Original Article

การตรวจสอบความใช้ได้ของวิธีการสกัดตัวอย่างแบบโซลิดเฟสเอ็กแทรกชันและตรวจวัดระดับความเข้มข้นของแอซิโทรมัยซินในพลาสมาด้วยโครมาโตกราฟีของเหลวสมรรถภาพสูงและตรวจวัดแบบอิเลคโทรเคมิคอล

Method Validation of a Solid Phase Extraction, High Performance Liquid Chromatography and Electrochemical Detection Method for Determination of Azithromycin in Human Plasma.

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Abstract

An effective and sensitive method for the determination of azithromycin in plasma with high performance liquid chromatography (HPLC) and electrochemical detection extracted by solid phase extraction has been developed and validated. Clarithromycin was used as an internal standard. Azithromycin was extracted from 1 ml of plasma by using an Oasis® HLB (Hydrophilic - Lipophilic Balance) solid-phase extraction cartridge. After eluting with 1 ml of methanol, the sample solution was evaporated to dryness, dissolved in 100 µl phosphate buffer-methanol-acetonitrile (60:20:20, v/v/v) and 40 µl injected into the HPLC system. The lower limit of quantitation (LLOQ) was 10 ng/ml without interfering peaks. The calibration curve was linear (r^2 = 0.9998) over the concentration range from 10 to 400 ng/ml. The accuracy and precision were in acceptable range according to criteria of guideline for Industry: Bioanalytical Method Validation of the US FDA (2001). 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 h at room temperature and 6 months stored at -80 °C. The post-preparative stability of spiked-samples was stable more than 24 h after preparation. The cartridge can be used two times providing deviation less than 4%. The method can be used for determination of azithromycin in plasma in pharmacokinetic study, bioavailability and bioequivalence.

Keywords: Azithromycin, solid-phase extraction, validation, HPLC, Oasis HLB

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บทคัดย่อ

การตรวจสอบความใช้ได้ของวิธีการวัดปริมาณแอซิโทรมัยซินในพลาสมา โดยการสกัดตัวอย่างแบบโซลิด-เฟสเอ็กแทรกชัน และตรวจวัดด้วยโครมาโตกราฟีของเหลวสมรรถภาพสูง และเครื่องตรวจวัดแบบอิเลคโทรเคมิคอล คลาริโทรมัยซินเป็นสารมาตรฐานภายใน แอซิโทรมัยซินถูกสกัดจากพลาสมา 1 มิลลิลิตร ผ่านคอลัมน์ยี่ห้อโอเอซิสเอชแอลบี (ไฮโดรฟิลิก-ไลโปฟิลิก บาลานซ์) ระเหยตัวอย่างให้แห้ง ละลายตัวอย่างด้วยสารละลายของฟอสเฟสบัฟเฟอร์-เมทานอล-อะซิโตนิไตล์ (60:20:20) ปริมาตร 100 ไมโครลิตร และฉีดสารละลายของแอซิโทรมัยซิน 40 ไมโครลิตร เข้าสู่เครื่องโครมาโตกราฟีของเหลวสมรรถภาพสูง ความเข้มข้นของแอซิ-โทรมัยซินที่วัดได้ต่ำสุดคือ 10 นาโนกรัมต่อมิลลิลิตร โดยปราศจาก พีครบกวนที่ตำแหน่งยาและสารมาตรฐานภายใน กราฟสารมาตรฐานช่วง 10 ถึง 400 นาโนกรัมต่อมิลลิลิตร มีค่าความเป็นเส้นตรง (r^2) เท่ากับ 0.9998 ความถูกต้องและแม่นยำอยู่ในเกณฑ์การยอมรับของ "guideline for Industry: Bioanalytical Method Validation" ปี 2001 ขององค์การอาหารและยา ประเทศสหรัฐอเมริกา (US FDA) ค่าเฉลี่ยเปอร์เซ็นต์การคืนกลับยาที่ระดับความเข้มข้น 30 100 และ 200 นาโนกรัมต่อมิลลิลิตร เท่ากับ 85.3±5.5%, 80.1±6.8% และ 82.9±2.5% ตามลำดับ มีความคงตัวอย่างน้อย 6 ชั่วโมง และ 6 เดือน เมื่อเก็บพลาสมาไว้ที่อุณหภูมิห้องและ -80°C ตามลำดับ และมีความคงตัว 24 ชั่วโมง หลังจากละลายตัวอย่างก่อนวัด เมื่อสกัดตัวอย่างผ่าน โอเอซิส เอชแอลบี จำนวน 2 รอบ มีค่าเบี่ยงเบนน้อยกว่า 5% ดังนั้น วิธีนี้ สามารถวัดปริมาณแอซิโทรมัยซินในพลาสมาได้อย่างมีความถูกต้องและแม่นยำเพื่อเป็นประโยชน์ในด้านการศึกษาเภสัชจลนศาสตร์ ชีวประสิทธิผลและชีวสมมูลของยาแอซิโทรมัยซิน

คำสำคัญ: แอซิโทรมัยซิน โซลิดเฟสเอ็กแทรกชัน ความใช้ได้ของวิธี HPLC-ECD โอเอซิสเอชแอลบี

Introduction

Azithromycin is an azalide antibiotic, derived from erythromycin containing a methyl-substituted nitrogen in the lactone ring (Hoepelmana and Schneide, 1995). It has been developed to be more stable to acid. Therefore, it exhibits better oral bioavailability and a more favorable pharmacokinetic behavior. It is a tissue-selective antibiotic and possesses a broad spectrum of activity against gram-positive and gram-negative bacteria (Hardy et al., 1988). Azithromycin does not have a specific UV chromophore. The UV detection gives only low sensitivity for determination of azithromycin in plasma. The sensitive methods that have been reported are based on high performance liquid chromatographic (HPLC) with various detector such as fluorescence detector, but pre-column derivatization of biological sample should be done before analysis (Bahrami et al., 2005; Bahrami and Mohammadi, 2006). Recently, HPLC with MS or MS/MS detector was the highest sensitivity of analytical methods for determination of azithromycin concentration in plasma (Hidy et al., 2002; Chen et al., 2007). Nevertheless, the instrument is not yet readily obtainable in most laboratories. The quantitative analysis of azithromycin in biological sample by using microbiological assays was reported (Riedel et al., 1992; Ca´rceles et al., 2005). However this method seemed to have high variation from microorganism.

HPLC with electrochemical detection (HPLC-ECD) has been widely used for determination of azithromycin in plasma due to its high sensitivity and precision without a derivatization process (Riedel et al., 1992; Kees et al., 1998 and Breitschwerdt et al, 1999). Kees et al. (1998) used cyanopropyl silica as 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 of protein with acetonitrile or methanol (Liu et al., 2007; Xue-Min et al., 2007) and liquid-liquid extraction by using organic solvents (Kees et al., 1998; Chen et al., 2006;). The extract resulting from liquid-liquid extraction indicated high recovery of azithromycin but was not clean (several potential interfering peaks) and formed an emulsion when reconstituted with the mobile phase (the analytical column can be blocked). The reconstituted sample which was extracted by solid phase extraction (SPE) seemed to obtain lower peak interferences, compared to those obtained from liquid-liquid extraction. A combination of SPE and HPLC with ECD was applied to analyze azithromycin plasma concentrations in animals with moderate sensitivity (Breitschwerdt et al., 1999). The objective of this study was to validate a simple, selective and sensitive HPLC-ECD method for determination of azithromycin in human plasma by using the SPE in order to apply this method for pharmacokinetics study, bioavailability and bioequivalence.

Materials and Methods

1. Chemicals and materials

Azithromycin and clarithromycin (internal standard, IS) were purchased from the United State Pharmacopeial Convention, Inc (USA). Methanol, acetonitrile, dibasic 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, USA). De-ionized water was obtained from a Milli-Q de-ionized (DI) water system (Millipore, Bedford, MA, USA). Drug-free plasma was obtained from Srinagarind Hospital (Khon Kaen University).

2. Equipment and chromatographic conditions

The modular isocratic HPLC system used in this study is consisted of a Spectrasystem P2000 pump (San Jose, CA, USA), a Waters^{TM} 717 (Waters, Milford, MA, U.S.A.) automatic sample injector and an amperometric detector Model 105 (Precision Instruments, France) with reference electrode (Ag/AgCl). The voltage was set at 1.2 V and current 10 nA range. Data acquisition was performed using Clarity^{TM} version 2.4.1.77 LC system software (Waters, Milford, MA, USA). The analytical column was an Eclipse XDB-CN^{TM} 5 µm, 150x4.6 mm (Agilent Technologies, Palo Alto, CA) protected by a guard column Xterra RP18, 3.9x20 mm (Waters, Milford, MA, USA). The mobile phase was acetonitrile-methanol-0.05M phosphate buffer, pH 6.0 (20:20:60, v/v/v) with a flow rate of 1.0 ml/min. The SPE cartridge used was a hydrophilic-lipophilic balanced copolymer extraction column (1 ml, 10 mg, Waters Oasis HLB, Waters, Milford, MA, USA). A SPE manifold (ASHCROFT, USA) was used to extract plasma. Plasma was forced through the columns by vacuum pump model DOA-V502-BN (GAST manufacturing Inc., MICH, USA). HPLC solvent was degassed by online degassing series 200 (Perkin-Elmer Corporation, Norwalk, CT). The mobile phase was filtered through 0.2 µm of nylon membrane. The HPLC column was maintained at 25±1 °C. Chemical substances were weighed by an electronic balance Model AL 204 (Mettler-Toledo Group). The centrifugation made with Labofuge 200 Hareus Sepatech centrifuge delivered with an integrated angle rotor (max speed 5300 rpm: 3030*g*).

3. Preparation of calibration curves

Stock solutions of azithromycin (500 µg/ml) and clarithromycin (30 µg/ml) were prepared freshly in methanol–water (70:30, v/v) as needed. Azithromycin stock solution was diluted with mobile phase to make a working solution at a concentration of 40 µg/ml. Calibration standards were prepared by spiking azithromycin working solution into drug-free human plasma to obtain concentrations of 10, 30, 50, 100, 200 and 400 ng/ml. The standard concentrations of spiked plasma samples were mixed for 20 s and left them for 1 h to equilibrate. Calibration curves were constructed using six calibration standards and calculated using peak area ratios of azithromycin to IS against the corresponding concentration. Calibration curves were generated by linear equation. The samples used for method validation prepared by spiking azithromycin working solution into drug-free human plasma to obtain concentrations of 30, 100 and 200 ng/ml. The calibration standards and the spiked samples were stored at -80 °C until analysis.

4. Sample preparation

Frozen spiked samples were thawed at room temperature, vortexed and then centrifuged for 3 min at 2700g in order to separate coagulates 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 out under vacuum, equilibrating with 2 ml of water, loading 1 ml of plasma sample and 30 µg/ml of IS 20 µl onto the Oasis HLB SPE columns, washing with 1 ml of DI water and then 1 ml of 5%v/v methanol in water, eluting with 1 ml of methanol (flow rate 0.5 ml/min in every step), evaporating the elute to dryness with nitrogen gas 20 lb^2/inch under a temperature of 25-30°C and reconstituting in 100 µl of mobile phase, mixing and sonicating briefly. Forty µl of Sample was injected into the HPLC-ECD system with 200 µl loop. Precision of injection was checked and the coefficient of variation (%C.V.) was less than 5%.

5. Method validation

The processes of method validation and their criteria followed the guideline for Bioanalytical Method Validation of the US FDA (2001). The concentration of azithromycin was calculated using peak area ratio of sample peak to internal standard. The standard curve was in the form of y=A+Bx, where y represents the plasma concentration of the analytes and x represents the ratio of the analysis peak area to the IS, calculated using linear regression. To evaluate linearity, plasma calibration curves were prepared and analyzed in 5 replicates at each calibration standard concentration. Correlation coefficient values (r^2) were calculated. Lower limit of quantification (LLOQ) was selected to be the minimum concentration where the precision was within ±20% and accuracy was within the range of 80-120%.

5.1 Selectivity

The interferences from other components such as decomposition products, metabolites and concomitant medication in the samples were investigated by the analysis of blank plasma samples from at least six different sources. The blank plasma chromatograms contained no interference to azithromycin and IS.

5.2 Accuracy, intra- and inter-day precision

Intra- and inter-day precisions were determined as %C.V. 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 the spiked samples 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 %C.V. of each concentration level should not exceed ± 15% and accuracy should be within the range of 85-115% (US FDA, 2001).

5.3 Recovery

The recoveries of azithromycin extraction at three levels of spiked samples 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 spiked samples level (n=5).

5.4 Stability

The short-term stability was examined by keeping two levels of the spiked samples (30 and 200 ng/ml, n=3) at room temperature and analyzing for 6 h. Auto-sampler stability of azithromycin was tested by analysis of two processed and reconstituted the spiked samples, those were stored in the auto-sampler tray for 24 h. The long-term stability of azithromycin in plasma was tested after storage of two levels of the spiked samples at -80°C for 24 weeks. Sampling times were at 0, 1, 3, 6, 9, 12 and 24 weeks.

6. The deviation of reused Oasis HLB cartridge

The percent deviation of duplicate extraction of Oasis HLB cartridges was determined. The percent deviation was calculated by an average concentration of the first extraction minus average concentration of second extraction, multiplied with 100 and divided by average concentration of the first extraction. The deviation at each QC levels should not exceed ±15% (US FDA, 2001).

Results

1. Method of SPE extraction

The important part of many chromatographic methods in the assay of biological samples is the elimination of interferences during the HPLC determination. A required selective extraction method was partially purified and concentrated the sample prior to their analysis. The chosen SPE column, Oasis HLB is a polymeric construction designed to have a Hydrophilic-Lipophilic Balance (HLB) that gives high recoveries and reproducibility for acidic, basic, and neutral compounds (Instruction sheet, Water Oasis). Azithromycin has been extracted from spiked plasma and purified by using Oasis HLB. Washing with 1 ml of DI water and 5%methanol provided acceptable recoveries and no interfering peaks to azithromycin or IS.

2. Method validation

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

2.1 Selectivity

Selectivity was assessed by comparing the chromatograms of six different sources of plasma

with the corresponding spiked plasma. There were no interfering peaks from blank human plasma (n=6) observed at the retention times of the analyses. The retention times of IS and azithromycin in spiked plasma are shown in Figure 1. The retention times of IS and azithromycin were about 14.0 and 16.6 min, respectively.

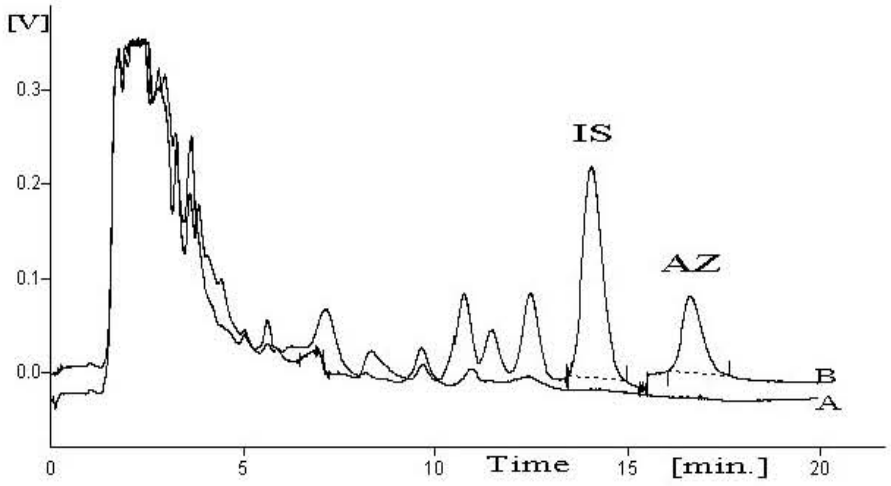

Figure 1 The overlay of chromatograms of a blank plasma (line A) and spiked plasma sample with azithromycin 200 ng/ml (AZ) and IS 30 µg/ml (line B) with mobile phase of acetonitrile-methanol-0.05M phosphate buffer, pH 6.0, (20:20:60,v/v/v); flow rate of 1.0 ml/min.

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

The r^2 of calibration curve was 0.9998 (n=5) which confirmed that the calibration curves were linear over the 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 of which accuracy and precision were in acceptable range. The accuracy was in the range of 80-120% (mean = 111.14%) and a precision less than 20% (mean = 5.85%).

2.3 Precision and accuracy

The intra-day, inter-day precision and accuracy for azithromycin were evaluated by assaying the three concentrations of azithromycin in spiked samples. In this assay, for 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 of 98.6-101.9% (Table 1). The results above demonstrate that the values are within the acceptable range and the method is accurate and precise.

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

Added (ng/ml)	Found* (ng/ml)	Intra-day, C.V. (%) *(n=5)*	Inter-day, C.V. (%) *(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

* Mean ± Standard deviation

2.4 Recovery and stability

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

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

Experimental condition	Initial concentration* (ng/ml)	Found* (ng/ml)	Deviation (%)
Auto-sampler 24 h storage at RT	30.51±0.16	30.57±0.35	-0.20
	199.9±2.4	199.8±1.1	0.02
6 h 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
24 weeks storage	33.1±1.4	29.26±0.55	11.5
at -80 °C (long-term)	206.9 ±9.3	194.7±4.3	5.9

* Mean ± Standard deviation

RT: room temperature

3. The deviation of reused Oasis HLB cartridge

The percent deviation of reused extraction of azithromycin in plasma by using Oasis HLB cartridge is shown in Table 3. The Oasis HLB cartridge can be reused in duplication with percent deviation of extraction less than 15% (range 0.23-3.4%).

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

Concentration (ng/ml) *(n=5)*	first extraction* (ng/ml)	second extraction* (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

* Mean ± Standard deviation

Discussion and conclusion

The HPLC method with ECD for determination of azithromycin in plasma is simple, selective and reproducible. Although HPLC with MS or MS/MS detection is highly sensitive with the lowest LLOQ of 1 ng/ml (Chen et al., 2007), the instruments are expensive and not yet readily available in most laboratories. SPE extraction was developed for the 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 of azithromycin and IS, with more than 80% recovery. Low albumin content and clear solution was presented in the reconstituted sample. Its advantages over the previously reported HPLC are: (i) obtained a clear reconstituted sample; (ii) the lower LLOQ (10 ng/ml), compared to that reported by Breitschwerdt *et al.* (1999) (30 ng/ml) and (iii) the duplicated extraction by using Oasis HLB cartridge was accurate. The method was fully validated according to the US FDA guidelines (2001) for Bioanalytical Method Validation. This method showed acceptable 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 h when run continuously. Sensitivity of the detector was reduced after running for 1 week continuously or about 250 sample injections. Therefore, the cell of detector should be cleaned with de-ionized water every week or after about 250 injections. The operating cost and SPE cartridge obstruction can be a limitation of this method.

In summary, the HPLC-ECD was sensitive method for determination of azithromycin in plasma and SPE was selected to provide a simple, clean-up and efficient sample preparation. This HPLC-ECD method validation can be applied for pharmacokinetics, bioavailability and bioequivalence studies in human.

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