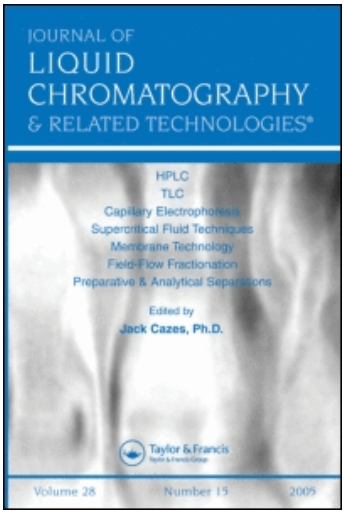

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AN ALTERNATIVE LIQUID CHROMATOGRAPHY-MASS SPECTROMETRIC METHOD FOR THE DETERMINATION OF AZITHROMYCIN IN HUMAN PLASMA AND ITS APPLICATION TO PHARMACOKINETIC STUDY

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AN ALTERNATIVE LIQUID CHROMATOGRAPHY-MASS SPECTROMETRIC METHOD FOR THE DETERMINATION OF AZITHROMYCIN IN HUMAN PLASMA AND ITS APPLICATION TO PHARMACOKINETIC STUDY

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□ *A simple, sensitive, selective, and reproducible method based on high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (LC/MS) was developed for the determination of a macrolide antibiotic azithromycin in human plasma. The internal standard (roxithromycin) was separated from azithromycin on a Hypersil Gold C_{18} column, with retention times of 10.71 and 13.67 min, respectively. The mobile phase consisted of a mixture of 20 mM ammonium acetate buffer (pH 5.2), acetonitrile and methanol (50:40:10, v/v/v), running through the column at a flow rate of 0.3 mL/min. The chromatographic analysis was operated at 25° C. Sample preparation was prepared by liquid-liquid extraction with a mixture of 7:3 (v/v) diethylether:dichloromethane. The precision of the method based on within-day repeatability and reproducibility (day-to-day variation) was below 5% (% coefficient of variations: % CV). Good accuracy was observed for both the intra-day or inter-day assays. Limit of quantification was accepted as 1 ng using 200 μL plasma samples. The mean recoveries for azithromycin and the internal standard were greater than 85%. The method was applied successfully to the investigation of the pharmacokinetics of azithromycin when given in combination with fosmidomycin as oral doses of 750 mg twelve hourly for 3 days in a total of 5 Thai male patients with acute uncomplicated falciparum malaria.*

Keywords azithromycin, human plasma, liquid chromatography, mass-spectrometry, pharmacokinetics

INTRODUCTION

Macrolide antibiotics, as exemplified by azithromycin (Figure 1a), are inhibitors of protein synthesis by specifically binding to the 50S subunit

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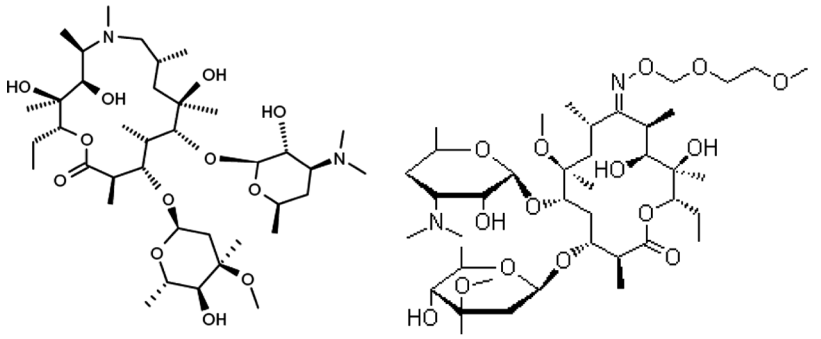

FIGURE 1 Chemical structures of (a) azithromycin and (b) the internal standard roxithromycin.

of the ribosomes in the apicoplast.[1] Azithromycin has been successfully used in combination with artemisinin derivatives and quinine for prophylaxis and treatment of malaria.[2–4] Fosmidomycin, has been shown to be an effective malarial blood schizontocide in addition to its wide spectrum of antibacterial activity. It possesses a novel mode of action through inhibition of 1-deoxy-D-xylose 5-phosphate (DOXP) reductoisomerase, an essential enzyme of the non-mevalonate pathway.[5–7] The combination of fosmidomycin and azithromycin represents an innovative approach to malaria chemotherapy through novel modes of action, coupled with the benefit of additive activity against *Plasmodium falciparum in vitro and in vivo*,[6] there are grounds for anticipating lack of cross resistance with existing drugs and protection against the development of resistance. Furthermore, the prospects for this combination are enhanced by the disparity in the half-lives of the two components affording an early therapeutic response and protection from recrudescent infections within the constraint of a three-day dosing regimen. Azithromycin confers a much improved pharmacokinetic profile and more favorable toxicological profile comparing with erythromycin.[7–9]

A number of analytical methods have been reported for the determination of azithromycin and other macrolides in human and animal biological fluids (plasma, serum, and tissues), water, and pharmaceutical products. These methods involve bioassay,[10] high performance liquid chromatography (HPLC) with ultraviolet,[11,12] electrochemical,[13–15] fluorescence,[16–18] and HPLC with mass-spectrometry (LC/MS)[19–24] detection. Among these methods, LC/MS is the most sensitive method with limit of quantification in the range of 1–5 ng/ml using 100–500 μL plasma or serum samples. The limit of quantification (LOQ) range from 0.05 to 20 ng/mL. The commonly used LC/MS/MS methods are based on electrospray ionization (EPI) tandem mass spectrometry[20,22,23] and atmospheric

pressure chemical ionization[19] for determination of azithromycin alone or simultaneously with other macrolides (erthythromycin, clarithromycin, roxithromycin). The sample clean-up and concentration procedures include either liquid-liquid extraction[20–23] or solid phase extraction.[13]

In the present study, we propose an alternative simple and sensitive LC/MS method with electrospray ionization for determination of azithromycin based on a single step and straight forward liquid-liquid extraction with high sensitivity down to 1 ng using 200 μL plasma sample. The method was applied for the investigation of the pharmacokinetics of azithromycin in Thai patients with acute uncomplicated falciparum malaria following a 3-days combination regimen of azithromycin and fosmidomycin.

EXPERIMENTAL

Chemicals

Azithromycin and the internal standard roxithromycin (99% pure) were purchased from Sigma Chemical., Co (St Louis, MO, USA). The following chemicals and solvents were obtained in the highest purity available: dichloromethane, diethylether, acetonitrile, and methanol (LAB-Scan LTD, Analytical Sciences, Thailand), ammonium acetate (BDH Laboratory supplies poole, England), ammonium hydroxide, ammonium acetate, and phosphoric acid acid (Sigma-Aldrich CO, USA). Demonized double distilled water was used for the preparation of working nicotine standard solutions.

Preparation of Standards

Stock solutions of azithromycin and roxithromycin (1,000 ng/μL) were prepared by dissolving 5 mg of the compound in 5,000 μL methanol and stored at −20°C until used. Working standard solutions were prepared by diluting the stock standard solutions with methanol. Standard solutions were stored at −20°C until analysis.

Seven aliquots of blank control plasma were spiked with azithromycin working solution in a serial dilution to obtain the standard calibration at the concentrations of 5, 10, 20, 50, 150, 250, and 500 ng/mL, with 250 ng internal standard (5 ng/μL roxithromycin).

Chromatography

Azithromycin and the internal standard were separated on a Hypersil Gold C_{18} reversed phase column (Thermo, 4.6 × 150 mm, 5 μm particle size). The HPLC system was operated under an isocratic mode at a flow-rate

of 0.3 mL/min. The mobile phase was a mixture of 20 mM ammonium acetate buffer (pH 5.2), acetonitrile, and methanol at a ratio of 50:40:10 (v/v/v). The HPLC system consisted of a solvent delivery system (SpectraSystem™ P4000 pump), and a vacuum membrane degasser (SpectraSystem™ SCM1000) was used to minimize gasses from the eluent flow prior to the introduction of a chromatographic sample into the mobile phase. For creating ions from analyses in solution, electrospray ionization was used to produce ions from solution into the gas phase and monitored the selected ions reaching the detector by measuring the ion current for one particular mass.

The mass spectrometer consisted of a Finnigan LCQ Deca XP Max plus ion trap detector equipped with the positive electron spray ionization (ESI) interface. The heated nebulizer was set at 300°C and pressure of 551 kPa; the flow-rates of auxiliary nitrogen gas and curtain gas were set at 70 and 15 arb, respectively. Mass results were plotted and processed by the LcQuan™ 2.0 (Thermo Electron Corporation, California, USA). Ions monitored in the selected reaction monitoring (SRM) mode were m/z 749.6 m/z for azithromycin and 837.6 m/z for roxithromycin. Argon was used as the collision gas and electron multiplier was set at 5,000 V.

Sample Preparation

Two hundred microliters (μL) of unknown plasma samples, or quality control samples were transferred to polypropylene tubes, and 50 μL internal standard (5 ng/μL) was added to each tube. After thoroughly mixing, methanol (50 μL) and 0.25 M carbonate-bicarbonate buffer pH 9.5 (250 μL) were added. After vortex mixing, the mixture was then extracted with 3 mL of the mixture of 7:3 (v/v) diethylether and dichloromethane by vortex mixing for 30 seconds. The upper organic phase was separated through centrifugation at 2,500 × g for 5 min at 4°C; the clear supernatant was transferred into a second set of 10-mL screw-capped Teflon tubes. Organic solvents were evaporated to dryness under stream of nitrogen at 40°C. The residue was reconstituted with 200 μL of the mobile phase, and 10 μL portion was injected onto the column.

Calibration Curves

The linearity of the method was observed in the expected concentration range, demonstrating its suitability for analysis. This LC/MS method was linear over the concentrations range of 5 to 1,000 ng/mL.

Calibration curves of azithromycin (5, 10, 50, 100, 200, 500, and 1,000 ng/mL) were prepared in the same day as sample analyses with varying

concentrations of azithromycin and a fixed concentration of roxithromycin (250 ng). Samples were analyzed as described previously.

Data Analysis

Concentrations of azithromycin were determined from the peak height ratios (peak heights of azithromycin/peak heights of internal standard), which corresponded to the known azithromycin concentrations in a calibration curve as described previously. Peak detection, peak height integration, and peak height ratio calculation were performed by the Millennium 2000 Chromatograph™ software.

Method Validation

Precision

The precision of the method based on *within-day repeatability* was determined by replicate analysis of six sets of samples spiked at four different concentrations of azithromycin (5, 50, 200, and 1,000 ng/mL plasma). The *reproducibility (day-to-day variation*) of the method was validated using the same concentration range of plasma as described previously, but only a single determination of each concentration was made on six different days. Coefficient of variation (CV) were calculated from the ratios of standard deviation (SD) to the mean and expressed as percentage.

Accuracy

Accuracy of the method was determined by replicate analysis of six sets of samples spiked at four different levels of azithromycin (5, 50, 200, and 1,000 ng/mL plasma) and comparing the difference between the spiked value and that actually found (theoretical value).

Recovery

The analytical recovery of sample preparation procedure for azithromycin was estimated by comparing the peak heights obtained from samples (plasma) prepared as described previously, with those measured with equivalent amounts of azithromycin in methanol. Triplicate analysis was performed at azithromycin and internal standard concentrations of 5, 50, and 200 ng/mL, and 250 ng/mL, respectively.

Selectivity

The selectivity of the assay was demonstrated by checking for the absence of (i) endogenous interferences at the retention times of azithromycin in

human blank plasma obtained from six different lots, and (ii) the interference by commonly used drugs, i.e., most antibacterials, antimalarials (mefloquine, quinine, artesunate), and dimenhydrinate, after subjecting them to sample preparation procedures.

Limit of Quantification

The limit of quantification (LOQ) of the assay procedure was determined from the lowest concentration of azithromycin (in spiked plasma sample) that produced a peak height ten times the baseline noise (absorbance unit full scale) in a 200 µL sample, which also produced acceptable accuracy (<20% of the nominal values) and precision (expressed as the coefficient of variation, CV <20%).

Stability

The stability of azithromycin was determined by storing spiked plasma samples (at the concentrations of 5, 50, and 200 ng/mL plasma; triplicate analysis for each concentration) in a −20°C freezer (Sanyo, Japan) for up to 1 month.

Quality Control

Quality control (QC) samples for azithromycin were made up in plasma using a stock solution separated from that used to prepare the calibration curve, at the concentrations of 5, 50, 200, and 1,000 ng/mL plasma. Samples were aliquoted into cryovials, and stored frozen at −20°C for use with each analytical run. The results of the QC samples provided the basis of accepting or rejecting the run. At least four of the six QC samples had to be within ±20% of their respective nominal values. Two of the six QC samples could be outside the ±20% of their respective nominal value, but not at the same concentration.

Application of the Method to Biological Samples

The method was applied to the investigation of pharmacokinetics o azithromycin when given in combination with fosmidomycin at 750 mg (250 mg *per* capsule Zithromax™, Pfizer, USA) given every twelve hours for three days in 5 Thai male patients with acute uncomplicated falciparum malaria (aged 25–42 years). Blood samples were collected from all patients at 0, 1, 2, 3, 4, 6, 8, 12, 14, 18, 24, 26, 30, 36, 38, 42, 48, 50, 54, 60, 62, 66, 72, 78, 84, 90, 96, and 108 hours after the first dose. The study was conducted at Hospital for Tropical Diseases and the study protocol was approved by the Ethics Committees of the Faculty of Tropical Diseases, Mahidol University,

Thailand. Written informed consents for study participation were obtained from all subjects who had been informed of the study protocol.

RESULTS AND DISCUSSION

Chromatographic Separation

Under the chromatographic condition previously described, the chromatograms of azithromycin and internal standard (roxithromycin) were free from any interference peak, with good resolution and sharp peaks. Blank plasma samples showed little noise fluctuation (Figure 2a). The retention times of azithromycin and roxithromycin were 10.71 and

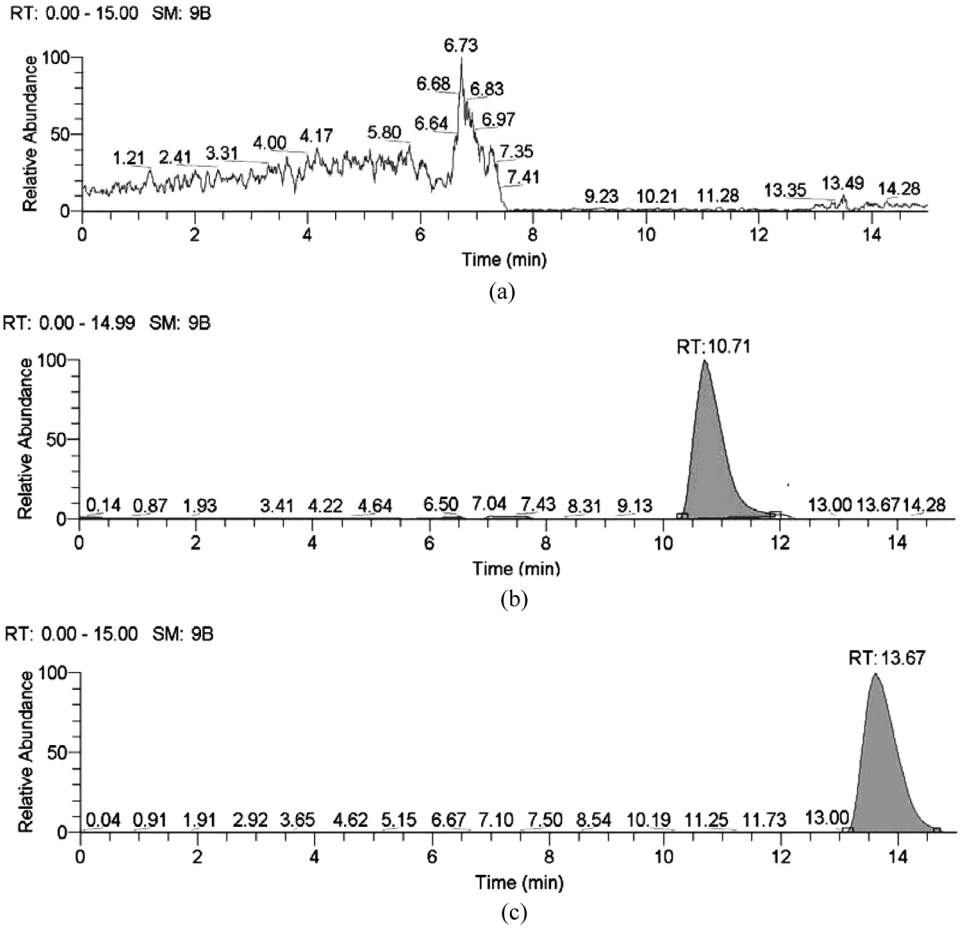

FIGURE 2 Chromatograms of (a) blank plasma and plasma spiked with (b) 50 ng/mL azithromycin and (c) 250 ng/mL roxithromycin (internal standard). The retention times for azithromycin and roxithromycin are 10.71 and 13.67 min, respectively.

13.67 min, respectively (Figure 2b, 2c). Azithromycin concentrations in unknown samples were determined by interpolating the peak height ratio of azithromycin and internal standard obtained with the calibration curves plotted.

Sample Preparation

Chromatograms of blank plasma and plasma spiked with azithromycin and roxithromycin at concentrations of 50 and 250 ng/mL are shown in Fig. 2a–c.

Calibration Curves

Plasma analysis was calibrated using a concentration range of 5–1,000 ng/mL. All calibration ranges yielded linear relationships with correlation coefficients ($r^2 \geq 0.9999$) or better (Figure 3). The linear regression equation obtained from the mean of the six calibration curves was $y = 0.00151x + 0.0184$, where y is the peak height ratio and x is the analyte concentration in ng.

Method Validation

Precision

Little variation of azithromycin assays was observed; coefficients of variation (CV) for six analysis at the concentration range observed were all below 5%. The intra-assay (within-day) and inter-assay (day-to-day) variation for azithromycin assay at the concentration range 5–1,000 ng/mL plasma are summarized in Table 1. Intra- and inter-day assay variation varied between 1.05 and 1.44%, and 0.89 and 2.06% (% CV), respectively.

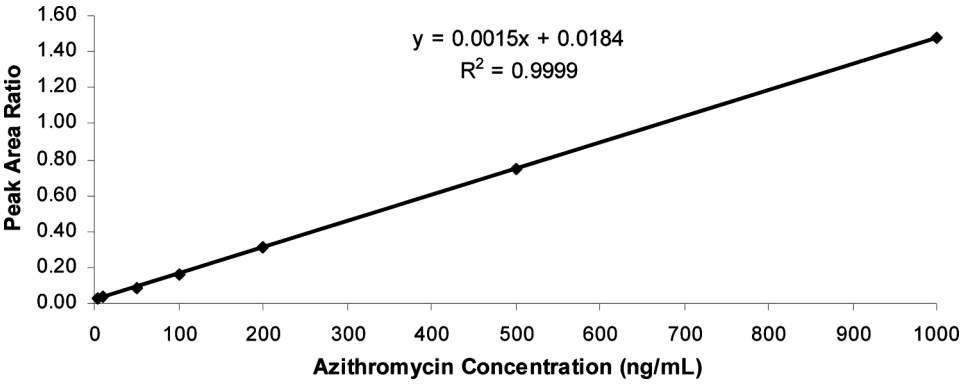

FIGURE 3 Mean calibration curve of azithromycin in plasma (n = 6).

TABLE 1 Inter-Day (Between Day) and Intra-Day (within Day) Validation of Azithromycin Concentrations

	Intra-Day Precision (n = 6)			Inter-Day Precision (n = 6)		
Concentration Added (ng/mL)	Concentration Measured (Mean ± SD; ng/mL)	CV (%)	Accuracy (DMV %)	Concentration Measured (Mean ± SD; ng/mL)	CV (%)	Accuracy (DMV %)
5.00	5.14 ± 0.06	1.17	2.83	5.34 ± 0.11	2.06	6.80
50.00	48.79 ± 0.51	1.05	−2.41	47.87 ± 0.60	1.25	−4.26
200.00	191.41 ± 2.76	1.44	−4.29	189 ± 189.49	0.89	−5.26
1,000.00	976.70 ± 5.48	0.56	−2.33	980.90 ± 3.44	0.35	−1.91

% CV: coefficient of variation; % DMV: deviation of mean value from the theoretical value.

Accuracy

Good accuracy was observed for both the intra-day or inter-day assays, as indicated by the minimal deviation of mean values found with measured samples from that of the theoretical values (actual amount added). The intra-assay (within-day) and inter-assay (day-to-day) accuracy for azithromycin assay at the concentration range 5–1,000 ng/mL plasma are summarized in Table 1. Intra- and inter-day assay accuracy, expressed as the mean deviation from the theoretical values varied between −4.29 and +2.83%, and −5.26 and +6.80%, respectively.

Recovery

The mean recoveries for azithromycin in plasma at the concentrations of 5, 50, and 200 ng/mL plasma were greater than 85% at all concentration. The recovery of the internal standard at the concentration of 250 ng/mL was 90%. The results indicate lack of interference from the sample preparation procedure. Further repeat analysis using 10 plasma samples from different sources at lower sensitivity, ensures the selectivity of the assay procedure.

Selectivity

Selectivity of the chromatographic separation was demonstrated by the absence of interferences from endogenous peaks in plasma at the retention times of azithromycin and the internal standard. Figures 2a–c illustrate typical chromatograms for blank plasma, and plasma spiked with azithromycin and internal standard.

Limit of Quantification

The limit of quantification (LOQ) in human plasma for azithromycin was accepted as 5 ng using 200 μL plasma.

TABLE 2 Storage Stability for Azithromycin in Spiked Plasma at Concentrations of 5, 50, and 200 ng/mL at 2 Weeks and 1 Month Follwing −20°C Storage (n = 3)

Storage Condition	Target Concentration (ng/mL)	Measured Concentration (ng/mL) (Mean ± SD; n = 3)	Difference (%)
Freshly prepared	5.0	5.46 ± 0.17	0.09
	50.0	47.05 ± 0.70	−0.06
	200.0	187.85 ± 0.80	−0.06
2 Weeks at −20°C	5.0	5.64 ± 0.27	0.13
	50.0	46.18 ± 1.20	−0.08
	200.0	186.17 ± 1.80	−0.07
1 Month at −20°C	5.0	5.15 ± 0.28	0.03
	50.0	49.02 ± 3.52	−0.02
	200.0	193.04 ± 6.72	−0.03

Stability

Azithromycin assay in plasma was found to be stable without decomposition of the drug after subjected to short-term freeze (stored in a −20°C freezer for a minimum of 1 month) (Table 2).

Quality Control

Three validated analysts conducted the analysis. A standard curve and quality control specimens were included with each analysis. Control samples with nominal concentration of 10, 50, 200, and 1,000 ng/mL plasma azithromycin were analyzed at the beginning and the end of the analytical run. All results were within the acceptable limit (+20% of their respective nominal values).

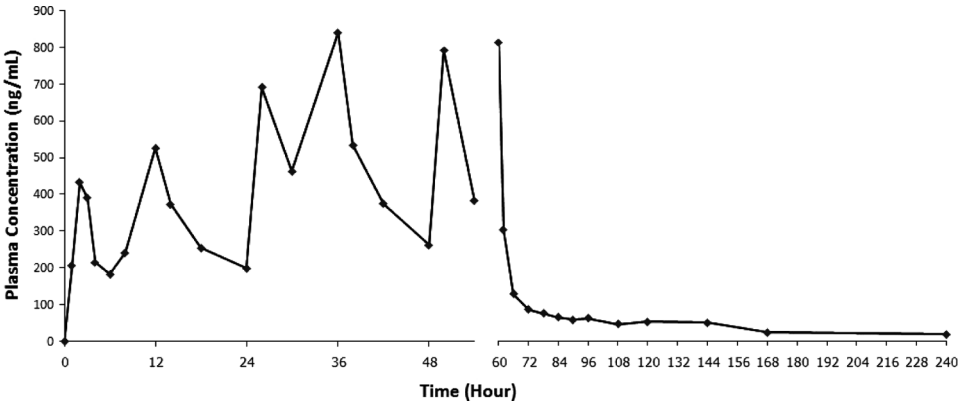

FIGURE 4 Mean plasma concentration-time profiles of azithromycin in 5 Thai male patients following a 3-days multiple dosing of 750 mg azithromycin given every 12 hours.

Application of Assay and Analysis of Specimens

The method appears to be robust and has been applied to the investigation of the pharmacokinetics of azithromycin when given in combination with fosmidomycin at 750 mg given every twelve hours for three days in patients with acute uncomplicated falciparum malaria. Figure 4 shows mean concentration-time profiles of azithromycin in 5 male and 5 female patients. Pharmacokinetics of azithromycin, following a 3-day multiple dosing in combination with fosmidomycin, is generally in agreement with that with report previously.[7,25] Mean (SD) C_{max} of 303 (12) ng/mL was achieved at 2 (0.1) hours (t_{max}), with a terminal phase elimination half-life at steady-stead ($t_{1/2z}$) of 33.2 (2.3) hours.

CONCLUSIONS

We have developed and validated a simple (a single step sample preparation with isocratic mode of separation), sensitive, selective, accurate, and robust for measuring of azithromycin in plasma using reverse phase liquid chromatography coupled to electrospray ionization-tandem mass-spectrometry (LC/MS). Only a single mass detection was applied to increase sensitivity of the assay as well as to reduce background interference. The procedure can be applied for measuring of azithromycin in plasma collected from a total of 5 patients with acute uncomplicated falciparum malaria. The advantage features of the developed method over the previously reported method for analysis of plasma or serum samples include the higher sensitivity (limit of quantification of 1 ng/mL plasma), and less complexity and thus shorter analysis time (single step liquid-liquid extraction). Sample preparation procedure is a single step and requires relatively small extraction volume of 250 μL. The high sensitivity of 1 ng/mL plasma was reported by Chen and co-workers[23] but requires larger volume of plasma sample (500 μL) and a higher capacity equipment, ultra performance liquid chromatography-electrospray ionization mass spectrometry (UPLC/MS/MS). The current method was fully validated according to the US FDA guidelines for bioanalytical method validation.[26] The method has been successfully applied to the pharmacokinetic study of azithromycin when given in combination with fosmidomyicn at multiple dosing in patients with acute uncomplicated falciparum malaria.

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