Original article **P42**

Liquid chromatography mass-spectrometry for determination of azithromycin in plasma and application for pharmacokinetic study

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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 azithromycin (AZM) in human plasma. The internal standard (IS: roxithromycin) was separated from AZM 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. 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 0.5 ng using 200 μL plasma samples. The mean recoveries for AZM and the IS were greater than 85%. The method was applied successfully to the investigation of the pharmacokinetics of AZM when given 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, liquid chromatography mass-spectrometry, pharmacokinetics

Introduction

The antibiotic azithromycin (AZM: Figure 1a), is an inhibitor of protein synthesis by specifically binding to the 50S subunit of the ribosomes in the apicoplast (1). It has been successfully used in combination with artemisinin derivatives and quinine for prophylaxis and treatment of malaria (2). The combination of AZM with fosmidomycin represents another 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* (3) and there are grounds for anticipating lack of cross resistance with existing drugs and protection against the development of resistance. Pharmacokinetic study of AZM is therefore, essential for dose optimization of the combination. A number of analytical methods have been reported for the determination of AZM in biological fluids including bioassay (3), high performance liquid chromatography (HPLC) with ultraviolet (4), electrochemical (5), fluorescence (6), and HPLC with mass-spectrometry (LC/MS) (7) detection. In the present study, we propose an alternative simple and sensitive LC/MS method with electrospray ionization for determination of AZM. The method was applied for the investigation of the pharmacokinetics of AZM in Thai patients with acute uncomplicated falciparum malaria fallowing a 3-days combination regimen of AZM and fosmidomycin.

Methods

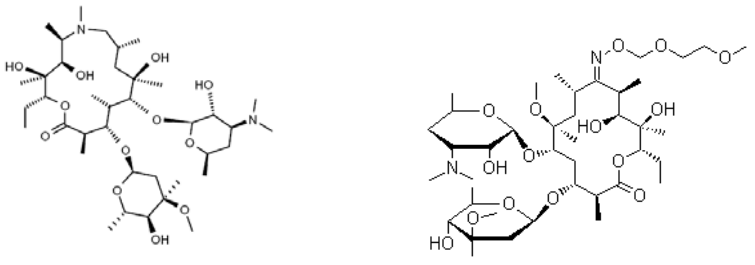
AZM and the internal standard roxithromycin (IS: Figure 1b) were separated on a Hypersil Gold C_{18} reversed phase column (Thermo, 4.6 x 150 mm, 5 μm particle size) with

the mobile phase consisting of a mixture of 20 mM ammonium acetate buffer (pH 5.2), acetonitrile and methanol at a ratio of 50:40:10 (v/v/v), running at a flow-rate of 0.3 mL/min. The mass spectrometer consisted of a Finnigan LCQ Deca XP Max plus ion trap detector equipped with the positive electron spray ionization (ESI) interface (temperature 300 °C, pressure 551 kPa; nitrogen gas flow 70 and 15 arb). Mass results were plotted and processed by the LcQuan^{TM} 2.0 (Thermo Electron Corporation, California, USA). Ions monitored in the selected reaction monitoring (SRM) mode were *m/z* 749.6 *m/z* for AZM; and 837.6 *m/z* for IS. Sample preparation was performed by liquid-liquid extraction as follow: 200 µL plasma, 25 ng IS, 50 µL methanol, 250 µL of 0.25 M carbonate-bicarbonate buffer pH 9.5, 3 mL of the mixture of 7:3 (v/v). Concentrations of AZM were determined from the peak height (PH) (Millennium 2000 Chromatograph$^{™}$) ratios (PH of AZM/PH of IS), which corresponded to the known AZM concentrations in a calibration curve. The developed method was validated for linearity, recovery, sensitivity, specificity, precision, and accuracy (8).

The validated method was applied for the investigation of pharmacokinetics of AZM 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

Selectivity of the bioassay system was demonstrated by the absence of interferences from endogenous substances and commonly used drugs. The retention time of AZM and IS were 10.71 and 13.67 min, respectively (Figure 2a, b). The calibration ranges yielded linear relationships with correlation coefficients of 0.990 or better. Good precision and accuracy for both the intra-assay (within-day) and inter-assay (day-to-day) was obtained (Table 1). The limit of quantification (L.O.Q.) in human plasma for AZM was accepted as 0.5 ng using 20 µl plasma. The mean recoveries for AZM and the IS were greater than 85%. AZM was stable when stored at -20^{o} C for up to 3 months. Figure 2 shows mean concentration-time profiles of AZM in 5 male patients. The advantage features of the developed method over the previously reported method include the higher sensitivity, simplicity and rapidity (single step liquid-liquid extraction), and requirement of relatively small extraction volume of 250 µL. Pharmacokinetics of AZM (mean$\pm$SD) following a 3-days multiple dosing are as follows: maximum concentration (C_{max}) 303 $\pm$12 ng/mL, time to maximum concentration (t_{max}) 2$\pm$0.1 hours, and terminal phase elimination half-life ($t_{1/2z}$) 33.2$\pm$2.3 hours.



(a) (b)

Figure 1 Chemical structures of (a) AZM and (b) the IS

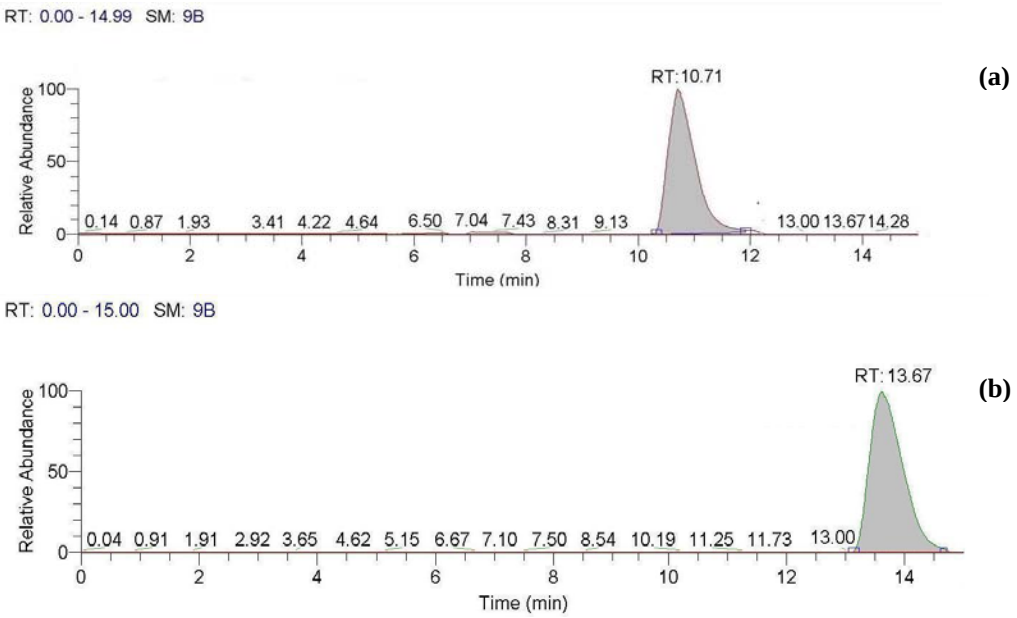


Figure 2 Chromatograms of plasma spiked with (a) 50 ng/mL AZM and (b) 250 ng/mL IS. The retention times for AZM and IS are 10.71 and 13.67 min, respectively.

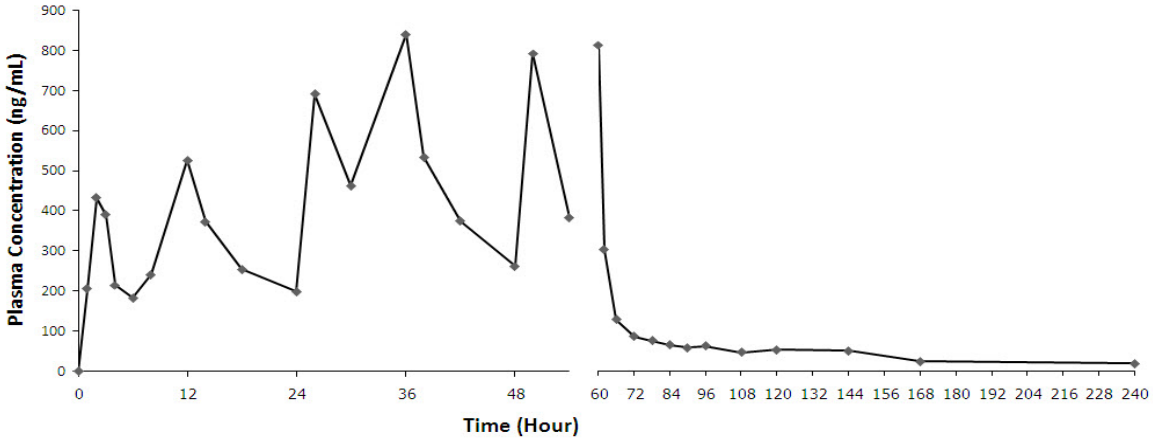


Figure 3 Mean plasma concentration-time profiles of AZM in 5 Thai male patients following a 3-days multiple dosing of 750 mg AZM given every 12 hours

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

Conclusion

The LC/MS method developed for determination of AZM in plasma is simple, accurate, sensitive, selective and reproducible for application for pharmacokinetics study of AZM in patients with malaria.

Acknowledgement

This investigation received financial support from the Commission on Higher Education, Ministry of Education of Thailand.

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