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

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Abstract. 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 minutes, 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/minute. Chromatographic analysis was carried out at 25ºC. Sample preparation was 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 intra-day and inter-day assays. The limit of quantification was acceptable at 0.5 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 5 Thai male patients with acute uncomplicated falciparum malaria.

Key word: azithromycin, chromalographic analysis, pharmacokinetic malaria

INTRODUCTION

Macrolide antibiotics, as exemplified by azithromycin (Fig 1a), are inhibitors of protein synthesis by specifically binding to the 50S subunit of the ribosomes in the apicoplast (Peters *et al*, 1992). Azithromycin has been successfully used in combination with artemisinin derivatives and quinine for prophylaxis and treatment of malaria (Heppner *et al*, 2005; Miller *et al*, 2006; Noedl *et al*, 2006). Fosmidomycin, has been shown to be an effective malarial

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blood schizontocide in addition to its wide spectrum of antibacterial activity. It posseses a novel mode of action through inhibition of 1-deoxy-D-xylose 5-phosphate (DOXP) reductoisomerase, an essential enzyme of the non-mevalonate pathway (Lode *et al*, 1996; Jomaa *et al*, 1999; Wiesner *et al*, 2003). The combination of fosmidomycin and azithromycin represents an innovative approach to malaria chemotherapy through novel modes of action, coupled with the benefit of activity against *Plasmodium falciparum in vitro* and *in vivo* (Wiesner *et al*, 2003); 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 constraints of a three-day dosing regimen. Azithromycin confers a much improved pharmacokinetic profile and more favorable toxicological profile compared to erythromycin (Lode *et al*, 1996; Bosnar *et al*, 2005; Kalilani *et al*, 2007).

A number of analytical methods have been used to determine the level of azithromycin and other macrolides in human and animal biological fluids (plasma, serum, tissues), water and pharmaceutical products. These methods involve bioassays (Riedel *et al*, 1992), high performance liquid chromatography (HPLC) with ultraviolet (Shaikh *et al*, 2008; Yang *et al*, 2009), electrochemical (Shepard *et al*, 1991; Kees *et al*, 1998; Supattanapong *et al*, 2008), fluorescence (Sastre *et al*, 1998; Bahrami *et al*, 2005, 2006), and HPLC with mass-spectrometry (LC/MS) (Fouda *et al*, 1995; Chen *et al*, 2006, 2007; Liu *et al*, 2007; Xue-Min *et al*, 2007; Xu *et al*, 2008) for detection. Among these methods, LC/MS is the most sensitive method with a limit of quantification in the range of 1-5 ng/ml using 100-500 μl plasma or serum. The limit of quantification (LOQ) range is from 0.05 to 20 ng/ml. The commonly used LC/MS/MS methods are based on electrospray ionization (EPI) tandem mass spectrometry (Chen *et al*, 2006, 2007; Xu *et al*, 2008) and atmospheric pressure chemical ionization (Fouda *et al*, 1995) 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 (Chen *et al*, 2006, 2007; Xue-Min *et al*, 2007; Xu *et al*, 2008) or solid phase extraction (Supattanapong *et al*, 2008).

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 a 200 μl plasma sample. The method was used to investigate the pharmacokinetics of azithromycin in Thai patients with acute uncomplicated falciparum malaria following a 3-day combination regimen of azithromycin and fosmidomycin.

MATERIALS AND METHODS

Chemicals

Azithromycin and the internal standard roxithromycin (99% pure) were purchased from Sigma Chemical, (St Louis, MO). The following chemicals and solvents were obtained in the highest purity available: dichloromethane, diethylether, acetonitrile and methanol (LAB-Scan, Analytical Sciences, Thailand), ammonium acetate (BDH Laboratory supplies poole, England), ammonium hydroxide, ammonium acetate and phosphoric acid (Sigma-

Aldrich, St Louis, MO). Deionized double distilled water was used for the preparation of working azithromycin 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 storing at -20ºC until used. Working standard solutions were prepared by diluting the stock standard solutions with methanol. Standard solutions were stored at -20ºC until analysis.

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

Chromatography

Azithromycin and the internal standard were separated on a Hypersil Gold C_{18} reversed phase column (Thermo, 4.6 x 150 mm, 5 μm particle size). The HPLC system was operated under an isocratic mode at a flow-rate of 0.3 ml/minute. 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) that was used to minimize gasses from the eluent flow prior to the introduction of a chromatographic sample into the mobile phase. For creating ions for analysis in solution, electrospray ionization was used to produce ions from solution into the gas phase. We 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 a positive electron spray ionization (ESI) interface. The head of the nebuliser was set at 300ºC and pressure at 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 with the LcQuan™ 2.0 (Thermo Electron Corporation, San Jose, CA). Ions monitored in the selected reaction monitoring (SRM) mode were 749.6 *m/z* for azithromycin and 837.6 *m/z* for roxithromycin. Argon was used as the collision gas and the electron multiplier was set at 5,000 V.

Sample preparation

Two hundred microliters (μl) of unknown plasma samples, or quality control samples, were transferred into polypropylene tubes, and 50 μl of 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 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 minutes at 4ºC; the clear supernatant was transferred into a second set of 10-ml screw-capped Teflon tubes. Organic solvents were evaporated to dryness under a stream of nitrogen at 40ºC. The residue was reconstituted with 200 μl of the mobile phase, and a 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 concentration range of 5 to 1,000 ng/ml.

Calibration curves for azithromycin (5, 10, 50, 100, 200, 500, and 1,000 ng/ml) were prepared on the same day as sample analyses with varying concentrations of azithromycin and a fixed concentration of roxithromycin (250 ng). Samples were analyzed as described above.

Data analysis

Concentrations of azithromycin were determined from the peak height ratios (peak height of azithromycin/peak height of internal standard), which corresponded to known azithromycin concentrations in the calibration curve as described above. Peak detection, peak height integration, peak height ratio calculation were performed with 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 with 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 above, 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. The accuracy of the method 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) and comparing the difference between the spiked value and that actually found (theoretical value).

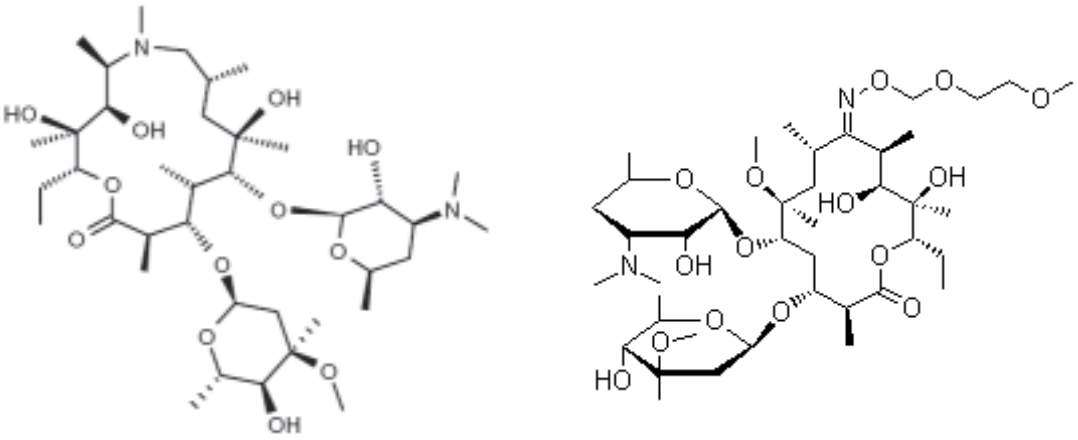
Recovery. The analytical recovery of sample preparation for azithromycin was estimated by comparing the peak heights obtained from samples (plasma) prepared as described above, with those measured with equivalent amounts of azithromycin in methanol. Triplicate analysis was performed with azithromycin 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 endogenous interferences at the retention times for azithromycin in human blank plasma obtained from six different lots, and interference by commonly used drugs, *ie*, 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 the spiked plasma sample) that produced a peak ten times higher than the baseline noise (absorbance unit full scale) in a 200 μl sample, which also produced acceptable accuracy (<20% of the nominal value) and precision (expressed as the coefficient of variation, CV < 20%).

Stability. The stability of azithromycin was determined by storing spiked plasma samples (at concentrations of 5, 50, and 200 ng/ml of plasma; triplicate analysis for each concentration) in a -20ºC 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 concentrations of 5, 50, 200, and 1,000 ng/ml plasma. Samples were



(a) (b)

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

aliquoted into cryovials, and stored at -20ºC for use with each analytical run. The results of the QC samples provided the basis for 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 investigation of the pharmacokinetics of azithromycin when given in combination with fosmidomycin at 750 mg (250 mg *per* capsule Zithromax™, Pfizer, NY) 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 the Hospital for Tropical Diseases and the study protocol was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Thailand. Written informed consent for study participation was obtained from each subject.

RESULTS

Chromatographic separation

Under the chromatographic conditions previously described, the chromatograms for azithromycin and the internal standard (roxithromycin) were free from any interference peak,with good resolution and sharp peaks. Blank plasma samples showed little noise fluctuation (Fig 2a). The retention times for azithromycin and roxithromycin were 10.71 and 13.67 minutes, respectively (Fig 2b, 2c). Azithromycin concentrations in unknown samples were determined by interpolating the peak height ratio of azithromycin and the 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, b and c.

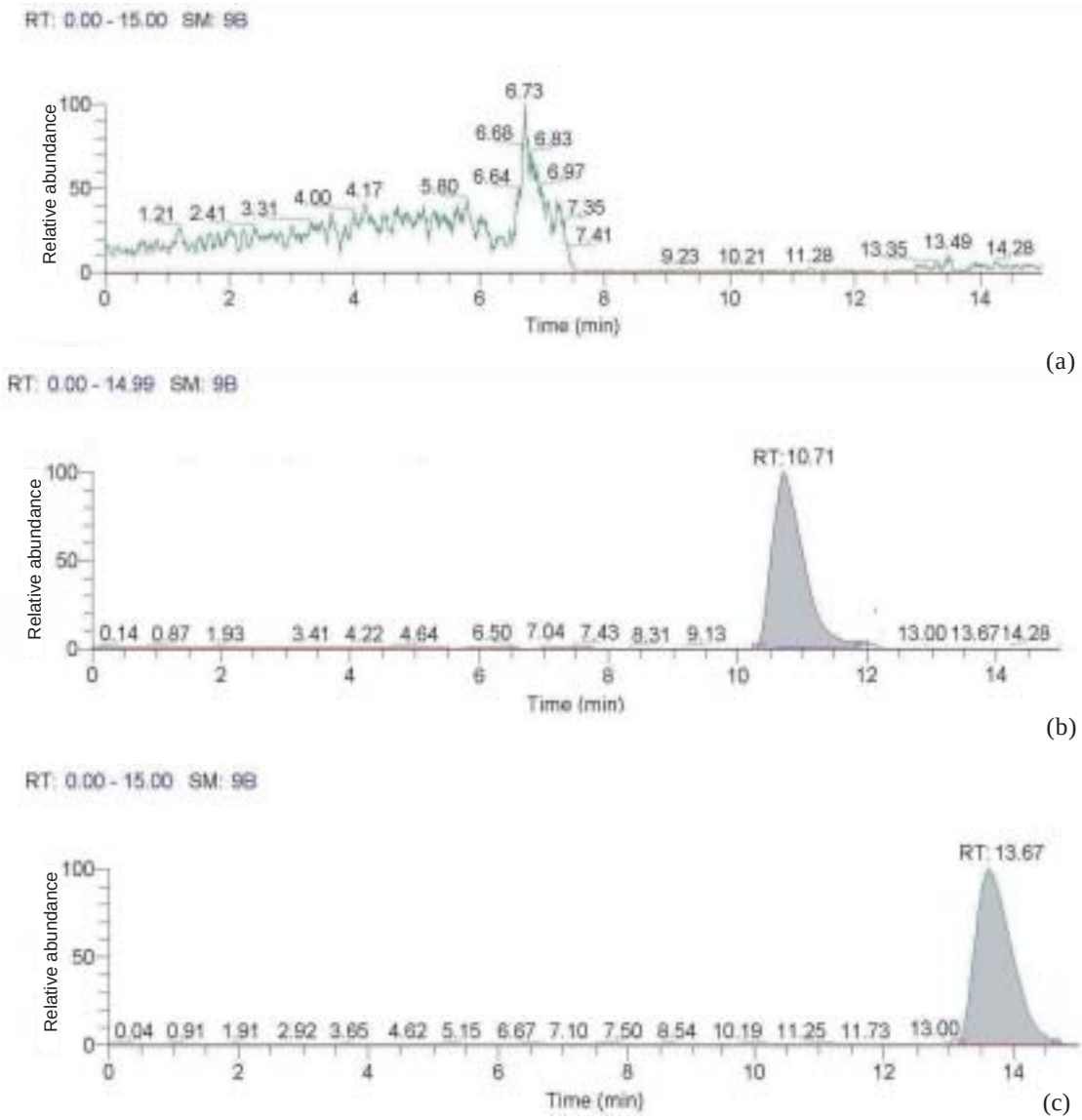


Fig 2–Chromatograms of (a) blank plasma and plasma spiked with (b) 50 ng/ml azithromycin and (c) 250 ng/ml roxithromycin (internal standard). The retention times for azithromycin and roxithromycin were 10.71 and 13.67 minutes, respectively.

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 of $R^2 \geq 0.9999$ or better (Fig 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 in azithromycin assays was observed; coefficients of variation (CV) for the 6 analyses at the concen-

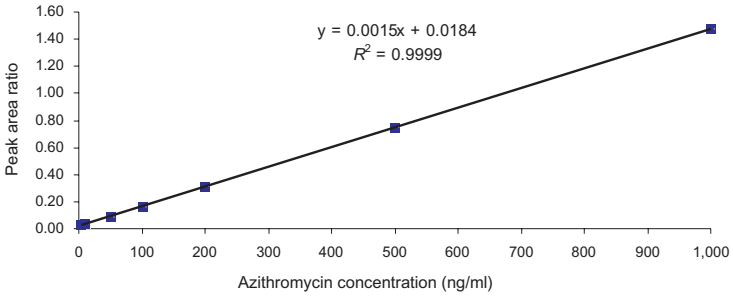


Fig 3–The mean calibration curve for azithromycin in plasma (n=6).

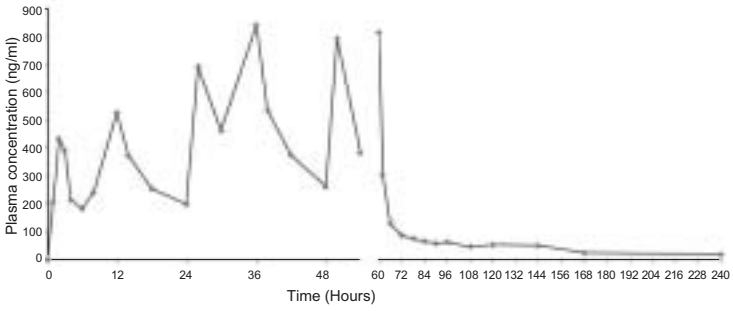


Fig 4–Mean plasma concentration-time profiles of azithromycin in 5 Thai male patients following 3-days multiple dosing of 750 mg azithromycin given every 12 hours.

tration range observed were all below 5%. The intra-assay (within-day) and inter-assay (day-to-day) variation for the azithromycin assays at concentration ranges of 5-1,000 ng/ml of plasma are shown in Table 1. Intra- and inter-day assay variation varied between 1.05 and 1.44%, and 0.89 and 2.06% (%CV), respectively.

Accuracy. Good accuracy was observed for both the intra-day and inter-day assays, as indicated by minimal deviation in the mean values found with measured samples compared to the theoretical values (actual amount added). The intra-assay (within-day) and inter-assay (day-to-day) accuracy for the azithromycin assays at concentration

Table 1
Inter-day (between day) and intra-day (within day) validation of azithromycin concentrations.

Concentration added (ng/ml)	Intra-day precision (n = 6)		Accuracy (% DMV)	Inter-day precision (n = 6)		Accuracy (% DMV)
	Concentration measured (mean ± SD; ng/ml)	% CV		Concentration measured (mean ± SD; ng/ml)	% CV	
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.

Table 2
Storage stability of azithromycin in spiked plasma at concentrations of 5, 50, and 200 ng/ml for 2 weeks and 1 month follwing storage at -20ºC (*n*=3).

Storage conditions	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 -20ºC	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 -20ºC	5.0	5.15 ± 0.28	0.03
	50.0	49.02 ± 3.52	-0.02
	200.0	193.04 ± 6.72	-0.03

ranges of 5-1,000 ng/ml of 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 concentrations of 5, 50, and 200 ng/ml plasma were greater than 85% at all concentrations. The recovery of the internal standard at a 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, ensured the selectivity of the assay procedure.

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

Limit of quantification. The limit of quantification (LOQ) in human plasma for azithromycin was 0.5 ng using 200 µl plasma.

Stability. The azithromycin assay in plasma was found to be stable without decomposition of the drug after being subjected to short-term freezing (-20ºC for a minimum of 1 month) (Table 3).

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

Application of assay and analysis of specimens. The method appears to be robust and was applied to the investigation of the pharmacokinetics of azithromycin when given in combination with fosmidomycin at 750 mg every twelve hours for three days in patients with acute uncomplicated falciparum malaria. Fig 4 shows the mean

concentration-time profiles for azithromycin in 5 male and 5 female patients. The pharmacokinetics of azithromycin following 3-days multiple dosing in combination with fosmidomycin are in agreement with those reported previously (Nahata *et al*, 1993; Lode *et al*, 1996). A 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.

DISCUSSION

We developed and validated a simple method (single step sample preparation with isocratic mode of separation) which is sensitive, selective, accurate, and robust for measuring 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 the sensitivity of the assay and reduce background interference. The procedure was applied to measuring azithromycin in plasma collected from 5 patients with acute uncomplicated falciparum malaria. The advantages of the method over previously reported methods of analysis of plasma or serum samples included a higher sensitivity (limit of quantification of 0.5 ng/ml plasma), less complexity and shorter analysis time (single step liquid-liquid extraction). Sample preparation is a single step and requires a relatively small extraction volume of 250 μl. A relatively high sensitivity of 1 ng/ml of plasma was reported by Chen *et al* (2007) but their method requires a larger volume of plasma sample (500 μl) and 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 (US FDA, 2001). The method has been successfully applied to the pharmacokinetic study of azithromycin when given in combination with fosmidomyicn as multiple doses in patients with acute uncomplicated falciparum malaria.

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