a working internal standard (IS) solution (0.4 μg/ml).

Plasma standards were prepared by spiking 25 μl of each working standard to 0.5 ml of drug-free plasma. The resulting standards were used to quantitate samples ranging in concentration from 1 to 200 ng/ml.

Quality control (QC) plasma samples at concentrations of 2.5, 25, and 150 ng/ml of fexofenadine, respectively, were prepared by adding 1 ml of 0.25, 2.5, and 15.0 μg/ml stock solution of fexofenadine, respectively, to 100 ml volumetric flasks, and filling the flask to volume with control human plasma. Aliquots (1 ml) of the resulting plasma were transferred to 3.6 ml Nunc cryotubes and stored at −20 °C.

2.6. Sample preparation

Half milliliter aliquots of plasma samples were spiked with 25 μl of the working internal standard solution and were diluted with 0.5 ml of acetate buffer (0.1 M, pH 4.0). Of the resulting solution, 0.5 ml of each sample were loaded to the wells of a Waters Oasis HLB (10 mg) 96-well plate, previously conditioned with 1 ml aliquots of methanol, water, and acetate buffer (pH 4.00). Following sample application, plate wells were washed with 1 ml aliquots of water. The drug and the internal standard were eluted from each extraction well with 250 μl of mobile phase. A 20 μl aliquot of the final extract was injected into the HPLC–MS/MS system.

2.7. Data acquisition and analysis

Data acquisition and analyses were performed using Analyst software (PE-Sciex). Standard curves were constructed by plotting the peak area ratios of fexofenadine to internal standard versus the concentration of fexofenadine. Unknown sample concentrations were calculated from the equation $y = mx+b$ as determined by the weighted $(1/x^2)$ linear regression analysis of the standard curve.

2.8. Extraction recovery and assessment of the matrix effect on ionization

Extraction recovery and the effect of the sample matrix on ionization were evaluated for fexofenadine using standards spiked at concentrations of 2, 20, and 200 ng/ml fexofenadine in five different lots of control plasma.

Recovery of the extraction was determined by comparing the absolute peak areas of the standards in human plasma extracted to control plasma extracted in the same manner and then spiked post extraction with the same concentration of the drug and internal standard.

Matrix enhancement/suppression of ionization was evaluated by comparing the absolute peak areas of control plasma extracted and then spiked with a known amount of drug, to neat standards injected directly in the same reconstitution solvent.

3. Results and discussion

3.1. MS/MS detection

Precursor ions for fexofenadine and IS were determined from Q1 spectra obtained during the infusion of neat solutions, via the heated nebulizer source, into the mass spectrometer operated in the positive ionization mode with the collision gas off. Under these conditions, protonated molecules at *m*/*z* 502 and 508 were predominately observed for fexofenadine and IS, respectively. Each of the precursor ions was subjected to collision induced dissociation in order to determine the resulting product ions. The full product spectra of the protonated molecular ion of fexofenadine and IS (Fig. 2) indicated that the two most prominent ions resulted from the loss of either one or two fragments with *m*/*z* 18 (*m*/*z* 484 and 466 for fexofenadine, *m*/*z* 490 and 472 for d_6-fexofenadine), which are most likely attributable to the loss of one or two molecules of water from the parent compound. To optimize the detection of the analytes, the operational parameters of the tandem mass spectrometer were optimized based on response from the MRM channels for fexofenadine (*m*/*z* 502 → 466) and d_6-fexofenadine (*m*/*z* 508 → 472).

3.2. Chromatographic conditions

Several analytical columns were screened in an effort to identify one which yielded sharp symmetrical peaks for the analytes with a mobile phase containing a relatively high organic solvent content. Experience has shown that these criteria are critical with respect to maximizing assay sensitivity. The use of a Restek Ultra IBD column with a mobile phase consisting of 90% acetonitrile and 10% 10 mM ammonium buffer with 0.1% formic acid at a flow rate of 0.5 ml/min was found to provide adequate retention and good peak shape for fexofenadine and its deuterated internal standard. It is interesting to note that the Ultra IBD column was found to exhibit pseudo-normal phase behavior; that is, retention of fexofenadine was found to increase with increasing mobile phase acetonitrile content. The pseudo-normal phase behavior that the Ultra IBD column exhibited allowed the analytes to be chromatographed with adequate retention using a mobile phase containing 90% acetonitrile. The high content of organic component in the mobile phase enhanced the efficiency of ionization for MS/MS detection. The proper choice of sample injection solvent was critical to ensure reproducible chromatography under these conditions. To ensure consistent retention and optimal peak shape, samples were injected in the HPLC mobile phase. Analyte and internal standard eluted with a retention factor (k') of 2.8 under these conditions, with the deuterated internal standard eluting slightly before the unlabeled analyte. The average peak width at the half height was about 0.2–0.3 min.

3.3. Sample preparation

To ensure reproducible chromatographic behavior for analyte and internal standard, it was desirable to identify solid phase extraction conditions that allowed the analytes to be eluted from the solid phase extraction plate with mobile phase. The use of a Waters Oasis HLB 96-well plate for the solid phase extraction was found to efficiently extract both analytes from plasma and met the criteria that mobile phase could be used directly as the eluant. The wash of the extraction plate with water alone was found to provide sufficient sample clean-up, while eliminating any sample loss.

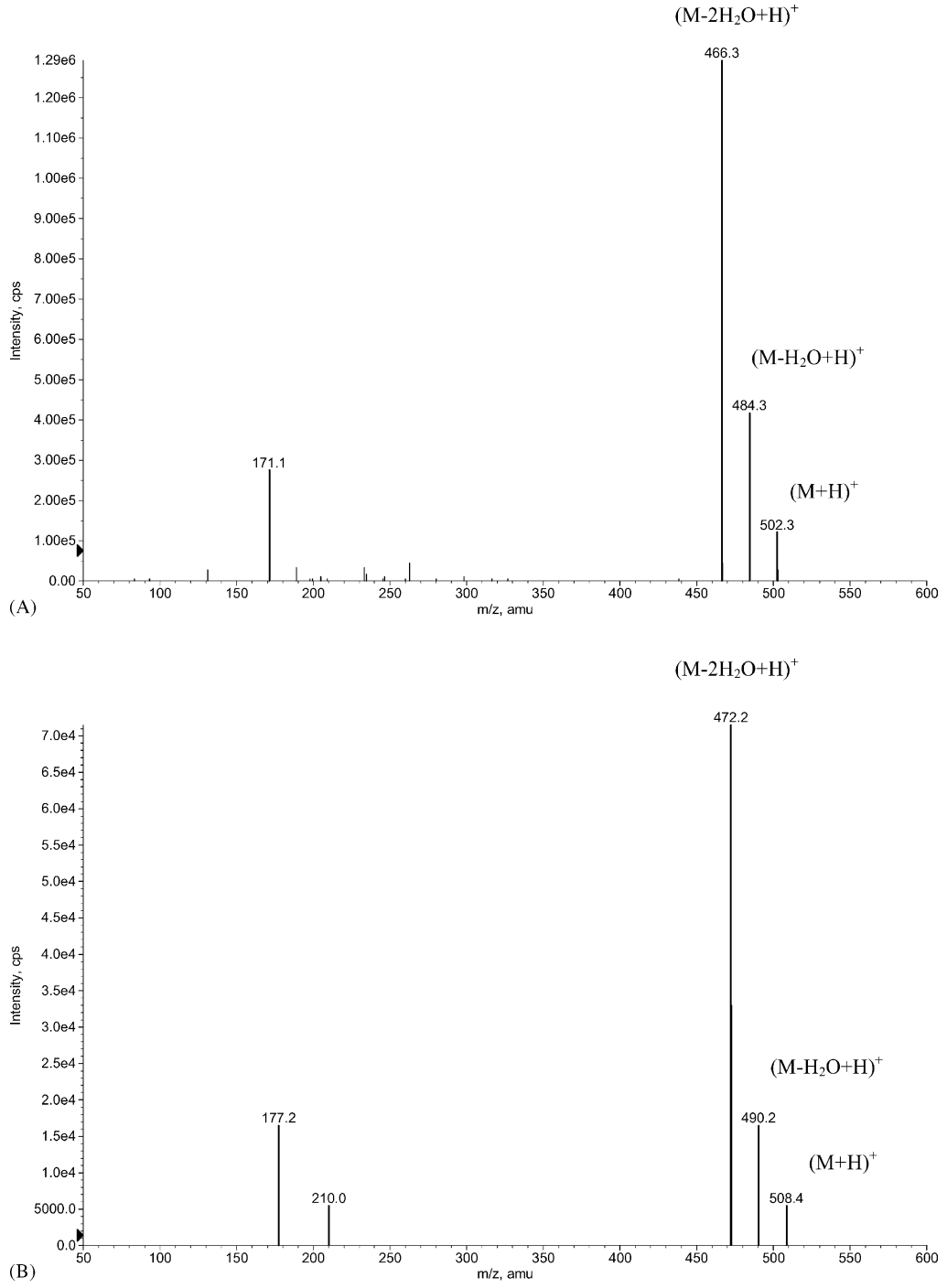

Fig. 2. Product ion mass spectra of fexofenadine (A) and d_6-fexofenadine (B).

3.4. Evaluation of analyte "cross-talk"

The "cross-talk" between channels used for monitoring both analytes was evaluated by the analysis of human plasma samples containing fexofenadine at the highest concentration on the standard curve and/or internal standard at the concentration used in the assay and monitoring the response in all other channels used for quantification. No response was observed from fexofenadine to d_6-fexofenadine (IS) or from d_6-fexofenadine (IS) to fexofenadine.

3.5. Assay selectivity

No peaks eluting at the retention times of the analyte or internal standard were detected in samples from five lots of human control plasma that were processed in accordance with the assay procedure. Representative chromatograms from a sample of control plasma and a plasma standard containing 2 ng/ml fexofenadine and 20 ng/ml are shown in Figs. 3 and 4, respectively.

3.6. Linearity

The coefficient of regression of the weighted least-square ($1/x^2$) regression calibration curve obtained by plotting the ratio of the peak areas of the analyte to the that of the internal standard versus analyte concentration was typically greater than 0.99 over the concentration range 1–200 ng/ml in plasma. The use of the weighted least-square regression resulted in less than 10% deviation between the nominal standard concentrations and experimentally determined standard concentrations calculated from the regression equations.

3.7. Extraction recovery and assessment of the matrix effect on ionization

Results of these assessments are shown in Table 1. Analyte recovery averaged approximately 73% over the standard curve range of the assay. A small absolute enhancement of ionization was observed in the control extracts to which analyte was added relative to analyte injected in mobile phase. The enhancement might be due to the presence of non-detectable endogenous compounds in the urine extract affecting the ionization of the analyte. However, since a stable isotope labeled internal standard was used in the assay, this effect would not be expected to affect the assay, as it should be compensated for by the internal standard. Lack of a relative matrix effect is confirmed by the excellent intra-day precision results (CV under 3.5% at all concentrations, Table 2) that were obtained using standard samples prepared in five different lots of control plasma.

Table 1
Extraction recovery and assessment of matrix effects on ionization during the determination of fexofenadine in human plasma

Standard concentration in plasma (ng/ml)	Mean extraction recovery[a] (%, $n = 5$)	Ionization enhancement[b] (%, $n = 5$)
2	74.6	123.0
20	72.5	126.8
200	72.7	129.8
20[c]	74.1	139.8

[a] Extraction recovery was calculated by dividing the mean peak area of analyte spiked before extraction by the respective mean peak area of analyte spiked after extraction and multiplying by 100.
[b] Matrix effect was calculated by dividing the mean peak area of analyte spiked after extraction by the mean peak area of the neat analyte standard and multiplying by 100.
[c] Internal standard.

3.8. Assay precision and accuracy

An assessment of intra-day variability was conducted by analyzing standard curve samples ($n = 5$ at each concentration) over the calibration range of

Table 2
Intra-day precision and accuracy data for the assay of fexofenadine in five lots of human plasma

Nominal concentration (ng/ml)	Mean analyzed concentration (ng/ml, $n = 5$)	Accuracy[a] (%)	Precision[b] (%)
1.0	1.01	101.3	1.6
2.0	1.95	97.7	1.3
5.0	5.00	100.0	2.1
10.0	10.01	100.1	2.5
20.0	19.76	98.8	2.7
50.0	50.32	100.6	1.3
100.0	101.5	101.5	3.4
200.0	200.0	100.0	1.4

[a] Expressed as [(mean observed concentration)/(nominal concentration)] × 100.
[b] Coefficient of variation of peak area ratios.

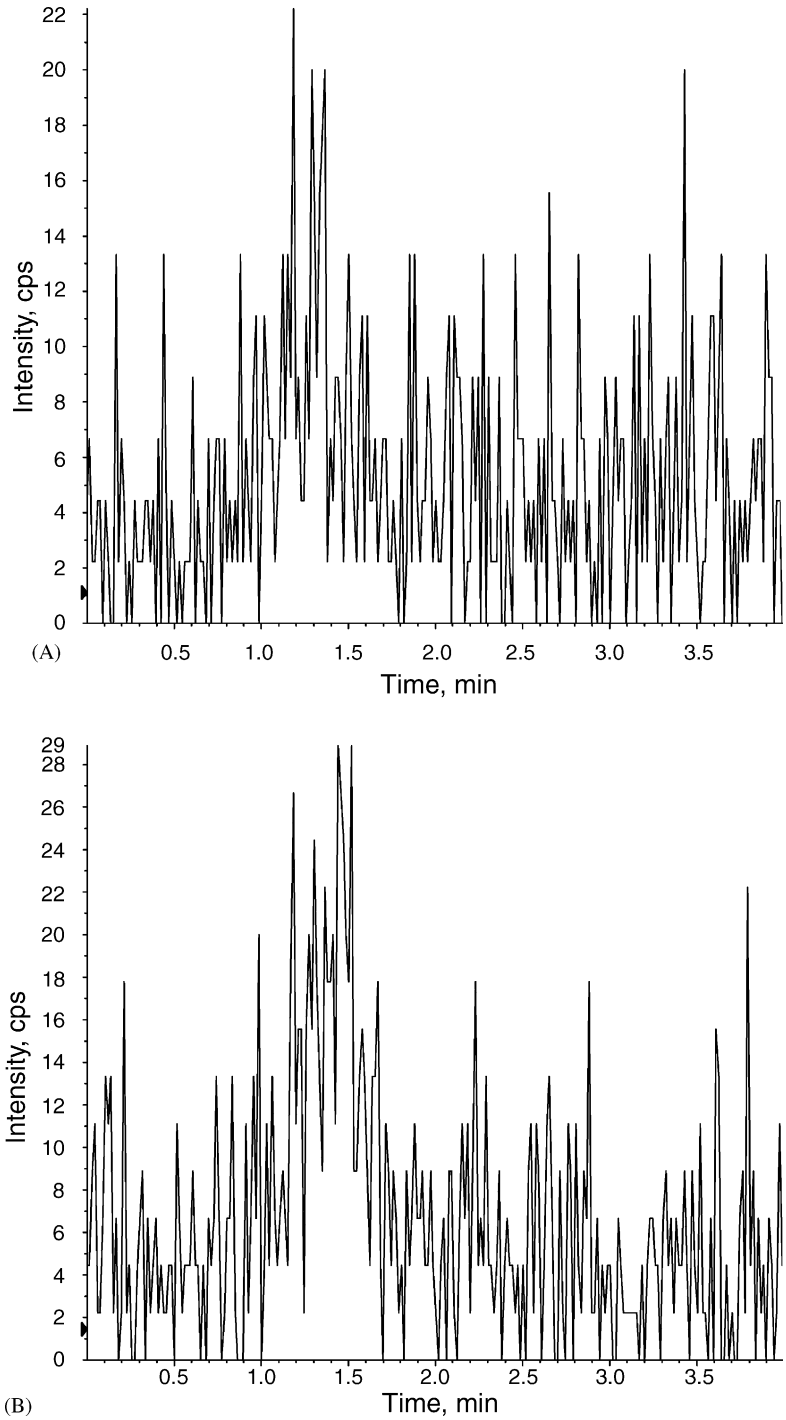

Fig. 3. Representative HPLC–MS/MS chromatogram of control human plasma. (A) Fexofenadine channel and (B) internal standard channel.

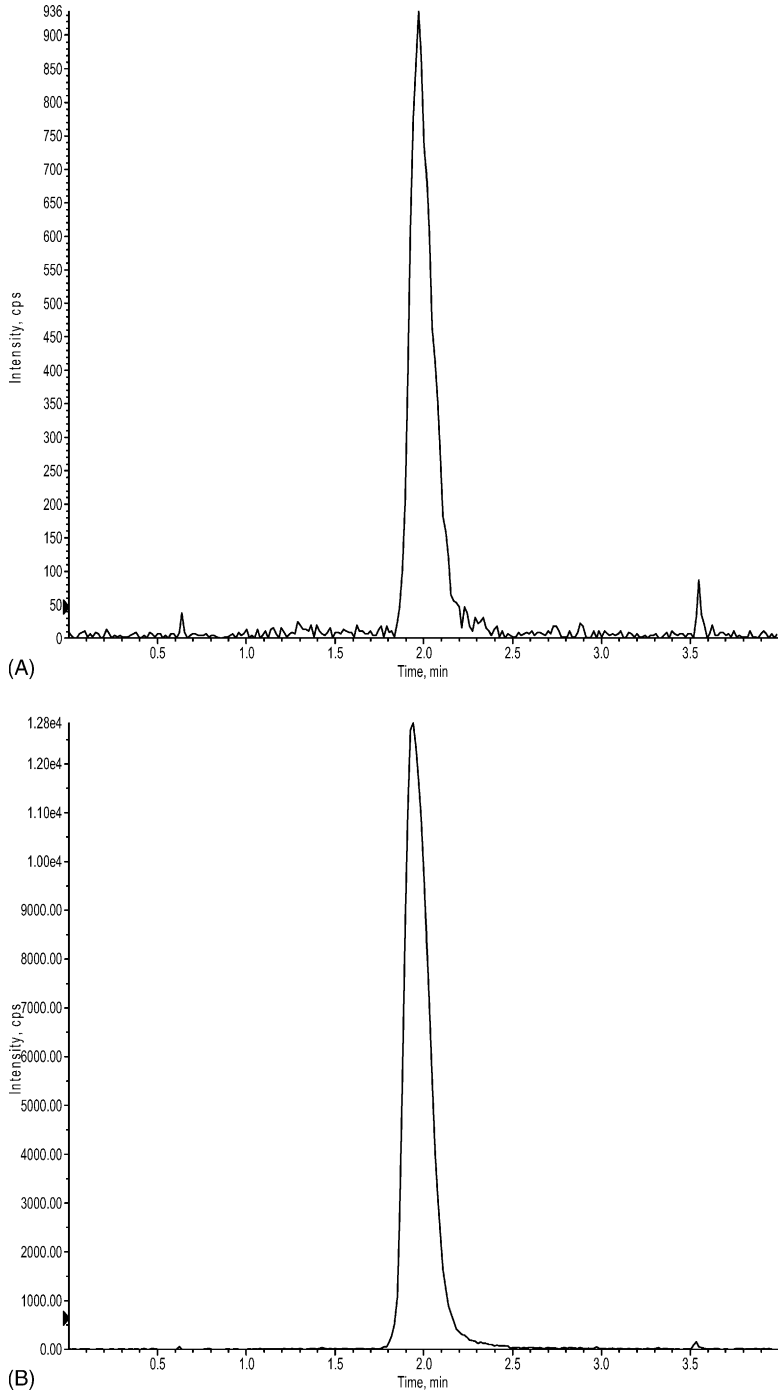

Fig. 4. Representative HPLC–MS/MS chromatogram of a standard sample containing 1 ng/ml fexofenadine and 20 ng/ml internal standard. (A) Fexofenadine channel and (B) internal standard channel.

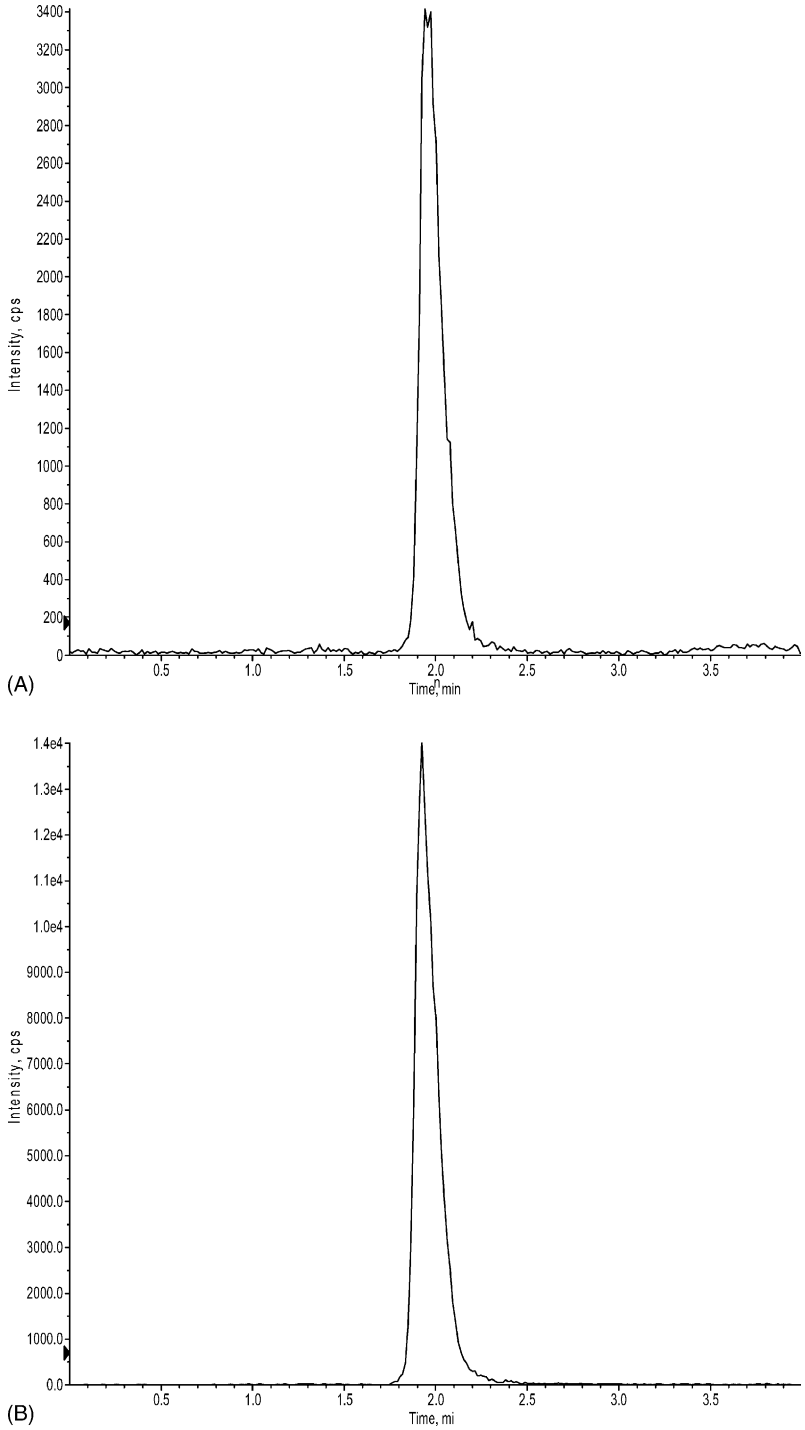

Fig. 5. Representative HPLC–MS/MS chromatogram of a plasma sample obtained from a subject 24 h following the administration of a single 60 mg dose of fexofenadine to which internal standard was added at a concentration of 20 ng/ml. The concentration of fexofenadine in the sample was equivalent to 3.92 ng/ml. (A) Fexofenadine channel and (B) internal standard channel.

Table 3
Assessment of freeze–thaw (F/T) stability of fexofenadine in human plasma

Nominal concentration (ng/ml)	Control found concentration, mean[a] (ng/ml, $n = 3$)	3 F/T cycles found concentration, mean[a] (ng/ml, $n = 3$)
2.50	2.43 (4.0)	2.43 (1.9)
25.0	24.7 (2.6)	23.2 (3.5)
150.0	142.3 (0.8)	139.7 (1.5)

[a] Numbers in parentheses are coefficients of variation (%CV).

1–200 ng/ml. These samples were prepared in five different lots of control plasma. The resulting assay precision and accuracy data are presented in Table 2. The intra-day precision of the assay, as measured by the coefficient of variation (%CV) was ≤3.4% for all points on the calibration curve. Assay accuracy was found to be within 2.3% of the nominal for all standards.

3.9. Quality control samples

Quality control samples containing fexofenadine were prepared at concentrations of 2.5 ng/ml (low QC), 25 ng/ml (middle QC) and 150 ng/ml (high QC). These samples were then frozen at −20 °C. Two quality control samples at each level were analyzed with each batch of clinical samples.

3.10. Freeze–thaw stability

Quality control samples ($n = 3$ at each concentration) were subjected to freeze–thaw cycles consisting of a thaw to reach room temperature (≥4 h), vortexing, and then refreezing (−20 °C) at least overnight and repeating for 3 cycles. Samples exposed to only 1 freeze–thaw cycle were used as the control samples. Results of the analysis are presented in Table 3. The results for the samples that were subjected to additional freeze–thaw cycles were within 6% of the controls, indicating that fexofenadine in the samples was stable toward repeated freeze–thaw cycles.

3.11. Assay application

The described assay has been successfully employed to quantitate fexofenadine in human plasma samples obtained from a volunteer following the administration of single 60 mg doses of the drug. A representative chromatogram of a plasma sample obtained 24 h following the administration of a 60 mg dose of fexofenadine is shown in Fig. 5.

4. Conclusion

The described method has been found suitable for the analysis of fexofenadine in human plasma samples obtained following the administration of single 60 mg doses. As such, the method is useful for assessing drug interactions between fexofenadine and co-administered medications. Sample preparation utilizes solid-phase extraction in the 96-well format, which is adaptable to high throughput automation. A stable labeled analog of fexofenadine is employed as internal standard; use of the stable label internal standard was found to adequately compensate for matrix effects on ionization.

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