SOLID PHASE EXTRACTION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE DETERMINATION OF AZITHROMYCIN IN HUMAN PLASMA

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Abstract. An effective, selective and sensitive method for the determination of azithromycin in plasma using high performance liquid chromatography-electrochemical detection (HPLC-ECD) extracted by solid phase extraction was developed and validated. Clarithromycin was used as an internal standard. Azithromycin was extracted from 1 ml of plasma using an Oasis HLB solid-phase extraction cartridge and the elution was evaporated to dryness, dissolved in 100 µl mobile phase and 40 µl auto-injected into an HPLC system with a 200 µl loop. The mobile phase was acetonitrile, methanol, phosphate buffer, 0.05M, pH 6.0 (20:20:60, v/v/v) with a flow rate of 1.0 ml/minute. The lower limit of quantitation (LLOQ) was 10 ng/ml without interfering peaks. The calibration curve was linear (r^2=0.9998) over a concentration range 10 to 400 ng/ml. The accuracy and precision were acceptable. The mean recoveries at 30, 100 and 200 ng/ml were 85.3±5.5%, 80.1±6.8% and 82.9±2.5%, respectively. Azithromycin was stable in plasma for at least 6 hours at room temperature and 6 months storage at -80ºC. The post-preparation stability of spiked samples was more than 24 hours after preparation. The cartridge can be used two times providing a deviation of less than 4%. This method has adequate sensitivity, specificity, accuracy and precision to measure azithromycin in human plasma and is free from interference from the plasma matrix. The validated method was to quantify azithromycin plasma concentrations in five healthy Thai volunteers after the administration of 500 mg azithromycin capsules.

INTRODUCTION

Azithromycin is an advanced-generation macrolide antibiotic derived from erythromycin and containing a methyl-substituted nitrogen in the lactone ring (Fig 1) (Hoepelmana and Schneide, 1995). Azithromycin possesses favorable pharmacokinetic (Bosnar *et al*, 2005) and toxicological profiles (Kalilani *et al,* 2007). It has shown intrinsic activity against *Plasmodium* spp *in vitro* (Noedl *et al*, 2001; Ohrt *et al,* 2002). Azithromycin has been successfully used in combination with artemisinin derivatives and quinine for prophylaxis and treatment of malaria (Krudsood *et al*, 2000; Noedl *et al*, 2001, 2006; Heppner *et al*, 2005; Miller *et al*, 2006). The high tissue concentration of azithromycin in the liver makes the drug potentially attractive as a causal prophylactic antimalarial agent (Girard *et al*, 1987; Andersen *et al,* 1994, 1995). Azithromycin has also been found to be an effective alternate to penicillin in the treatment of less severe cases of leptospirosis (Ghouse *et al*, 2006). Its efficacy is not inferior to that of doxycycline for the treatment of leptospirosis and scrub typhus (Phimda *et al*, 2007). Plasma azithromycin concentrations not only provide useful information about its systemic availability, but also its potential therapeutic use for malaria and leptospirosis treatment, when

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compared with the inhibitory concentrations (Ohrt *et al,* 2002; Murray *et al,* 2004).

Azithromycin does not have a specific UV chromophore and thus UV detection gives only low sensitivity for the determination of azithromycin in plasma. The methods that have been reported are based on high performance liquid chromatography (HPLC) with fluorescence detection after pre-column derivatization (Bahrami *et al*, 2005; Bahrami and Mohammadi, 2006), or microbiological assay (Riedel *et al*, 1992; Cárceles *et al*, 2005). HPLC with electrochemical detection (HPLC-ECD) has been widely used due to its high sensitivity and precision without need of a derivatization process. Kees *et al* (1998) used cyanopropyl silica for the stationary phase of an analytical column which proved to be at least as efficient, and of better selectivity than reverse phase C_{18}. The common methods that have been used for sample extraction are direct precipitation protein with acetonitrile or methanol (Liu *et al*, 2007; Xue-Min *et al*, 2007) and liquid-liquid extraction using organic solvents (Kees *et al*, 1998; Chen *et al*, 2006). The extract resulting from liquid-liquid extraction indicated a high recovery of azithromycin but was not clean (several potential interfering peaks) and formed an emulsion when reconstituted with the mobile phase. Recently, an analytical method with the highest sensitivity using solid phase extraction (SPE) for azithromycin plasma sample preparation and HPLC with MS/MS detection was reported (Ke *et al*, 2002). However, this method is not readily available for most laboratories. A combination of SPE and HPLC with ECD was applied to analyze azithromycin plasma concentrations in animals with moderate sensitivity (Breitschwerdt *et al*, 1999). It is likely that the application of SPE could provide lower peak interferences and inter-analyst variation, compared to those obtained from liquid-liquid extraction. The objective of this study was to develop and validate a simple, selective and sensitive HPLC-ECD method for determination of azithromycin in human plasma using the SPE.

MATERIALS AND METHODS

Chemicals and materials

Azithromycin dihydrate (USP Reference standard, purity 93.8%, Lot I0F079) and clarithromycin, as the internal standard (IS), (USP Reference standard, purity 97.7%, Lot G0D356) were purchased from the United States Pharmacopeal Convention (USA), with a USP certificate of analysis. The chemical structure of azithromycin and the IS are shown in Fig 2. Methanol, acetonitrile, and the dibasic

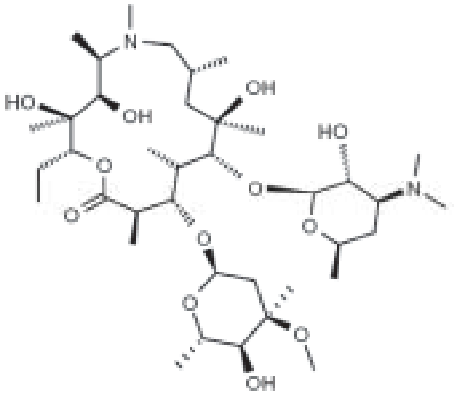

Fig 1–Structure of azithromycin (Andreotti *et al*, 2007).

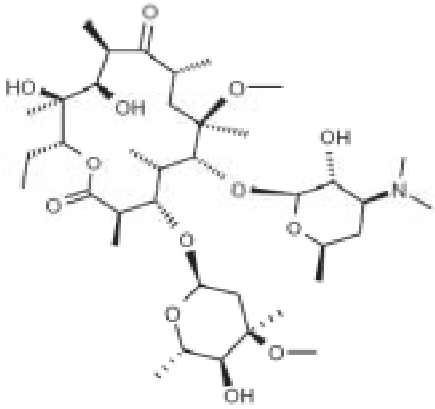

Fig 2–Structure of clarithromycin (Andreotti *et al*, 2007).

potassium phosphate tri-hydrate were all HPLC grade and purchased from BDH Chemicals (Poole, Dorset, UK); potassium di-hydrogen phosphate was purchased from Sigma (Sigma Chemical, St Louis, MO). De-ionized water was obtained from a Milli-Q de-ionized (DI) water system (Millipore, Bedford, MA). Drug-free plasma was obtained from the Blood Bank of Srinagarind Hospital (Khon Kaen University).

Equipment and chromatographic conditions

The modular isocratic HPLC system consisted of a Spectrasystem P2000 pump (San Jose, CA), a Waters™ 717 (Waters, Milford, MA) automatic sample injector and an amperometric detector Model 105 (Precision Instruments, France) with reference electrode (Ag/AgCl) and voltage values set at 1.2V and 10 nA. Data acquisition was performed using Clarity™ version 2.4.1.77 LC system software (Waters, Milford, MA). The analytical column was an Eclipse XDB-CN™ 5 µm, 150 x 4.6 mm (Agilent Technologies, Palo Alto, CA) protected by a guard column Xterra RP18, 3.9 x 20mm (Waters, Milford, MA). The mobile phase a was acetonitrile-methanol-phosphate buffer, 0.05M, pH 6.0 (20:20:60, v/v/v) with a flow rate of 1.0 ml/minute. The SPE cartridge used was a hydrophilic-lipophilic balanced copolymer extraction column (1 ml, 10 mg, Waters Oasis HLB, Waters, Milford, MA). A solid phase extraction manifold (Ashcroft) was used to extract plasma and force solvent through the columns by vacuum pump, model DOA-V502-BN (GAST Manufacturing, MI). The HPLC solvent mobile phase was filtered through a 0.2 µm nylon membrane, degassed by sonication, then online degassed with a series 200 degasser (Perkin-Elmer Corporation, Norwalk, CT). The temperature of the HPLC column was kept at 25ºC.

Preparation of calibration curves

Fresh preparations of stock solutions of azithromycin (500 µg/ml) and clarithromycin (30 µg/ml) were prepared in methanol-water (70:30, v/v). The azithromycin stock solution was diluted with a mobile phase to make a working solution at a concentration of 40 µg/ml. Calibration standards were prepared by spiking the azithromycin working solution into pooled drug-free human plasma from the Blood Bank of Srinagarind Hospital (total volume 500 ml) to obtain concentrations of 10, 30, 50, 100, 200 and 400 ng/ml. The samples were mixed for 20 seconds and equilibrated for 1 hour and stored frozen in aliquots at -80ºC. Calibration curves were constructed using the six calibration standards and calculated using peak area ratios for azithromycin for the corresponding concentration. Linear calibration curves were generated by a linear regression equation with r^2 not less than 0.995 over the standard concentration.

Sample preparation

Frozen plasma samples were thawed at room temperature, vortexed and then centrifuged for 3 minutes at 2,700*g* in order to separate coagulated proteins and lipids from the plasma matrix. The processes of sample extraction were: conditioning the cartridge with 2 ml of methanol, allowing the cartridge to dry under vacuum, equilibrating with 2 ml of water, loading 1 ml of plasma sample and IS 20 µl of 30 µg/ml onto the Oasis HLB SPE columns, washing with 1 ml of deionized (DI) water and then 1 ml 5% v/v methanol in water, eluting with 1 ml of methanol (flow rate 0.5 ml/minute in every step), evaporating the elute to dryness with nitrogen gas 20 lb^2/inch at a temperature of 25-30ºC and reconstituting in 100 µl of mobile phase, mixing and sonicating briefly. Forty µl of sample was auto-injected into the HPLC-ECD system with a 200 µl loop. Precision of injection was checked and the coefficient of variation (%CV) was less than 5%.

Method validation

The processes of method validation and their criteria followed the guidelines for Bioanalytical Method Validation of the US FDA (2001).

Lower limit of quantification (LLOQ). The lower limit of quantification (LLOQ) of the assay procedure was selected to be the lowest concentration of azithromycin (in five replicates of spiked pooled drug-free human plasma) that produced a peak area ten times the base line noise at a sensitivity of 0.01 V, where the %CV was within 20% and accuracy was within the range 80-120% (US FDA, 2001).

Selectivity. The interference from the endogenous matrix by other components, such as decomposition product metabolites in drug-free human plasma samples from the Blood Bank of Srinagarind Hospital from at least six different donors were analyzed individually. In addition, concomitant medication or commonly used drugs (paracetamol, diclofenac sodium, ibuprofen, clindamycin hydrochloride, loratadine and ambroxol hydrochloride) in the samples was investigated by spiking the drugs into drug-free plasma. Selectivity was assessed by comparing the chromatograms of the drug-free plasma from the six different donors, the plasma spiked with the concomitant drugs, and the plasma spiked with azithromycin and IS.

Accuracy, intra- and inter-day precision. Intra- and inter-day precision were determined as %CV. The accuracies were calculated by dividing the measured concentrations by true concentrations and expressing as a percentage. Intra-day precision and accuracy were calculated using replicate *(n=5)* determinations of spiked pooled human plasma at three different concentrations of azithromycin (30, 100 and 200 ng/ml) during a single analytical run. The reproducibility (inter-day precision) of the method was validated using the same three different spiked plasma concentrations as described above on three separate days. The %CV for each concentration level did not exceed 15% and accuracy was within the range of 85-115% (US FDA, 2001).

Recovery. The recoveries of azithromycin extraction at three different concentrations of the spiked pooled plasma (30, 100 and 200 ng/ml) were evaluated by assaying the samples as described above and comparing the peak areas of azithromycin with the IS, and then comparing with those obtained from direct injection of the azithromycin dissolved in methanol at the same concentrations. The mean recoveries were calculated at each concentration (*n*=5).

Stability. The short-term stability was examined by keeping two concentrations of the spiked pooled plasma (30 and 200 ng/ml, *n*=3) at room temperature and analyzed at 6 hours. The auto-sampler stability of azithromycin was tested by analysis of the two processed and reconstituted samples as described above, which were stored in the auto-sampler tray for 24 hours. The long-term stability of azithromycin in plasma was tested after storage of the spiked pooled plasma at the same concentrations as described above at -80ºC for 24 weeks (sampling time: 0, 1, 3, 6, 9, 12 and 24 weeks).

Preparation of quality control samples. Quality control (QC) samples were prepared in plasma using a stock solution separated from that used to prepare the calibration curve, at the three different concentrations of the spiked pooled plasma (30, 100 and 200 ng/ml). The QC samples were aliquoted into cryovials and stored frozen at -80ºC for use with each analytical run. The result of the QC samples gave the basis for accepting or rejecting the run. At least four of the six QC samples had to be within ± 15% of their nominal concentration (US FDA, 2001).

The deviation of reused Oasis HLB cartridges

The percent deviation using the Oasis HLB cartridges twice was determined. The percent deviation was calculated by the average concentration of the first extraction minus the average concentration of second extraction, multiplied by 100 and divided by the average

concentration of the first extraction. The deviation for each of the three concentrations of spiked pooled plasma (30, 100 and 200 ng/ml, *n=3*) could not be more than ±5%.

Application of the method to biological samples

The method was applied to the investigation of the plasma concentration-time profile of azithromycin following administration of 500 mg azithromycin in a capsule. The volunteers were healthy Thais (three males and two females), aged between 20 and 45 years with a body mass index of 18 - 24. The study was approved by the Ethics and Scientific Review Committee of Khon Kaen University. Written informed consent was obtained from the volunteers prior to study participation. Blood samples (5 ml) were contained in heparinized-coated plastic tubes at the following time points: 0, 0.5, 1, 2, 2.5, 3, 4, 6, 9, 12, 24 and 48 hours after dosing. Blood samples were centrifuged at 2,700*g* for 10 minutes. Plasma samples were collected and stored at -80ºC until analyzed using the presented method.

RESULTS

Method of SPE extraction

The important part of many chromatographic methods in the assay of biological samples is the elimination of interferences during HPLC determination. A selective extraction method used to purify and concentrate the samples prior to analysis is required. The chosen SPE column, Oasis HLB, is of polymeric construction designed to have a hydrophilic-lipophilic balance (HLB) that gives a high recovery rate and good reproducibility for acidic, basic, and neutral compounds (Instruction sheet, Waters Oasis). Azithromycin was extracted from the spiked pooled plasma and purified using Oasis HLB. Washing with 1 ml of DI water and 5% methanol provided acceptable recoveries and no peaks interfering with determination of azithromycin or IS.

Method validation

The chromatographic system is appropriate for determination of azithromycin in plasma and can separate azithromycin and IS from interfering peaks. Sensitivity can be increased by applying higher detector voltages. The voltage of E=1.2V was sufficiently sensitive and can detect an azithromycin plasma concentration of 10 ng/ml. The %CV of auto injection at 500 ng/ml in the mobile phase was 4.5%.

Selectivity

There was no interference from endogenous matrix components on individual analysis of drug-free human plasma samples (*n*=6) observed at the retention times for analyses. The study showed that paracetamol, diclofenac sodium, ibuprofen, clindamycin hydrochloride, and ambroxol hydrochloride were detected from the described analytical method but did not give interference with the analysis for azithromycin or IS. Their retention times were earlier than those for azithromycin and IS, *ie* ranging between 3 and 9 minutes. Loratadine was not detected. The retention times for IS and azithromycin in spiked plasma are shown in Fig 3 (line A). The retention times for IS and azithromycin were about 14.0 and 16.6 minutes respectively.

Linearity of calibration curves and lower limits of quantification (LLOQ)

The correlation coefficient of the calibration curve was r^2=0.9998 (*n*=5) which confirmed that the calibration curves were linear over a concentration range of 10 to 400 ng/ml, and the equation of the standard curve was $y = 0.001+0.0023x$. The LLOQ was 10 ng/ml for which an acceptable accuracy (*n=5*) was in the range 80-120% (111.14%) and a precision below 20% (5.85%).

Precision and accuracy

The intra-day and inter-day precision and accuracy for azithromycin were evaluated by assaying the three concentrations of azithromycin in spiked pooled plasma samples. In this

assay, at each concentration, the intra-day precision was 5.7% or less, and the inter-day precision was 6.9% or less. The accuracy was in range 98.6-101.9% (Table 1). The results demonstrate the values were within the acceptable range and the method is accurate and precise.

Recovery and stability

The mean recoveries determined for each of the three concentrations (30, 100 and 200 ng/ml) of azithromycin were 85.3±5.5%, 80.1±6.8% and 82.9±2.5% (*n*=5), respectively. All stability determinations were carried out using 30 and 200 ng/ml azithromycin plasma samples. No significant degradation occurred under any of the experimental conditions. The stability data are shown in Table 2. The stability of the analyte specified in the US FDA criterion (2001) was that the decrease from initial value should not exceed 15%. The results of the percentage deviation for each of the experimental conditions showed that azithromycin was stable in plasma for at least 6 hours at room temperature (RT) and stable at -80ºC for at least six months. After reconstituting with mobile phase, it was stable for at least 24 hours for auto-sampler injection.

Quality control samples

The control samples with concentrations of 30, 100 and 200 ng/ml plasma azithromycin were analyzed at the beginning and the end of the analytical run. All the results were within acceptable limits (± 15% of their nominal value).

The deviation of reused Oasis HLB cartridges

The percent deviation of extraction of azithromycin in plasma by reusing Oasis HLB cartridges is shown in Table 3. The Oasis HLB cartridge can be reused in duplication with a percent deviation of extraction of not more

Table 1
Accuracy and precision for the determination of azithromycin in plasma.

Added (ng/ml)	Found[a](ng/ml)	Intra-day, CV (%) (*n*=5)	Inter-day, CV (%) (*n*=5, 3 days)	Accuracy (%) (*n*=5)
30	29.6±1.3	4.51	6.35	98.6
100	101.9±5.8	5.65	6.85	101.9
200	200.5±9.8	4.88	5.03	99.4

[a] Mean ± Standard deviation

Table 2
Stability of azithromycin plasma samples (*n*=3).

Experimental condition	Initial concentration[a] (ng/ml)	Found[a] (ng/ml)	Deviation(%)
Auto-sampler samples, 24 hour at RT	30.51 ± 0.16	30.57 ± 0.35	-0.20
	199.9 ± 2.4	199.8 ± 1.1	0.02
Plasma sample, 6 hour storage at RT (short-term)	33.1 ± 1.4	34.2 ± 3.8	3.6
	206.9 ± 9.3	213.3 ± 8.1	3.1
Plasma sample, 24 weeks storage at -80ºC (long-term)	33.1 ± 1.4	29.26 ± 0.55	11.5
	206.9 ± 9.3	194.7 ± 4.3	5.9

[a] Mean ± Standard deviation

than 5% (range 0.23-3.4%).

Application of assay and analysis of specimens

To demonstrate the clinical applicability of the method, plasma concentration levels of azithromycin were determined in five healthy Thai volunteers following a single dose of 500 mg azithromycin by capsules. The plasma concentration-time profiles for azithromycin are shown in Fig 4.

DISCUSSION

The HPLC method with ECD for the determination of azithromycin in plasma is simple, selective and reproducible. Although HPLC with MS or MS/MS detection is highly sensitive, with the lowest reported LLOQ of 1 ng/ml (Chen *et al*, 2007), the instruments are expensive and not readily available in most laboratories. SPE extraction was developed for sample preparation. Washing the sample with 1 ml of water and 5% methanol was sufficient to give an acceptably clean chromatogram without interfering peaks near the retention times for azithromycin and IS, with more than 80% recovery. The reconstituted sample presented low albumin con-

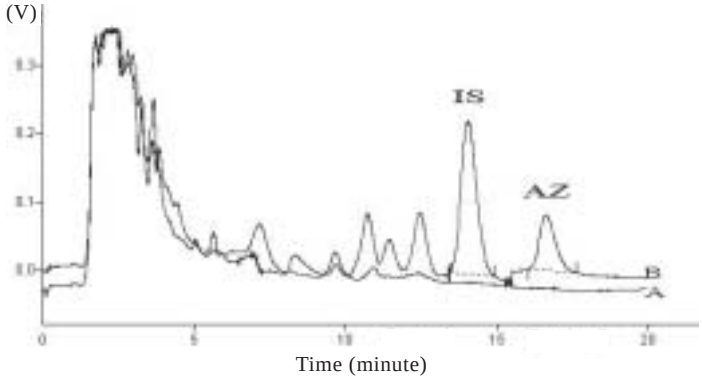

Fig 3–The overlay of chromatograms of a drug-free plasma (line A) and a spiked plasma sample with azithromycin 200 ng/ml (AZ) and IS 30 µg/ml (line B) with mobile phase of acetonitrile-methanol-0.05M phosphate buffer pH 6.0, (20:20:60,v/v/v); flow rate of 1.0 ml/min.

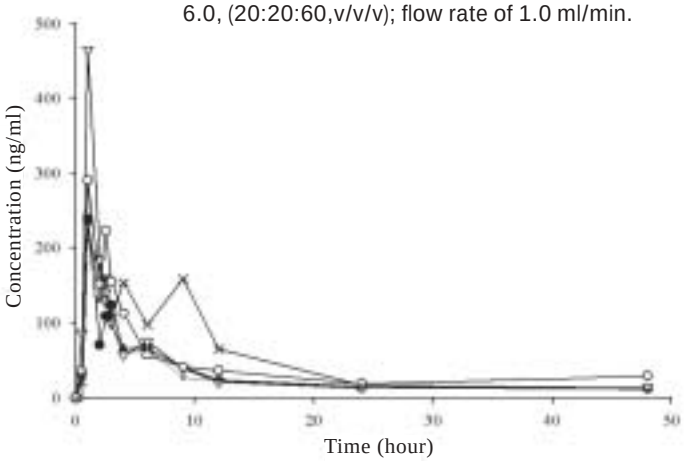

Fig 4–Representative data showing plasma concentration-time profiles of 5 healthy Thai volunteers after the administration of 500 mg azithromycin capsules.

Table 3
The percent deviation of extraction of azithromycin in plasma using Oasis HLB cartridge two times.

Concentration (ng/ml) *(n=5)*	First extraction[a] (ng/ml)	Second extraction[a] (ng/ml)	Deviation (%)
30	27.26 ± 0.82	27.0 ± 2.4	0.85
100	98.4 ± 2.0	95.1 ± 1.3	3.4
200	195.3 ± 2.2	194.8 ± 2.1	0.23

[a] Mean ± Standard deviation

tent and a clear solution. Its advantages over the previously reported HPLC are: (1) it obtained a clear reconstituted sample, (2) a lower LLOQ (10 ng/ml) compared to that reported by Breitschwerdt *et al* (1999) (30 ng/ml) and (3) the Oasis HLB cartridge still had accuracy with duplicate extractions. The method was fully validated according to US FDA guidelines (2001) for Bioanalytical Method Validation. This method had good accuracy, precision, selectivity and linearity. The amperometric detector had low background noise at high voltage and the waiting time for stability was not more than 1 hour when run continuously. The detector recovered after about 2 hours at a flow rate of 1 ml/minute after flushing with methanol, and an overnight pause. The eluent was based on a phosphate buffer because the addition of ammonium acetate or tetra-butyl ammonium to the mobile phase has been reported to increase background noise (Kees *et al*, 1998). The sensitivity of the detector was reduced after running for 1 week continuously or about 250 sample injections. Therefore, the cell detector should be cleaned with DI water every week or after about 250 injections. The mobile phase could be recycled for at least 4 days.

In summary, SPE was selected to provide simple, clean and efficient sample preparation. The LLOQ of the HPLC method with ECD was 10 ng/ml. This method has been successfully applied to the determination of plasma azithromycin concentrations, and demonstrated that the method was reproducible, specific, precise and accurate.

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REFERENCES

Andersen SL, Ager AL, McGreevy P, Schuster BG, Ellis W, Berman J. Efficacy of azithromycin as a causal prophylactic agent against murine malaria. *Antimicrob Agents Chemother* 1994; 38: 1862-3.

Anderson SL, Berman J, Kuschner R*, et al.* Prophylaxis of *Plasmodium falciparum* malaria with azithromycin administered to volunteers. *Ann Intern Med* 1995; 123: 771-3.

Andreotti D, Bientinesi I, Biondi S, *et al*. A novel ketolide class: Synthesis and antibacterial activity of a lead compound. *Bioorg Med Chem Lett* 2007; 17: 5265-9. Epub 2007 Feb 9.

Bahrami G, Mirzae S, Kiani A. High performance liquid chromatographic determination of azithromycin in serum using fluorescence detection and its application in human pharmacokinetic studies. *J Chromatogr B* 2005; 820: 277-81.

Bahrami G, Mohammadi B. A new on-line, in-tube pre-column derivatization technique for high performance liquid chromatographic determination of azithromycin in human serum. *J Chromatogr B* 2006; 830: 355-8.

Bosnar M, Kelneric Z, Munic V, Erakovic V, Parnham MJ. Cellular uptake and efflux of azithromycin, erythromycin, clarithromycin, telithromycin, and cethromycin. *Antimicrob Agents Chemother* 2005; 49: 2372-7.

Breitschwerdt EB, Papich MG, Hegarty BC, Gilger B, Hancock SI, Davidson MG. Efficacy of doxycycline, azithromycin, or trovafloxacin for treatment of experimental Rocky Mountain spotted fever in dogs. *Antimicrob Agents Chemother* 1999; 43: 813-21.

Cárceles CM, Font A, Espuny A, Fernández-Varón E, Serrano JM, Escudero E. Pharmacokinet-

ics of azithromycin after intravenous and intramuscular administration to goats. *J Vet Pharmacol Ther* 2005; 28: 51-5.

Chen B-M, Liang Y-Z, Chen X, Liu S-G, Deng F-L, Zhou P. Quantitative determination of azithromycin in human plasma by liquid chromatography-mass spectrometry and its application in a bioequivalence study. *J Pharm Biomed Anal* 2006; 42: 480-7.

Chen L, Qin F, Ma Y, Li F. Quantitative determination of azithromycin in human plasma by ultra performance liquid chromatography-electrospray ionization mass spectrometry and its application in a pharmacokinetic study. *J Chromatogr B Analyt Technol Biomed Life Sci* 2007; 855: 255-61. Epub 2007 May 26.

Ghouse M, Maulana AB, Mohamed Ali MG, Sarasa VD. A two-year study of the efficacy of azithromycin in the treatment of leptospirosis in humans. *Indian J Med Microbiol* 2006; 24: 345-6.

Girard AE, Girard D, English AR, *et al.* Pharmacokinetic and *in vivo* studies with azithromycin (CP-62,993), a new macrolide with an extended half-life and excellent tissue distribution. *Antimicrob Agents Chemother* 1987; 31: 1948-54.

Heppner DG Jr, Walsh DS, Uthaimongkol N, *et al*. Randomized, controlled double-blind trial of daily oral azithromycin in adults for the prophylaxis of *Plasmodium vivax* malaria in western Thailand. *Am J Trop Med Hyg* 2005; 73: 842-9.

Hoepelmana IM, Schneide MME. Azithromycin: the first of the tissue-selective azalides. *Int J Antimicrob Agents* 1995; 5: 145-67.

Kalilani L, Mofolo I, Chaponda M, *et al*. A randomized controlled pilot trial of azithromycin or artesunate added to sulfadoxine-pyrimethamine as treatment for malaria in pregnant women. *PLoS ONE* 2007; 2: e1166.

Ke J, Lewis J, Hidy BJ. Quantitation of azithromycin in human plasma via HPLC with MS/MS detection. Orlando, Florida: Proceedings of the 50th ASMS Conference on Mass Spectrometry and Allied Topics, 2002.

Kees F, Spangler S, Wellenhofer M. Determination of macrolides in biological matrices by high performance liquid chromatography with electrochemical detection. *J Chromatogr A* 1998; 812: 287-93.

Krudsood S, Silachamroon U, Wilairatana P, *et al*. A randomized clinical trial of combinations of artesunate and azithromycin for treatment of uncomplicated *Plasmodium falciparum* malaria in Thailand. *Southeast Asian J Trop Med Public Health* 2000; 31: 801-7.

Liu F, Xu Y, Huang J, Gao S, Guo Q. Sensitive liquid chromatography/mass spectrometry assay for the quantification of azithromycin in human plasma [Abstract]. *Biomed Chromatogr* 2007; 21: 1272-8.

Miller RS, Wongsrichanalai C, Buathong N, *et al*. Effective treatment of uncomplicated *Plasmodium falciparum* malaria with azithromycin-quinine combinations: a randomized, dose-ranging study. *Am J Trop Med Hyg* 2006; 74: 401-6.

Murray CK, Ellis MW, Hospenthal DR. Susceptibility of Leptospira serovars to antimalarial agents. *Am J Trop Med Hyg* 2004; 71: 685-6.

Noedl H, Krudsood S, Chalermratana K, *et al*. Azithromycin combination therapy with artesunate or quinine for the treatment of uncomplicated *Plasmodium falciparum* malaria in adults: a randomized, phase 2 clinical trial in Thailand. *Clin Infect Dis* 2006; 43: 1264-71. Epub 2006 Oct 12.

Noedl H, Wernsdorfer WH, Krudsood S, *et al*. Antimalarial activity of azithromycin, artemisinin and dihydroartemisinin in fresh isolates of *Plasmodium falciparum* in Thailand. *Acta Trop* 2001; 80: 39-44.

Ohrt C, Willingmyre GD, Lee P, Knirsch C, Milhous W. Assessment of azithromycin in combination with other antimalarial drugs against *Plasmodium falciparum in vitro*. *Antimicrob Agents Chemother* 2002; 46: 2518-24.

Phimda K, Hoontrakul S, Suttinont C. Doxycycline versus azithromycin for treatment of leptospirosis and scrub typhus. *Antimicrob Agents Chemother* 2007; 51: 3259-63. Epub 2007 Jul 16.

Riedel K-D, Wildfeuer A, Laufen H, Zimmermann T. Equivalence of a HPLC assay and a bioassay of azithromycin in human serum samples. *J Chromatogr* 1992; 576: 358-62.

US Department of Health and Human Services Food and Drug Administration (US FDA). Guidance for industry: bioanalytical method validation. *FDA/CDER/CVM*, 2001. [Cited 2006 May 2]. Available from: URL: http://www.fda.gov/cder/guidance/4252fnl.pdf

Xue-Min Z, Jie L, Juan G, Quan-Sheng Y, Wen-Yan W. Determination of azithromycin in human plasma by LC-MS-MS and its pharmacokinetics. *Pharmazie* 2007; 62: 255-7.