Journal of Chromatography B, 879 (2011) 2189–2193

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Determination of azatadine in human plasma by liquid chromatography/tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 14 March 2011
Accepted 31 May 2011
Available online 12 June 2011

Keywords:
Azatadine
LC–MS/MS
Human plasma
Pharmacokinetics

ABSTRACT

A sensitive method using liquid chromatography with tandem mass spectrometric detection (LC–MS/MS) was developed and validated for the analysis of antihistamine drug azatadine in human plasma. Loratadine was used as internal standard (IS). Analytes were extracted from human plasma by liquid/liquid extraction using ethyl acetate. The organic phase was reduced to dryness under a stream of nitrogen at 30 °C and the residue was reconstituted with the mobile phase. 5 μL of the resulting solution was injected onto the LC–MS/MS system. A 4.6 mm × 150 mm, I.D. 5 μm, Agilent TC-C_{18} column was used to perform the chromatographic analysis. The mobile phase consisted of ammonium formate buffer 0.010 M (adjusted to pH 4.3 with 1 M formic acid)/acetonitrile (20:80, *v*/*v*) The chromatographic run time was 5 min per injection and flow rate was 0.6 mL/min. The retention time was 2.4 and 4.4 min for azatadine and IS, respectively. The tandem mass spectrometric detection mode was achieved with electrospray ionization (ESI) iron source and the multiple reaction monitoring (MRM) (291.3 → 248.2 *m*/*z* for azatadine, 383.3 → 337.3 *m*/*z* for IS) was operated in positive ion modes. The low limit of quantitation (LLOQ) was 0.05 ng/mL. The intra-day and inter-day precision of the quality control (QC) samples was 8.93–11.57% relative standard deviation (RSD). The inter-day accuracy of the QC samples was 96.83–105.07% of the nominal values.

1. Introduction

Azatadine maleate (6-11-dibydro-11- [1-methyl-4-piperclylidene]-5H {5, 6} cyclohepta {1, 2-b} pyridine maleate {1:2}), a first generation antihistaminic with anticholinergic, antiserotonin and antianaphylactic activity, which blocks the effects of the naturally occurring chemical histamine in human body and can be used to treat sneezing, runny nose, itching, watery eyes, hives, rashes and other symptoms of allergies [1–3]. Azatadine maleate tablet (Opitimine®) was developed by Schering Canada Inc. and was put into US's market after the FDA's approval in 2002. Recently, the azatadine maleate tablet has been developed and approved to conduct clinical trial in China. To date, only pharmacokinetic parameter of elimination half-life values and T_{max} in white people was mentioned [4]. Therefore, it is urgent to investigate the pharmacokinetic properties in Asian people, especially in Chinese people. The aim of our study was to develop a method to determine azatadine in human plasma and to assess the pharmacokinetic properties in healthy Chinese volunteers. Two methods have been reported for the determination of azatadine in biological samples, which involved a gas–liquid chromatographic method [5] and a HPLC method [6]. However, the limit of detection was about 100 ng/mL and 10 ng/mL, respectively, and could not meet the requirement of azatadine phamacokinetic study. Taking into consideration the low levels of azatadine in human blood, a new, simple, specific and highly sensitive LC–MS/MS method was developed, which has a lower LLOQ (0.05 ng/mL) and was efficient in analyzing large numbers of plasma samples obtained from pharmacokinetic studies after 2 mg single dose oral administration of azatadine maleate.

2. Experimental

2.1. Chemicals and reagents

Azatadine maleate (purity 101.13%) was supplied by Libang Pharmaceutical Co. Ltd. (Shaanxi, China). Loratadine (purity 99.8%) as an internal standard (IS) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Water of HPLC grade was deionized and purified using a Milli-Q system (Millipore, Bedford, MA, USA). Methanol and ace-

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doi:10.1016/j.jchromb.2011.05.050

tonitrile of HPLC grade were purchased from Fisher scientific (St. Louis, MO, USA). Ethyl acetate of HPLC grade was from Tianjin Baishi Chemical industry Co. Ltd. (Tianjin, China), and other chemicals and reagents are of analytical grade. Heparinized blank human plasma was obtained from Xijing hospital (Xi'an, China).

2.2. LC–MS/MS instrumentation and analytical conditions

The sample was separated on an Agilent TC-C_{18} column (4.6 mm × 150 mm, I.D. 5 μm. Agilent Technologies, USA). The mobile phase consisted of ammonium formate buffer 0.010 M (adjusted to pH 4.3 with 1 M formic acid): acetonitrile (20:80, *v*/*v*), the inject volume was 5 μL and the flow rate was 0.6 mL/min. The analysis of azatadine was performed using an Agilent 1200 series HPLC and an Agilent 6410 Triple Quadrupole mass spectrometer equipped with an electrospray ionization source (Agilent Technologies, USA). The tandem mass spectrometric detection was achieved with electrospray positive ionization using multiple reaction monitoring (MRM). The mass transition was 291.3 → 248.2 *m*/*z* for azatadine, 383.3 → 337.3 *m*/*z* for IS, respectively. Other working parameters of the mass spectrometer were as follows: dwell time 200 ms, gas flow 12 L/min, gas temperature 350 °C, nebulizer pressure 55 psi, fragmentor voltage 115 V (azatadine) and 100 V (IS), and collision energy 14 eV (azatadine) and 22 eV (IS). All data were acquired and processed on the MassHunter workstation (Agilent Technologies, USA).

2.3. Preparation of standard solutions

Primary stock solution of azatadine was prepared by dissolving 17.78 mg azatadine maleate (amount to 10 mg azatadine) in 10 mL methanol. Standard solutions at concentration of 10.0 μg/mL, 1.0 μg/mL, 100 ng/mL, 10.0 ng/mL and 1.0 ng/mL were prepared by serially dilution of the stock solutions with methanol. The internal standard stock solution was prepared by dissolving 10.0 mg loratadine in 10 mL methanol and was diluted with methanol to give final concentrations at 200 ng/mL. All solutions were stored at 4 °C.

2.4. Preparation of calibration curves and quality control samples

Appropriate amount of stock solutions of azatadine and IS were spiked into 1 mL blank plasma to obtain final concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2, and 5 ng/mL for the analyte and 200 ng/mL for IS. QC samples were made using stock solution. Three levels of QC samples in plasma were 0.15 ng/mL (low), 0.4 ng/mL (medium) and 4 ng/mL (high) for the analyte. All samples were stored for in a −70 °C freezer and were not opened until analysis.

2.5. Samples preparation

Plasma samples were thawed at room temperature. 50 μL IS (200 ng/mL) solution was added to 1 mL plasma sample and vortex-mixed for 30 s and then 50 μL sodium hydroxide (1 mol/L) was added in and mixed. The mixture was extracted with 5 mL of ethyl acetate, vortex-mixed for 3 min, and then centrifuged at 4000 r/min for 8 min. The upper organic layer was separated and evaporated to dryness using a gentle stream of nitrogen in the water-bath maintained at 30 °C. The residuum was dissolved with 120 μL of mobile phase, vortex-mixed for 1 min, centrifuged at 16,000 r/min for 3 min, and a 5 μL of the supernatant was then injected onto the LC–MS/MS for analysis.

2.6. Method validation

A thorough and complete method validation of azatadine determination in human plasma was done following the US FDA guidelines. The method was validated for specificity, sensitivity, linearity, accuracy and precision, recovery, matrix effect and stability.

The specificity of the method was checked by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Each blank plasma sample was tested using the extraction procedure and chromatographic conditions described above to ensure no interference of azatadine and IS from plasma.

The linearity was observed from four calibration curves prepared and run on four different days over the range of 0.05–5.0 ng/mL for azatadine. Calibration curves ($Y = aX + b$) were represented by plotting the peak area ratios (Y) of azatadine to IS versus the concentrations (X) of the calibration standards. A weighted linear regression model (weighting factor $1/x$) was used to fit the calibration line. The acceptable correlation coefficients were 0.99 or better, and that each back-calculated standard concentration must be within 15% deviation from the nominal value except at the LLOQ, which was defined as the lowest concentration in the standard curve at which the relative standard deviation (SD) ($n = 5$) was within 20% and accuracy was within 100 ± 20%. The curves were established using eight samples independent of standards.

To evaluate the accuracy and precision of the method, each concentrate of QC samples were assayed in sets of five replicates on three consecutive days. Each day included a set of calibration standards. The accuracy was required to be within ±15%, and the intra- and inter-day precisions not to exceed 15%.

In order to evaluate extraction recovery, the mean peak-area of the extracted QC samples was compared to the mean peak-area of the extracted blanks spiked with the corresponding neat solutions. The number of replicates for each concentration was five.

Stability experiments were performed to evaluate the stability of the analyte in plasma under different conditions. Plasma samples were subject to store at ambient temperature for 6 h, to long-term storage conditions (−20 °C) for 5 weeks and 12 weeks and to three freeze–thaw stability studies. The autosampler stability was conducted by re-analyzing the samples after store in autosampler condition for 12 h. All stability studies were conducted at two QC levels (0.15 and 4 ng/mL as low and high values) with three determinations for each.

The matrix effect was defined as the direct or indirect alteration or interference in response due to the presence of unintended or other interfering substances in the samples. It was evaluated by comparing the peak area of the analytes dissolved in the blank plasma sample's precipitated solution with that of the analytes dissolved in the mobile phase. Three different concentration levels of azatadine (0.15, 0.4 and 4 ng/mL) were evaluated by analyzing five samples at each level. If the peak area ratio is less than 85% or more than 115%, a matrix effect is implied.

2.7. Pharmacokinetic study

The pharmacokinetics of azatadine maleate was studied in healthy Chinese subjects according to the requirements of the guideline for the conduct of pharmacokinetic study of chemical medicines issued by the State Food and Drug Administration of China (Guideline of SFDAC) and the Declaration of Helsinki for biomedical research involving human subjects. The protocol and associated informed consent statements were reviewed and approved by the Committee on Human Rights Related to Human Experimentation at Xijing Hospital. Written informed consents were obtained from all subjects before the study. Twenty male and female healthy volunteers who aged from 26 to 38, body mass index (BMI) ranged 19–24 kg/m^2 were enrolled in the study. All volunteers have passed a obtaining of complete medical history

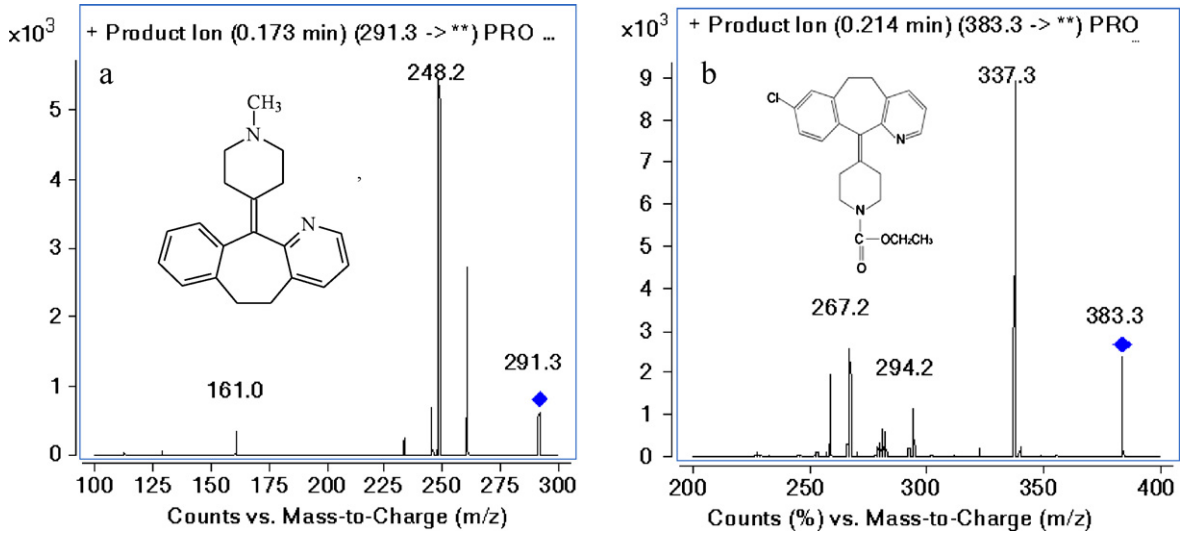

Fig. 1. Product ion mass spectra of (a) maleate azatadine $[M+H]^+$ and (b) loratadine $[M+H]^+$.

and physical examination before participating in the study. All subjects were confirmed abstinence from other medications, alcohol, tobacco and caffeinated products. After a single oral administration of 2 mg of azatadine maleate tablet, blood samples (5 mL) were collected at 0 h (pre-dose) and 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 24.0, 36.0 and 48.0 h (post-dose). The samples were transferred to heparinized tube and centrifuged at 3000 × *g* for 10 min. Plasma was separated and stored at −70 °C until analysis.

The plasma concentrations of azatadine versus time profiles were acquired for each subject. The plasma concentration–time data were analyzed by standard non-compartmental model with the help of the Drug and Statistics software (DAS software, a recommended pharmacokinetic program in China, version 2.1.1). The individual C_{max} (maximum plasma concentration) and t_{max} (time to reach C_{max}) were directly obtained from the plasma concentration versus time data. The other pharmacokinetic parameters were obtained by the software.

3. Results and discussion

3.1. Method development

3.1.1. Mass spectrometry

The electrospray ionization of aztadine and the IS produced the $[M+H]^+$ ions at 291.3 and 383.3 under positive ionization conditions. These molecular ions undergo fragmentation in the collision cell, and the product ion mass spectra of azatadine and the IS are shown in Fig. 1. With the experimental conditions used in these experiments, azatadine and IS show an intense product ion at *m*/*z* 248.2 and 337.31, respectively. The results showed that the most sensitive mass transition were *m*/*z* 291.3 → 248.2 for azatadine and *m*/*z* 383.3 → 337.3 for IS. The MRM state file parameters were the optimized values for the sensitivity and specificity required for azatadine.

3.1.2. Liquid chromatography

Chromatographic analysis of azatadine and IS was initiated under isocratic conditions with an aim to develop a simple separation process with a short run time. Both SB-C_{18} and TC-C_{18} columns were tested to achieve ideal separation. Compared with the SB-C_{18} column, the separation by TC-C_{18} column showed better peak shape. Feasibility of various mixture(s) of solvents using different buffers such as ammonium acetate, ammonium formate and formic acid, along with altered flow rates (in the range of 0.2–0.8 mL/min) were tested to identify the optimal mobile phase that produced the best sensitivity, efficiency and peak shape. The peak which belongs to azatadine was vulnerable to the change of water phase pH. It was found that a mixture of acetonitrile/0.010 M ammonium formate buffer (adjusted to pH 4.3 with 1 M formic acid) (20:80, *v*/*v*) could achieve this purpose and was finally used as the mobile phase. A flow rate of 0.6 mL/min permitted a run time of 5 min.

3.1.3. Sample preparation

To achieve better extraction efficacy and less interference, various sample processing approaches including direct protein precipitation and liquid–liquid extraction with different solvents were, respectively investigated. After comprehensively evaluating extraction efficiency and total processing time, one step liquid–liquid extraction with 5 mL of ethyl acetate was used. No interference was observed from any endogenous or exogenous plasma matrix.

3.2. Specificity and linearity

Under the optimized chromatographic conditions and sample processing procedure, the retention times of azatadine and I.S. were approximately 2.4 min and 4.4 min, respectively. Each run can be completed within 5 min. The chromatograms showed a clear and excellent separation between azatadine, I.S. and endogenous interferences from plasma. Representative chromatographs are shown in Fig. 2. The calibration curve for azatadine exhibited a good linearity in the concentration range of 0.05–5 ng/mL. The linear equation (mean ± SD, $n = 4$) was $Y = -0.001804 + 0.16875X$ ($r = 0.9997$) (Y: peak area ratio of aztadine to I.S., X: concentrations of azatadine). A weighing factor of $1/x$ of concentration was chosen to achieve homogeneity of variance.

Table 1
The intra-and inter day precision and accuracy of azatadine spiked in human plasma.

Concentration (ng/mL)	Precision (CV%)		Accuracy (mean ± SD, %)	
	Intra-day	Inter-day	Intra-day	Inter-day
0.15	9.06	9.17	105.07 ± 9.5	101.69 ± 9.2
0.4	9.00	8.93	100.16 ± 9.0	100.45 ± 9.1
4	11.57	9.09	96.83 ± 11.2	100.15 ± 9.1

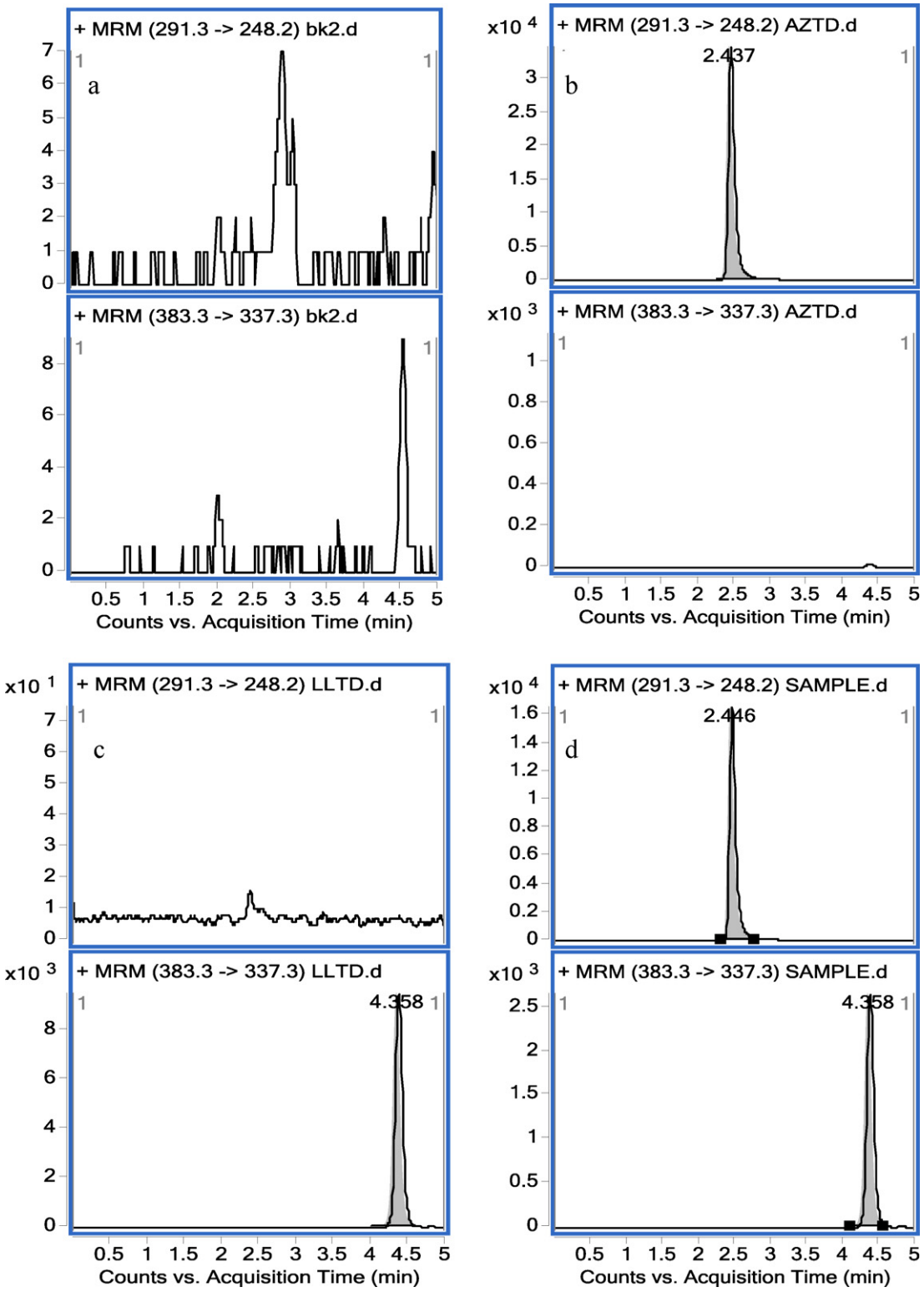

Fig. 2. Representative MRM chromatograms of (a) blank plasma; (b) plasma sample spiked with azatadine maleate at 10 ng/mL; (c) plasma sample spiked with loratadine at 10 ng/mL; (d) plasma sample after administration.

3.3. Sensitivity (LLOQ), accuracy and precision

The LLOQ was around 0.05 ng/mL, which was sensitive enough for pharmacokinetic study of oral administration of azatadine maleate in human. The accuracy and RSD of LLOQ were 88.1–107.9% and 8.2% for azatadine, respectively ($n = 5$). The accuracy and precision was estimated by assaying the quality control samples (low, medium and high concentration) in five applications. As show in Table 1, the intra- and inter-day precisions were less than 11.57% and 9.17%, and the accuracy was 96.83–105.07% for azatadine.

Table 2
The stability of azatadine under different conditions (n = 15 mean, %).

Concentration (ng/mL)	At room temperature for 6 h	Freeze–thaw stability	At 25 °C in the autosampler for 12 h	At −20 °C for 5 weeks	At −20 °C for 12 weeks
0.15	109.18	99.83	91.28	108.70	103.28
4	103.39	94.54	94.22	99.23	104.02

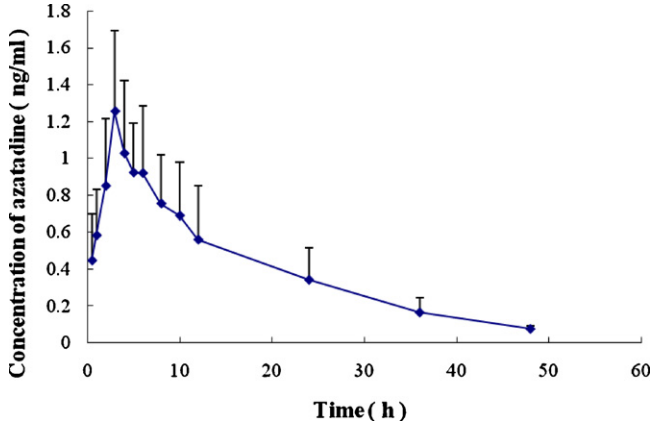

Fig. 3. Mean plasma concentration (+SD) versus time curve of azatadine maleate tablet administration at dose of 2–20 mg volunteers.

3.4. Recovery and matrix effect

The extraction recovery of azatadine was calculated by analyzing five replicates at 0.15, 0.4 and 4 ng/mL. The extraction recoveries of the assay were 70.9 ± 2.4%, 75.0 ± 8.2% and 74.9 ± 8.6%, respectively. The matrix effects (mean ± SD%) for plasma determined at concentrations of 0.15, 0.4 and 4 ng/mL for azatadine were (92.8 ± 4.1%). No significant matrix effects were evident.

3.5. Stability

Results of the stability tests are summarized in Table 2. Azatadine was stable in human plasma after being placed at ambient temperature for 6 h, in the autosampler at 25 °C for 12 h, after being stored at −20 °C for 5 weeks, 12 weeks and through three freeze–thaw cycles.

3.6. Application to biological samples

The LC–MS/MS method developed in this study yielded satisfactory results for the quantitation of azatadine in human plasma and has been successfully applied to the pharmacokinetic study of oral administration of azatadine maleate tablet. The mean plasma concentration versus time curve is presented in Fig. 3. The major pharmacokinetic parameters of azatadine were calculated by non-compartment model based on statistical moment and presented in Table 3.

Table 3
Main pharmacokinetic parameters of azatadine maleate following single dose of 2 mg to Chinese volunteers (n = 20, mean ± SD).

Parameters	Single dose of 2 mg azatadine
C_{max} (ng/mL)	1.4 ± 0.2
$T_{1/2}$ (h)	10.9 ± 2.5
V1 (L)	1722.4 ± 816.0
CL (L/h)	107.5 ± 37.3
$AUC_{(0-48)}$ (ng h/mL)	18.9 ± 6.8
$AUC_{(0-\infty)}$ (ng h/mL)	20.5 ± 6.6

4. Conclusion

A rapid, sensitive and specific HPLC/MS/MS method was developed and validated for the determination of total azatadine in human plasma for the first time. The method was successfully applied to characterize the pharmacokinetics of azatadine in human after oral administration of azatadine maleate tablet. A liquid–liquid extraction procedure was taken to extract azatadine from human plasma, followed by chromatography with tandem mass spectrometry detection. The method showed good sensitivity, repeatability, precision and accuracy, as well as recovery for azatadine. Compared with other methods for quantifying azatadine reported in the literature, the present LC–MS/MS method is much more sensitive, simpler and faster, with higher specificity and wider linear range.

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