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Quantitative determination of azithromycin in human plasma by ultra performance liquid chromatography–electrospray ionization mass spectrometry and its application in a pharmacokinetic study

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Abstract

A selective, rapid and sensitive ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) method was developed for the quantitative determination of azithromycin in human plasma and its application in a pharmacokinetic study. With roxithromycin as internal standard, sample pretreatment involved a one-step extraction with diethyl ether of 0.5 mL plasma. The analysis was carried out on an ACQUITY UPLC™ BEH C_{18} column (50 mm × 2.1 mm, i.d., 1.7 μm) with gradient elution at flow rate of 0.35 mL/min. The mobile phase was 50 mM ammonium acetate and acetonitrile. The detection was performed on a triple-quadrupole tandem mass spectrometer by multiple reaction monitoring (MRM) mode via electrospray ionization (ESI). Linear calibration curves were obtained in the concentration range of 1–1000 ng/mL, with a lower limit of quantification of 1 ng/mL. The intra- and inter-day precision (RSD) values were below 15% and accuracy (RE) was −1.3% to 5.7% at all QC levels. The method was applicable to clinical pharmacokinetic study of azithromycin in healthy volunteers following oral administration.

Keywords: Azithromycin; UPLC–ESI-MS/MS; Human plasma; Pharmacokinetic

1. Introduction

Azithromycin is a semisynthetic macrolide antibiotic of the erythromycin group with a 15-membered azalactone ring. Azithromycin is derived from erythromycin, however, it differs chemically from erythromycin in that a methyl-substituted nitrogen atom is incorporated into the lactone ring (Fig. 1).

Azithromycin appears to bind to the same receptor as erythromycin and has a very high tissue-to-blood concentration ratio with a half-life of 2–4 days in most tissues, which may partly explain its outstanding antibiotic performance [1,2]. Azithromycin has been shown to be effective in patients with acute bronchitis, pneumonia, sinusitis, pharyngitis, tonsillitis and otitis media. It is very effective against upper and lower respiratory tract infections, sexually transmitted disease, skin and soft tissue infections.

Several microbiological assays have been used in bioavailability study of azithromycin [3–6]. However, these assays tended to lack specificity. Several HPLC methods have also been described for the determination of azithromycin in human plasma using various methods of detection, such as UV detection [7,8], electrochemical detection [9–17], and fluorescence detection after derivatization [18–22]. However, azithromycin does not have a specific UV chromophore, therefore, UV detection is only of low sensitivity for determination of azithromycin in biological matrix. Fluorescence detection requires complicated sample pretreatment involving pre-column derivatization of the analyte. Thus, LC–MS [23] and LC–MS/MS [24,25] were used in the determination of azithromycin with lower limit of quantification (LLOQ) of around 2 ng/mL [23–25,28]. The reported methods required either a complicated extraction [23] and/or derivatization procedure [18–22], or long analysis time [23,25], and may not well meet the requirement of desired throughput, speed and sensitivity in biosample analysis.

This paper describes a fast, selective and highly sensitive approach, which enables the determination of azithromycin at 1 ng/mL in plasma with good accuracy using ultra performance

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liquid chromatography coupled to tandem mass spectrometry (UPLC–MS/MS). This method was fully validated and applied to the pharmacokinetic study in healthy volunteers after oral administration of azithromycin tablets.

2. Experimental

2.1. Reagents and chemicals

Azithromycin reference standard (99.2% of purity) and roxithromycin (internal standard, I.S., 99% of purity) (Fig. 1) were obtained from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, PR China). Acetonitrile, methanol and ammonium acetate (HPLC grade) were purchased from Dikma (Richmond Hill, NY, USA). All Other chemicals were of analytical grade. Sodium hydroxide and diethyl ether were purchased from Yuwang (Chemical Reagent Plant, Shandong, China). Water was purified by redistillation and filtered through 0.22 μm membrane filter before use.

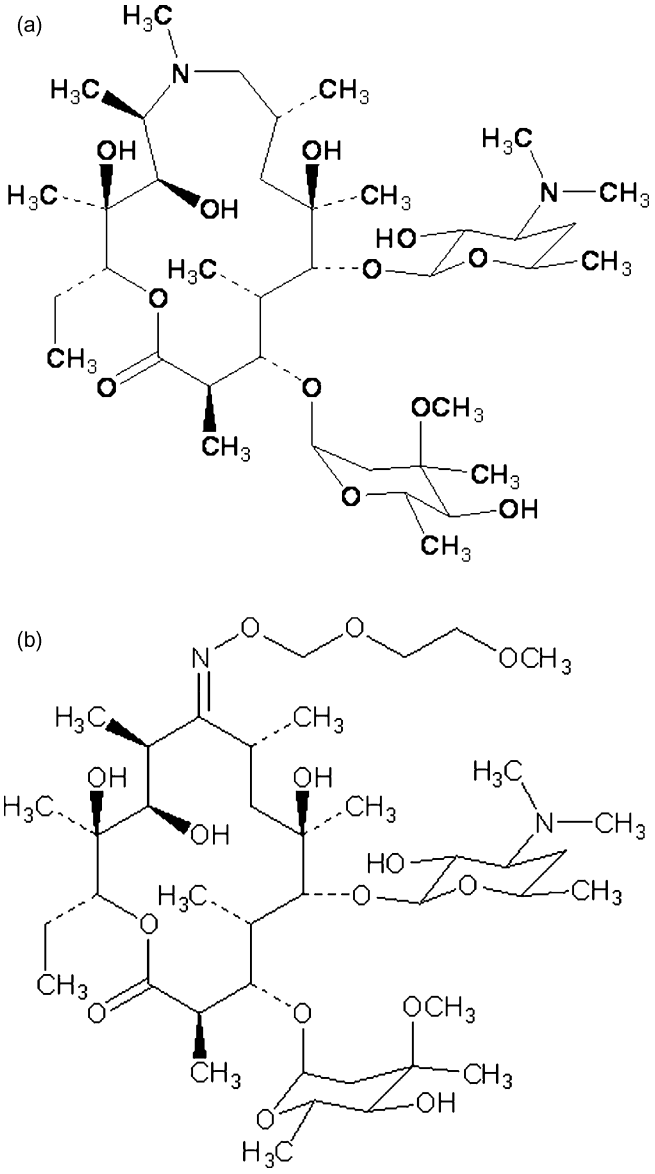

Fig. 1. Chemical structures of azithromycin and roxithromycin (I.S.).

2.2. Apparatus and operation conditions

2.2.1. Liquid chromatography

The chromatography was performed on ACQUITY^{TM} UPLC system (Waters Corp., Milford, MA, USA) with cooling autosampler and column oven enabling temperature control of analytical column. An ACQUITY UPLC^{TM} BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm; Waters Corp., Milford, MA, USA) was employed. The column temperature was maintained at 40 °C and chromatographic separations were achieved with a gradient elution. Mobile phase A was 50 mM ammonium acetate aqueous, and mobile phase B was acetonitrile. The gradient started at 40% mobile phase B and changed linearly to 90% mobile phase B in 1.5 min, after being maintained at 90% B for 0.5 min it then returned to the initial condition. The flow rate was set at 0.35 mL/min. The auto-sampler was conditioned at 4 °C and the injection volume was 5 μL using partial loop mode for sample injection.

2.2.2. Mass spectrometry

Triple-quadrupole tandem mass spectrometric detection was carried out on a Micromass® Quattro micro^{TM} API mass spectrometer (Waters Corp., Milford, MA, USA) with an electrospray ionization (ESI) interface. The ESI source was set in positive ionization mode. Quantification was performed using multiple reaction monitoring (MRM) of the transitions of *m/z* 749 → 591 for azithromycin, *m/z* 837 → 679 for roxithromycin (I.S.), respectively, with scan time of 0.10 s per transition. The optimal MS parameters were as follows: capillary 1.2 kV, cone 35 kV, source temperature 110 °C and desolvation temperature 300 °C. Nitrogen was used as the desolvation and cone gas with a flow rate of 400 and 30 L/h, respectively. Argon was used as the collision gas at a pressure of approximately 2.61×10^{-3} mbar. The optimized collision energy for both azithromycin and roxithromycin was 25 eV. The product ion spectra of azithromycin and roxithromycin are shown in Fig. 2. All data collected in centroid mode were acquired and processed using MassLynx^{TM} NT 4.1 software with QuanLynx^{TM} program (Waters Corp., Milford, MA, USA).

2.3. Preparation of standards and quality control samples

Standard stock solutions of azithromycin and roxithromycin were both prepared in methanol at the concentration of 200 μg/mL and 100 μg/mL, respectively. The internal standard solution was diluted with methanol to 1000 ng/mL. And the azithromycin solution was then serially diluted with methanol to provide working standard solutions of desired concentrations. All the solutions were stored at −20 °C.

Calibration standards were prepared by spiking 0.5 mL of blank human plasma with working standard solutions of azithromycin. The effective concentrations in standard plasma samples were 1.0, 2.0, 10.0, 50.0, 100, 200, 500, 1000 ng/mL. One calibration curve was constructed on each analysis day

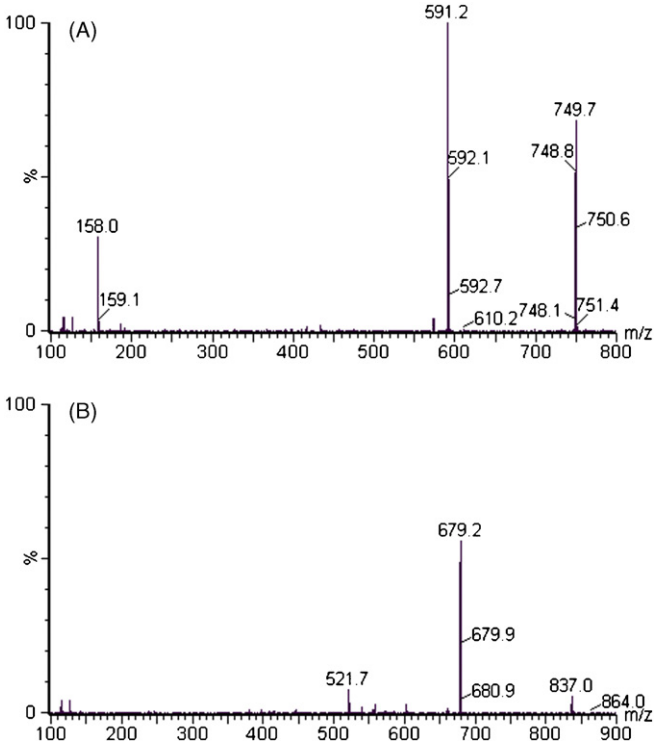

Fig. 2. Product ion spectra of azithromycin (A) and roxithromycin (B).

using freshly prepared calibration standards. The quality control samples (QCs) were prepared with blank plasma at LLOQ, low, middle and high concentrations of 1.00, 2.00, 200, 800 ng/mL and stored at −20 °C after preparation. The standards and quality controls were extracted on each analysis day with the same procedure for plasma samples as described below.

2.4. Plasma sample preparation

To a 0.5 mL aliquot of plasma sample in 10 mL clean glass tubes, 100 μL of internal standard (1000 ng/mL) and 400 μL of 0.05 mol/L sodium carbonate solution were added. The samples were vortexed for 60 s and 3 mL of diethyl ether were added. The mixture was vortex-mixed for another 60 s, then shaken on a mechanical shaker for 10 min. After centrifugation at 3500 × *g* for 10 min, the upper organic layer was then transferred into another set of clean glass tubes and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 200 μL of acetonitrile:water (70:30, v/v), and transferred to 700 μL glass vials, and an aliquot of 5 μL was injected into the UPLC–MS/MS system for analysis.

2.5. Method validation

Validation runs were conducted on three consecutive days. Each validation run consisted of a minimum of one set of calibration standards and six replicates of LLOQ and QC plasma samples at three concentrations. The results from LLOQ and QC plasma samples in three runs were used to evaluate the precision and accuracy of the method developed.

2.5.1. Selectivity

Selectivity was studied by comparing chromatograms of six different batches of blank plasma obtained from six subjects with those of corresponding standard plasma samples spiked with azithromycin and roxithromycin (1000 ng/mL) and plasma sample after oral doses of azithromycin tablets.

2.5.2. Linearity and lower limit of quantification (LLOQ)

Calibration curves were prepared by assaying standard plasma samples at eight concentrations of azithromycin ranging 1–1000 ng/mL. The linearity of each calibration curve was determined by plotting the peak area ratio (y) of azithromycin to roxithromycin (I.S.) versus the nominal concentration (x) of azithromycin. The calibration curves were constructed by weighted ($1/x^2$) least square linear regression.

The lower limit of quantification is defined as the lowest concentration on the calibration curve, it was validated using an LLOQ sample for which an acceptable accuracy (RE) within ±20% and a precision (RSD) below 20% were obtained.

2.5.3. Precision and accuracy

For determining the intra-day accuracy and precision, a replicate analysis of QC plasma samples of azithromycin was performed on the same day. The run consisted of a calibration curve and six replicates of each LLOQ, low, mid, and high concentration quality control samples. The inter-day accuracy and precision were assessed by analysis of three batches on different days. The precision was expressed as the relative standard deviation (RSD) and the accuracy as the relative error (RE).

2.5.4. Extraction recovery and matrix effect

The extraction recovery of azithromycin was determined by calculating the peak areas obtained from blank plasma samples spiked with analyte before extraction with those from blank plasma samples, to which analyte was added after extraction. According to the guidance of USFDA [30], recovery experiments should be performed at three concentrations (low, medium, and high). So this procedure was repeated for five replicates at three concentrations of 2.00, 200 and 800 ng/mL.

In order to evaluate the matrix effect on the ionization of analyte, i.e. the potential ion suppression or enhancement due to the matrix components. Azithromycin at three concentration levels were added to the extract of 0.5 mL of blank plasma, dried and reconstituted with 200 μL of mobile phase, the corresponding peak areas (A) were compared with those of the azithromycin standard solutions dried directly and reconstituted with the same mobile phase (B). The ratio ($A/B \times 100$)% was used to evaluate the matrix effect. The matrix effect of internal standard was also evaluated using the same method.

2.5.5. Stability

2.5.5.1. Freeze and thaw stability. The effect of three freeze and thaw cycles on the stability of plasma samples containing azithromycin was determined by subjecting five aliquots of QC samples at low, mid and high concentration unextracted quality control samples to three freeze–thaw cycles. After completion of

the three cycles, the samples were analyzed and the experimental concentrations were compared with the nominal values.

2.5.5.2. Long-term stability. Five aliquots of QC samples at low, mid and high concentration unextracted QC samples were stored at −20 °C for 30 days. Then, the samples were processed and analyzed and the concentrations obtained were compared with the nominal values.

2.5.5.3. Short-term stability. Five aliquots of QC samples at low, mid and high concentration unextracted QC samples were kept at ambient temperature (25 °C) for 4 h in order to determine the short-term stability of azithromycin in human plasma. Then, the samples were processed and analyzed. The concentrations obtained were compared with the nominal values of QC samples.

2.5.5.4. Post-preparation stability. In order to estimate the stability of azithromycin in the prepared sample, five aliquots of QC samples at low, mid and high concentration were kept in an autosampler maintained at 4 °C for about 4 h. Then, the samples were analyzed and the concentrations obtained were compared with the nominal values.

2.6. Application to pharmacokinetic study

The method was applied to determine the plasma concentrations of azithromycin from a clinical trial in which 20 healthy male volunteers received azithromycin tablet (containing 500 mg azithromycin each). The pharmacokinetic study was approved by the local Ethics Committee and all volunteers gave their signed informed consent to participate in the study according to the principles of the Declaration of Helsinki. Blood samples were collected before and 0.50, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0, 48.0, 72.0, 96.0 and 120.0 h post-dosing. Samples were centrifuged and plasma was separated and stored at −20 °C until analyzed.

The maximum plasma concentration (C_{max}) and their time were noted directly. The elimination rate constant (k_e) was calculated by linear regression of the terminal points of the semi-log plot of plasma concentration against time. Elimination half-life ($t_{1/2}$) was calculated using the formula $t_{1/2} = 0.693/k_e$. The area under the plasma concentration-time curve (AUC_{0-t}) to the last measurable plasma concentration (C_t) was calculated by the linear trapezoidal rule. The area under the plasma concentration–time curve to time infinity ($AUC_{0-\infty}$) was calculated as: $AUC_{0-\infty} = AUC_{0-t} + C_t/k_e$.

3. Results and discussion

3.1. Selection of IS

The best internal standard in LC–MS assay is a deuterated form of the analyte. In our laboratory, no deuterated azithromycin was available, therefore, a compound being structurally or chemically similar to the analyte was considered. In LC–MS/MS the I.S. should also have similar chromatographic and mass spectrometric behaviours to the analyte, and mimic the analyte in any sample preparation steps. Roxithromycin was chosen as the internal standard for the assay because of its similarity of structure, retention time and ionization to azithromycin.

3.2. Chromatography and mass spectrometry

The separation and ionization of azithromycin and roxithromycin were affected by the composition of mobile phase. Therefore, the selection of mobile phase components was critical. Ammonium acetate was employed to supply the ionic strength. With buffers of lower strength, the peak shapes were not satisfactory, whereas with higher strength there was an improvement in the peak shapes. It was found that a mixture of 50 mM ammonium acetate buffer–acetonitrile could achieve this purpose and was finally adopted as the mobile phase. This binary mobile phase is simpler than those reported in the literatures [23–25], which contained methanol, acetonitrile and buffer or with addition of formic acid [26]. It is similar to the mobile phase reported by Abuin et al. [27] for the analysis of macrolide antibiotics in river water except for a different concentration of ammonium acetate.

Gradient elution is usually used in order to extend column life and elute the analyte rapidly. The use of small particles of stationary phase allowed UPLC to push the limits of both peak capacity (due to higher efficiency) and speed of analysis (due to higher linear velocities) without compromising resolution. However, the column with sub-2 μm particles was more easily blocked. Therefore, gradient elution was used to provide a better peak shape and increase the column life.

Two channels were used for recording the response, channel 1 for roxithromycin with a retention time of 1.26 min, and channel 2 for azithromycin with a retention time of 0.81 min. As shown in Fig. 3, both azithromycin and roxithromycin were well separated with excellent peak shapes.

The very narrow chromatographic peaks with a peak width about 6 s, produced by UPLC™ indicated an increase in the chromatographic efficiency which produced a fast separation. Both azithromycin and roxithromycin were rapidly eluted with retention times less than 1.5 min, and the total run time was 3.0 min per sample. The analysis time for azithromycin in the literatures [23,25,27] which used HPLC–MS and HPLC–MS/MS were about 5 min, 6 min and 4 min, respectively. The short analysis time may meet the requirement for high sample throughput in bioanalysis.

The increasing column efficiency in UPLC also resulted in a high sensitivity. The LLOQ in the present method is 1 ng/mL in plasma lower than those reported in the literature [23–25,28] used HPLC–MS and HPLC–MS/MS, which were higher than 2 ng/mL in human plasma.

3.3. Selection of extraction method

As azithromycin is a lipophilic compound, liquid–liquid extraction was applied to extract the analyte. Several extraction solvents such as ethyl acetate, diethyl ether and hexane were investigated, and it was found that diethyl ether extracted the analyte more efficiently. Moreover, the boiling point of diethyl ether

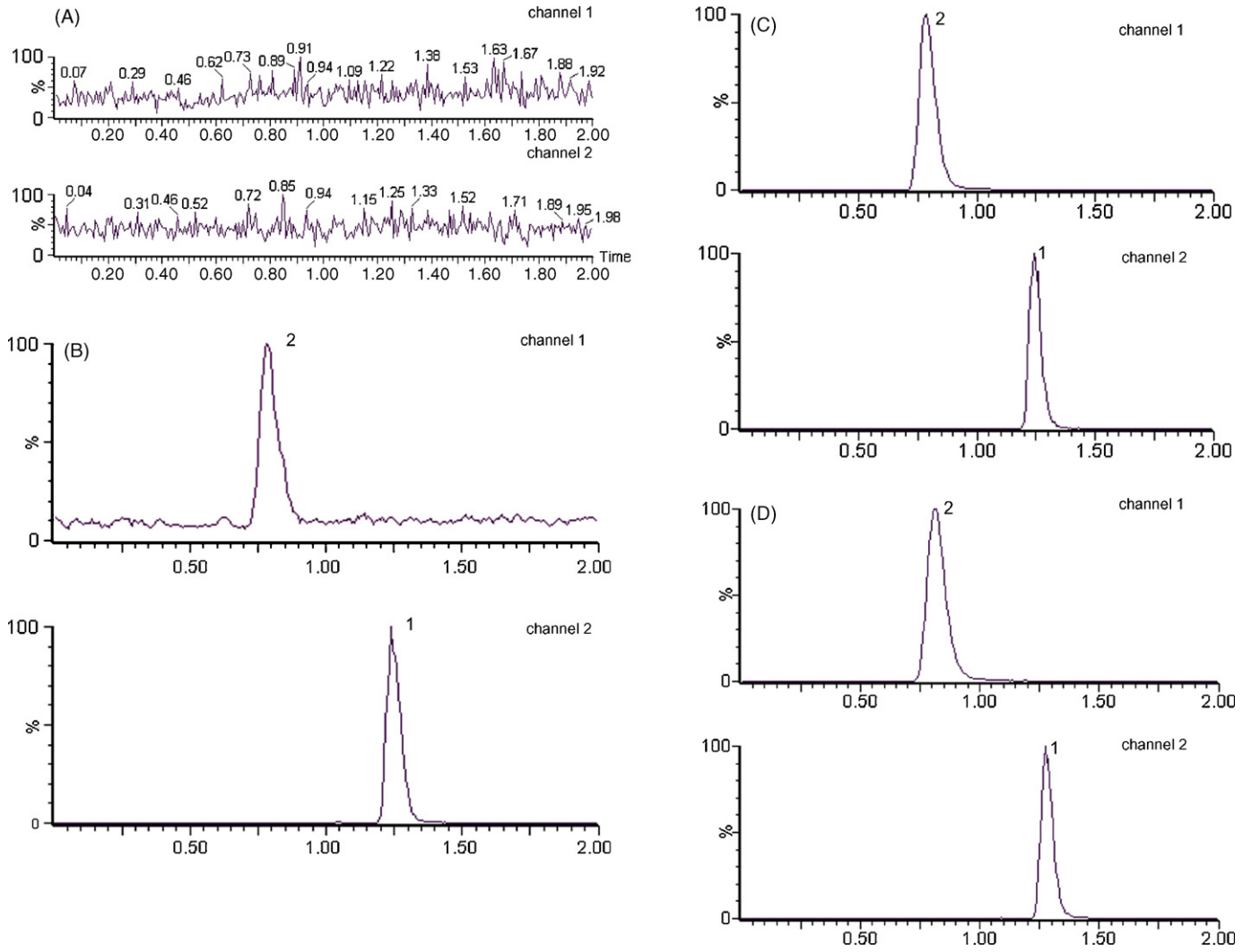

Fig. 3. Representative MRM chromatograms of azithromycin (peak 1, channel 2) and roxithromycin (peak 2, channel 1) in human plasma samples. (A) A blank plasma samples; (B) a blank plasma sample spiked with azithromycin at the LLOQ of 1 ng/mL and roxithromycin (1000 ng/mL); (C) a blank plasma sample spiked with azithromycin at 200 ng/mL and roxithromycin (1000 ng/mL); (D) a plasma sample from a volunteer 1.0 h after oral administration of azithromycin. The retention times of azithromycin and rothxiromycin were 0.81 and 1.26 min, respectively.

is lower, so it was evaporated to dryness more quickly. Therefore, we chose diethyl ether as the extraction solvent. This extraction method is more convenient than the reported method [23], which required methyl *tert*-butyl ether–hexane (50:50, v/v). And the mean recovery of azithromycin in our method was 90.8%, which was higher than 81.2% in the literature [23].

3.4. Method validation

3.4.1. Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. As shown in Fig. 3A, no interference from endogenous substance was observed at the retention time of azithromycin and roxithromycin.

3.4.2. Linearity and LLOQ

The standard calibration curves for azithromycin were linear over the concentration range of 1–1000 ng/mL ($r^2 > 0.99$) by using weighted least square linear regression analysis with a weigh factor of $1/x^2$. A typical equation for the calibration curves was: $y = 7.103 \times 10^{-3}x + 8.294 \times 10^{-4}$, $r = 0.9991$.

The lower limit of quantification for azithromycin was 1 ng/mL with 5 μL injected onto the UPLC column with precision and accuracy presented in Table 1 with RE within ±20% and RSD lower than 20%. A corresponding chromatogram is given in Fig. 3B. Compared with the previous method regarding the determination of azithromycin in human plasma, the present method gave a higher sensitivity with an LLOQ of 1 ng/mL (that is 12.5 pg on column with the injection volume of 5 μL). The high sensitivity could be attributed to the extra resolution and peak sharpness produced by the UPLC chromatographic system and the improved ionization efficiency under the mass spectrometric conditions.

3.4.3. Precision and accuracy

The data of intra-day and inter-day precision and accuracy for the method are listed in Table 1. The intra-day precision for

Table 1
Precision and accuracy for the determination of azithromycin in human plasma (intra-day: $n = 6$; inter-day: $n = 6$ series per day, 3 days)

Added C (ng/mL)	Found C (ng/mL)	Intra-run RSD (%)	Inter-run RSD (%)	Accuracy RE (%)
1.00 (LLOQ)	1.00 ± 0.06	5.7	4.0	−0.2
2.00 (low)	1.97 ± 0.17	8.4	4.7	−1.3
200.0 (middle)	204 ± 11.7	5.2	7.3	2.0
800.0 (high)	846 ± 43.3	4.0	9.4	5.7

low, mid and high QC levels of azithromycin were 8.4%, 5.2% and 4.0%, respectively, and that of inter-day analysis were 4.7%, 7.3%, 9.4%, respectively, with an accuracy (RE) within −1.3% to 5.7%. The precision and accuracy of the present method conform to the criteria for the analysis of biological samples according to the guidance of USFDA [30] where the precision (RSD) determined at each concentration level is required not exceeding 15% and accuracy (RE) within ±15% of the actual value.

3.4.4. Extraction recovery and matrix effect

The extraction recoveries of azithromycin from human plasma were 92.1 ± 5.0%, 87.9 ± 2.9%, and 92.3 ± 4.5% at concentration levels of 2.00, 200 and 800 ng/mL, respectively, and the mean extraction recovery of roxithromycin was 80.6 ± 2.7%.

In terms of matrix effect, all the ratios $(A/B \times 100)$% defined as in Section 2 were between 85% and 115%, which means no matrix effect for azithromycin and roxithromycin in this method.

3.4.5. Stability

The stock solution of azithromycin and roxithromycin were found to be stable at room temperature for 4 h and at 4 °C for 25 days. The results from all stability tests presented in Table 2 demonstrated a good stability of azithromycin over all steps of the determination. The method is therefore proved to be applicable for routine analysis.

3.5. Pharmacokinetic application

The present method was successfully applied to the pharmacokinetic study of azithromycin after oral administration in healthy male volunteers. Mean plasma concentration–time curve of azithromycin in single dose study is shown in Fig. 4.

After administration of a single dose of 500 mg azithromycin, the C_{max} and T_{max} were 565.9 ± 207.6 ng/mL and 1.9 ± 0.6 h, respectively. Plasma concentration declined with the $t_{1/2}$ of 50.1 ± 5.0 h. The AUC_{0-t} and $AUC_{0-\infty}$ values obtained were 4536.0 ± 1018.8 ng h/mL and 5243.3 ± 1257.3 ng h/mL,

Table 2
Stability of azithromycin in human plasma at three QC levels ($n = 5$)

Stability	Accuracy (mean ± SD) (%)		
	2.00 (ng/mL)	200 (ng/mL)	800 (ng/mL)
Short-term stability	104.0 ± 0.2	96.5 ± 6.2	99.3 ± 2.7
Long-term stability	98.3 ± 0.1	106.5 ± 1.2	97.0 ± 4.7
Freeze–thaw stability	104.1 ± 0.1	102.3 ± 7.8	101.6 ± 5.7
Post-preparative stability	100.0 ± 0.1	99.3 ± 4.1	100.0 ± 2.8

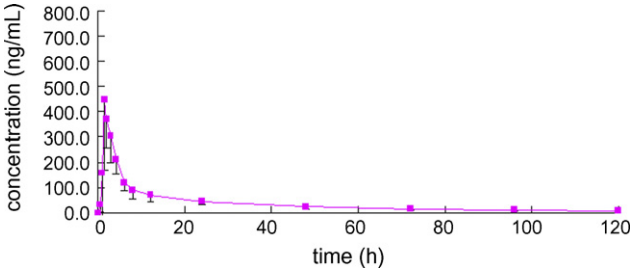

Fig. 4. Mean plasma concentration–time curve of azithromycin in 18 volunteers after a single oral dose of azithromycin.

respectively. The results were similar to the reported pharmacokinetic parameters with the same dosage form and dosage [29], indicating the applicability of this method to the pharmacokinetic study of azithromycin.

4. Conclusion

A sensitive, selective and rapid UPLC–ESI-MS/MS method for the determination of azithromycin in human plasma is described. Compared with the published methods, the sharp peaks produced by UPLC are of particular advantage when coupled to electrospray mass spectrometry, reducing ion suppression and offering superior sensitivity with an LLOQ of 1 ng/mL, satisfactory selectivity and short run time of 3.0 min. The method has been successfully applied to a pharmacokinetic study of azithromycin given in tablet form to healthy volunteers.

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