Azithromycin Quantitation in Human Plasma by High-Performance Liquid Chromatography Coupled to Electrospray Mass Spectrometry: Application to Bioequivalence Study

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Abstract

A sensitive and specific liquid chromatography–electrospray ionization mass spectrometry method is developed and validated for the identification and quantitation of azithromycin in human plasma. After the addition of the internal standard and 1.0M sodium hydroxide solution, plasma samples are extracted with a methylene chloride–ethyl acetate mixture (20:80, v/v). The organic layer is evaporated under a stream of nitrogen at 40°C. The residue is reconstituted with 200 µL of the mobile phase. The compounds are separated on a prepacked Shimadzu Shim-pack VP-ODS C18 (5 µm, 150 mm x 2.0 mm) column using a mixture of acetonitrile–water (65:35) (0.5% triethylamine, pH was adjusted to 6.2 with acetic acid) as the mobile phase. Detection is performed on a single quadrupole mass spectrometer by selected ion monitoring mode via electrospray ionization source. The method is fully validated and linear calibration curves are obtained in the concentration ranges from 5 to 2000 ng/mL. The intra- and interbatch relative standard deviations at four different concentration levels are all < 10%. The limit of detection and quantitation are 2 ng/mL and 5 ng/mL, respectively. The proposed method enables the unambiguous identification and quantitation of azithromycin for pharmacokinetic, bioavailability, or bioequivalence studies.

Introduction

Azithromycin ([2R-[2R*,3S*,4R*,5R*,8R*,10R*,11R*,12S*,13S*,14R*]]-13-[[2,6-Dideoxy-3-C-methyl-3-O-methyl-α-L-ribo-hexopyranosyl]oxy]-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,14-heptamethyl-11-[[3,4,6-trIDEOXY-3-(dimethylamino)-β-D-xylo-hexopyranosyl]oxy]-1-oxa-6-azacyclotetradecan-15-one) is a macrolide antibiotic derived from erythromycin. It differs chemically from erythromycin in that an ethyl-substituted nitrogen atom is incorporated into the lactone ring (as can be seen in Figure 1) which results in improved acid stability and oral bioavailability compared with erythromycin.

Azithromycin plays a leading role in the treatment or prophylaxis of several diseases such as opportunistic infections in AIDS, toxoplasmosis, pediatric infections, and respiratory tract infections (1–5).

Determination of the pharmacokinetic profile of azithromycin is important for gaining a better understanding of its mechanism of action, and for ensuring a more efficient therapeutic application. Therefore, several methods have been reported for the determination of azithromycin in plasma.

High-performance liquid chromatography (HPLC) with UV (6), fluorescence (7), or electrochemical (8–13) detection have been the widely used methods to quantitate azithromycin in biological liquids, but these methods were of low sensitivity and not sufficient for pharmacokinetic studies at low plasma concentrations.

As an effort to reduce the time required for drug testing in biological fluids, our laboratory has been continually investigating the applications of LC–mass spectrometry (MS) in biological analysis. The aim of the present study was to combine a fast HPLC technique with MS in order to validate a robust and repro-
ducible reversed-phase LC–MS method for azithromycin determination in human plasma, and to dramatically increase sample throughput. This method was validated to ensure the proper quantitation of azithromycin in human plasma down to the concentration limit of 5 ng/mL. At the same time, it was expected that the method would be efficient in analyzing large numbers of plasma samples, supporting pharmacokinetic, bioavailability, or bioequivalence studies after therapeutic doses of azithromycin.

**Experimental**

**Chemicals and reagents**

Azithromycin orally disintegrating test tablets were supplied by Jinling Pharmaceutical Co., Ltd (Nanjing, P.R. China). Azithromycin orally disintegrating tablets were purchased from The United Laboratories Ltd. (Zhuhai, P.R. China). Acetonitrile was chromatographic pure grade and purchased from Merck (Darmstadt, Germany). Other chemicals were all of analytical grade. Deionized water was distilled before use. Other reagents were used as received.

**Instrumentation and operating conditions**

LC was performed on a Shimadzu LC-10AD HPLC system consisting of an autosampler (SIL-10ATc). The HPLC was coupled to a Shimadzu LC–MS-2010A triple quadrupole MS with an electrospray ionization (ESI) interface. Data acquisition and processing were accomplished using Shimadzu LC–MS solution software for LC–MS-2010 HPLC–MS.

Chromatographic separation was carried out at 40°C with a Shimadzu Shim-pack VP-ODS C18 (5 µm, 150 mm × 2.0 mm) column. The mobile phase consisted of acetonitrile–water [0.5% triethylamine (TEA), pH was adjusted to 6.2 by acetic acid] = 65:35 (v/v) and was set at a flow rate of 0.2 mL/min. The ESI source was set at the positive ionization mode. The [M+H]+, m/z 749.35 for azithromycin and [M+H]+, m/z 837.35 for roxithromycin were selected as detecting ions, respectively. The MS operating conditions were optimized as follows: drying gas, 1.5 L/min; CDL temperature, 280°C; block temperature, 230°C; and probe voltage, +4.5 kv.

**Preparation of stock solutions**

Primary stock solutions of azithromycin for preparation of standards and quality controls (QC) were prepared with accuracy weighing. The primary stock solutions were prepared in solvent (acetonitrile–water = 65:35, v/v) at a concentration of 1.0 mg/mL and were stored at 4°C. The internal standard (IS) stock solution was prepared by dissolving 10.0 mg of roxithromycin in 10 mL solvent [acetonitrile–water (65:35), v/v], producing a concentration of 1.0 mg/mL, and was stored at 4°C. Working solutions of azithromycin were prepared daily in solvent (acetonitrile–water = 65:35, v/v) by appropriate dilutions at 1.0, 10, and 100 µg/mL.

**Calibration curves**

Calibration curves were prepared by spiking different samples of 0.2 mL blank plasma each with the proper volume of one of the previously mentioned working solutions to produce a calibration curve point equivalent to 5, 10, 25, 50, 100, 250, 500, 1000, and 2000 ng/mL of azithromycin. Each sample also contained 1 µg of the IS. In each run, a plasma blank sample (no IS) was also analyzed. Calibration curves were prepared by determining the best-fit of peak area ratios (peak area of analyte/peak area of IS) versus concentration, and fitted to the equation \( R = bC + a \) by unweighted least-squares regression.

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Preparation of QC samples

QC samples were prepared at four different concentration levels: low limit, middle level, and high level. QC samples were prepared daily by spiking different samples of 0.2 mL blank plasma each with the proper volume of the corresponding standard solution to produce a final concentration equivalent to 10, 50, 500, and 2000 ng/mL of azithromycin and 1 µg of internal standard.

Extraction procedure

QC, calibration curve, and clinical plasma samples were extracted employing a liquid–liquid extraction technique. To each tube containing 0.2 mL plasma, 1 µg of IS, 80 µL of 1.0 M sodium hydroxide solution, and 3 mL methylene chloride–ethyl acetate mixture (20:80, v/v) solution were added and vortexed for 3 min. Afterwards, samples were centrifuged for 10 min at 13800 × g. The organic layer was evaporated under a stream of nitrogen at 40°C. The residue was redissolved in 200 µL of the mobile phase. An aliquot of 10 µL was injected into the LC–MS system.

Method validation

The method validation assays were carried out according to the currently accepted US Food and Drug Administration (FDA) bioanalytical method validation guidance (14). The following parameters were considered.

The method’s specificity was tested by screening six different batches of healthy human blank plasma. Each blank sample was tested for interference using the proposed extraction procedure and chromatographic/spectroscopic conditions, and compared with those obtained with an aqueous solution of the analyte at a concentration near to the lower limit of quantitation.

The matrix effect on the ionization of analytes was evaluated by comparing the peak area of analytes resolved in blank sample (the final solution of blank plasma after extraction and reconstitution) with that resolved in mobile phase. Three different concentration levels of azithromycin (10, 50, 500, and 2000 ng/mL) were evaluated by analyzing six samples at each level. The blank plasma used in this study was six different batches of healthy human blank plasma. At ratios < 85% or > 115%, an exogenous matrix effect was implied.

Linearity was tested for the range of concentrations 5–2000 ng/mL. For the determination of linearity, standard calibration curves of at least seven points (non-zero standards) were used. In addition, a blank plasma sample was also analyzed to confirm the absence of interferences; this sample was not used to construct the calibration function. The acceptance criterion for correlation coefficient was 0.99 or more, otherwise the calibration curve would be rejected. Five replicate analyses were done.

The precision of the assay was determined from the QC plasma samples by replicate analyses of four concentration levels of azithromycin (10, 50, 500, and 2000 ng/mL). Within-batch precision and accuracy were determined by repeated analysis of the group of standards on one day (n = 5). Between-batch precision and accuracy were determined by repeated analysis on three consecutive days (n = 5 series per day). The concentration of each sample was determined using a standard curve prepared and analyzed on the same day.

The extraction yield (or absolute recovery) was determined by comparing the azithromycin/IS peak area ratios obtained following the outlined extraction procedure (the procedure was a little different from the outlined extraction procedure for QC, calibration curve, and clinical plasma samples; that is, IS was added to the organic layer after the extraction of azithromycin) with a procedure obtained from those which contained the same amount of azithromycin in extracted plasma but not extracted after addition of the drug. This procedure was repeated for the four different concentrations of azithromycin added; namely 10, 50, 500, and 2000 ng/mL.

The limit of quantitation (LOQ) was defined as the lowest drug concentration that can be determined reproducibly (coefficient of variation < 20%) and accurately (percent error < 20%). The LOQ of this method was 5 ng/mL.

Short-term temperature stability: 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 (approximately 6 h). Samples were analyzed as mentioned previously.

Post-preparative stability: The autosampler stability was conducted by reanalyzing extracted QC samples kept under the autosampler conditions (4°C) for 12 h.

Freeze and thaw stability: QC plasma samples containing azithromycin were tested after three freeze (–20°C) and thaw (room temperature) cycles.

Long-term stability of azithromycin in human plasma was studied for a period of 8 weeks employing QC samples at three different levels. If after the stability study, the analyte was found to be unstable at –20°C, then it would be stored at –70°C.

The stability of azithromycin and internal
standard working solutions were evaluated by testing their validity for 6 h at room temperature. The stability of working solutions was expressed as percentage recovery.

A calibration curve was generated to assay samples in each analytical run and was used to calculate the concentration of azithromycin in the unknown samples in the run. The calibration was analyzed in the middle of each run. In order to monitor the accuracy and precision of the analytical method, a number of QC samples were prepared to ensure that the method continues to perform satisfactorily. The QC samples in duplicate at three concentrations (10, 50, 500, and 2000 ng/mL) were prepared and analyzed with processed test samples at intervals based on the total number of samples per batch.

**Clinical study design**

**Subjects**

This was an open, randomized, balanced, two-period crossover study in 20 healthy Chinese men. Each volunteer received in random order a single oral dose of 500 mg azithromycin orally disintegrating test tablets or orally disintegrating reference tablets in cycle. Blood samples (5 mL) for the assay of plasma concentrations of azithromycin were collected at 0, 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 48, 72, 96, and 144 h after oral administration of the tablets. They were put into lithium heparin tubes and immediately centrifuged at 2000 g for 10 min. The plasma obtained was frozen at –20°C in coded polypropylene tubs until analysis.

**Pharmacokinetic analysis**

Pharmacokinetic analysis was performed by means of a model independent method using a BAPP 2.2 computer program. The elimination rate constant (λ₂) was obtained as the slope of the linear regression of the log transformed plasma concentration values versus time data in the terminal phase. The elimination half-life (T1/2) was calculated as 0.693/λ₂. The area under the curve to the last measurable concentration (AUC₀–t) was calculated by the linear trapezoidal rule. The area under the curve extrapolated to infinity (AUC₀–∞) was calculated as AUC₀–t + Cᵣλ₂, where Cᵣ is the last measurable concentration.

**Statistical analysis**

To assess the bioequivalence between the two formulations, AUC₀–t, AUC₀–∞, and Cₘₐₓ were considered as the primary variables. Two-way analysis of variance (ANOVA, BAPP2.2 computer program) for crossover design was used to assess the effect of formulations, periods, sequences, and subjects on these parameters. The difference between two related parameters was considered statistically significant for p-value equal to or less than 0.05. Parametric 90% confidence intervals based on the ANOVA of the mean test/reference (T/R) ratios of AUCs and Cₘₐₓ were computed. Wilcoxon’s signed rank test was utilized to compare Tₘₐₓ.

**Results and Discussion**

**Selection of IS**

It is necessary to use an IS to get high accuracy when a mass spectrometer is used as an HPLC detector. Roxithromycin was adopted in the end because of its similarity of structure (Figure 1), retention, and ionization to the analyte.

**Sample preparation**

Liquid–liquid extraction was necessary and important because this technique can purify the sample. Ethyl acetate, methylene chloride–ethyl acetate mixture (20:80, v/v), and isopropanol–n-hexane (5:95, v/v) were all tested for extraction and methylene chloride–ethyl acetate mixture (20:80, v/v) was finally adapted because of its high extraction efficiency. NaOH (1.0M, 0.2 mL) was added to the plasma in order to accelerate the drugs’ dissociation from the plasma and reduce interference, because most endogenous are of acidic nature.

In our studies of optimization, the vortex times 1, 2, 3, 4, and 5 min were all estimated; we found that the vortex time influenced the extraction efficiency significantly and the extraction was fully completed after 3 min. A 3 min vortex time was subsequently used in our experiments.

**Separation and specificity**

Positive ion ESI mass scan spectrum of azithromycin and IS
was shown in Figures 2 and 3, respectively. The major ions observed were \([\text{M+H}]^+, \text{[M+Na]}^+, \text{[M+K]}^+\) peaks at \(m/z\) 749.35, 771.25, and 787.25 for azithromycin and \([\text{M+H}]^+, \text{[M+Na]}^+, \text{[M+K]}^+\) peaks at \(m/z\) 837.35, 859.35, and 875.35 for roxithromycin. The ions of \([\text{M+H}]^+, m/z\) 749.35 for azithromycin and \([\text{M+H}]^+, m/z\) 837.35 for roxithromycin were finally selected for the SIM(+) mode for their stability and high intensity.

The SIM(+) chromatograms extracted from supplemented plasma are depicted in Figure 4. As shown, the retention times of azithromycin and IS were 4.0 and 4.8 min, respectively. In our studies, we found that the concentrations of TEA in the mobile phase had a significant effect on the peak shapes of azithromycin and roxithromycin. Finally, 0.5% of TEA in the mobile phase was proved to be enough to ensure that azithromycin and roxithromycin had symmetrical peaks without tailing.

The total HPLC–MS analysis time was 6.0 min per sample. No interferences of the analyte were observed because of the high selectivity of the SIM technique. No ion suppression effects were observed under the developed sample preparation and chromatographic conditions. Figure 5 shows an HPLC chromatogram for a blank plasma sample indicating no endogenous peaks at the retention times of azithromycin or IS (roxithromycin). The SIM(+) chromatograms obtained from an extracted plasma sample of a healthy volunteer who participated in a bioequivalence study conducted on 20 persons are depicted in Figure 6.

The purpose of these investigations was to develop a specific and sensitive assay for the determination of the macrolide antibiotic azithromycin. HPLC–ESI-MS has several advantages for the analysis of azithromycin. The combination of HPLC (under the isocratic conditions described) with ESI-MS leads to short retention time and yields both high selectivity and sensitivity. ESI is a “gentle” ionization technique that produces high mass-to-charge \([\text{M+H}]^+\) precursor ions with minimal fragmentation of the analyte.

**Method validation**

The method exhibited a good linear response for the range of concentrations from 5 to 2000 ng/mL. The function of mean calibration curve is \(R = 0.0036C + 0.0043\) with a coefficient of determination \((r)\) of 0.9973.

Data are presented in Table I for the intra- and inter-batch precision of the method for azithromycin as determined from the QC samples run at the concentrations of 10, 50, 500, and 2000 ng/mL.

The lower limit of quantitation for azithromycin was proven to be 5 ng/mL, and the lower limit of detection (LLOD) for azithromycin was 2 ng/mL. Figure 7 shows the chromatogram of an extracted sample that contained 2 ng/mL of azithromycin (LLOD).

The extraction recovery (Table II) determined for azithromycin was shown to be consistent, precise, and reproducible. The mean recoveries of the low, mid, and high QC levels were 92.71, 89.07, 86.64, and 87.12%, respectively; whereas the precision (RSD) was 5.46, 6.19, 4.56, and 4.88%, respectively.

**Stability**

Table III summarizes the short-term stability, freeze and thaw stability, long-term stability, and post-preparative stability data of azithromycin.
Short-term stability indicated reliable stability behavior under the experimental conditions of the regular runs. The results of freeze and thaw stability indicated that the analyte is stable in human plasma for three cycles of freeze and thaw, when stored at –20°C, and thawed to room temperature. The post-preparative stability of QC samples shows that azithromycin was stable when kept at 4°C in the autosampler for 24 h. The findings from long-term stability indicate that storage of azithromycin’s plasma samples at –20°C is adequate when stored for 8 weeks, and no stability-related problems would be expected during routine analysis of the samples for pharmacokinetic, bioavailability, or bioequivalence studies.

The stability of working solutions was tested and established at room temperature for 6 h. Based on the obtained results, these working solutions were stable within 6 h.

Results of pharmacokinetic and relative bioavailability study

The method was applied to analyze plasma samples obtained from 20 healthy volunteers who each received a single dose of 500 mg azithromycin preparations in the bioequivalence study.

The mean timed plasma concentrations of azithromycin are shown in Figure 8. Pharmacokinetic parameters are shown in Table IV. ANOVA for these parameters, after log-transformation of the data, showed no statistically significant difference between the two formulations either in periods, formulations, or sequence, with p value greater than 0.05. The 90% confidence intervals also demonstrated that the ratios of $AUC_{0-\infty}$, $AUC_{0-\infty}$, or $C_{\text{max}}$ of the two formulations lie within the FDA acceptable range of 80%–125%.

The mean ratio of $AUC_{0-\infty}/AUC_{0-\infty}$ for test and reference formulations of 93.5% and 93.11%, respectively, indicating that the sampling time was adequate. The 90% confidence limits for $AUC_{0-\infty}$, $AUC_{0-\infty}$, and $C_{\text{max}}$ as well as the results of the Schuurmann’s two one-sided t-tests are shown in Table V. The 90% CI for $AUC_{0-\infty}$, $AUC_{0-\infty}$, and $C_{\text{max}}$ were within the bioequivalence acceptable range of 80% to 125%. Furthermore, the results of the Schuurmann’s t-test indicated that the lower and upper limits of the calculated t-test were greater than the critical t-value. Therefore, the two formulations can be considered bioequivalent with regard to the extent and rate of absorption.

Conclusion

The proposed method of analysis provided a sensitive and specific assay for azithromycin determination in human plasma. A simple liquid–liquid extraction procedure and a short run time can curtail the test’s time, which is important for large sample batches. It was shown that this method is suitable for the analysis of azithromycin in plasma samples collected for pharmacokinetic, bioavailability, or bioequivalence studies.

References


