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Short communication

High performance liquid chromatographic determination of azithromycin in serum using fluorescence detection and its application in human pharmacokinetic studies

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Abstract

A fast and sensitive high-performance liquid chromatographic method for determination of azithromycin in human serum using fluorescence detection was developed. The drug and an internal standard (clarithromycin) were extracted from serum using *n*-hexan and subjected to pre-column derivatization with 9-fluorenylmethyl chloroformate as labeling agent. Analysis was performed on a phenyl packing material column with sodium phosphate buffer containing 2 ml/l triethylamine (pH 5.9) and methanol (29:71, v/v) as the mobile phase. The standard curve was linear over the range of 10–500 ng/ml of azithromycin in human serum. The means between-days precision were from 13.3% (for 10 ng/ml) to 2% (500 ng/ml) and the within-day precision from 11.9 to 1.7% determined on spiked samples. The accuracy of the method was 100.7–107.2% (between days) and 100.3–107.8% (within day). The limit of quantification was 10 ng/ml. This method was applied in a bioequivalence study of four different azithromycin preparations in 12 healthy volunteers.

Keywords: Reverse phase chromatography; HPLC; Azithromycin; Serum; Bioequivalence study; Macrolide antibiotics

1. Introduction

Azithromycin [9-de-oxo-9a-aza-9a-methyl-9a-homoerythromycin A dehydrate], is a macrolide antibibiotic. It is chemically related to erythromycin with enhanced spectrum and potency against some bacteria, longer elimination half life, superior stability in acid environment and higher tissue concentrations [1]. Low plasma concentration is achieved following administration of azithromycin, thus quantification methods of the drug in pharmacokinetic studies and monitoring of its efficacy needs to be sensitive and specific. The analysis of the drug is complicated, because azithromycin has only a weak UV absorbance in the wavelength range of less than 220 nm. High-performance liquid chromatography (HPLC) with UV detection has been used for analysis of the drug in in-vitro studies including bulk samples, raw materials and acid degradation studies [2–4]. In biological samples several HPLC methods using electrochemical detection have been developed for determination of azithromycin [5–12]. A sensitive HPLC method using atmospheric pressure chemical ionization mass spectrometric detection has been reported for the analysis of azithromycin in plasma. This method requires only a sample volume of 50 μl [13]. Quantitative determination of azithromycin in human serum using 9-fluorenylmethyl chloroformate (FMOC-Cl) as a fluorescent labeling agent has been reported by Torano and Guchelaar [14]. In their method however, the limit of quantitation (LOQ) is 92 ng/ml which is not enough for human pharmacokinetic studies using low dosages. Also due to the simultaneous determination of all macrolides the retention time is 20 min, which is time consuming. Based on their method a new sensitive, rapid and simple assay for the determination of azithromycin in human serum has been developed. In the present method the sensitivity has been improved and the run time has been reduced. This method was applied for analysis of the drug in

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a bioequivalence study following oral administration of four different azithromycin preparations in 12 healthy volunteers.

2. Experimental

2.1. Chemicals

Azithromycin and clarithromycin hydrochloride (I.S.) were from Sigma (St. Louis, MO, USA) and kindly provided by Tehran Shimi pharmaceutical company (Tehran, Iran). Methanol (HPLC grade), hexan, boric acid, potassium chloride, potassium hydroxide, potassium dihydrogen phosphate, triethylamine, sodium carbonate, sodium bicarbonate, phosphoric acid and glycine were purchased from Merck (Darmstadt, Germany). FMOC-Cl was obtained from Sigma (St. Louis, MO, USA). All reagents used were of analytical grade except methanol which was HPLC grade. Water was glass-double distilled and further purified for HPLC with a Maxima purification system (USF ELGA, England).

2.2. Preparation of standard solutions

Stock solutions of azithromycin and the I.S. were prepared by dissolving the compounds in acetonitrile at concentrations of 1000 and 25 μg/ml, respectively. The azithromycin stock solution was further diluted with acetonitrile to working solutions ranging from 10 to 5000 ng/ml. A borate buffer (0.1 M) was prepared by dissolving 0.625 g of boric acid and 0.750 g of potassium chloride in 100 ml water and adjusting the pH to 7.4 with 0.2 M potassium hydroxide solution. A 500 μl/ml solution of FMOC-Cl was prepared in acetonitrile. Stock solution of glycine (4 mg/ml) was prepared in water. All solutions were stored at 4 °C and were stable for at least 4 weeks.

2.3. Chromatography

The HPLC system consisted of two pumps (LC-10AD), a column oven (CTO-10A), a spectrofluorometric detector (RF-551) operated at an excitation wavelength of 260 and emission wavelength of 315 nm, a degasser (DGU-3A) and a data processor (C-R4A) all from Shimadzu, Kyoto, Japan. The analysis was performed on a phenyl column (150 mm × 6 mm i.d.) which was packed with 5 μm particles (Shimpack-CLC-Phenyl) and operated at 62 °C. The mobile phase consisted of methanol–0.05 M sodium phosphate buffer (71/29, v/v) containing 2 ml/l triethylamine and adjusted to a pH of 5.9 with phosphoric acid. The eluent was filtered, degassed and pumped at a flow rate of 2.5 ml/min.

2.4. Sample preparation and derivatization

To 1 ml serum samples 100 μl of I.S., and 5 ml hexan were added. After mixing for 30 s on a vortex mixer and centrifugation (5 min at 6000 × *g*), the organic phase was removed and evaporated to dryness under a stream of nitrogen at 45 °C. The residue was subjected to derivatization with FMOC-Cl as previously described [14] with some modifications. Briefly 160 μl FMOC-Cl (500 μl/ml in acetonitrile) and 40 μl of borate buffer (pH 7.4) were added and after vortex mixing for 10 s the samples were kept at 50 °C for 40 min. The reaction was stopped by adding 10 μl glycine (0.1 M) and, after 1 min, 20 μl of the reaction mixture was injected in to the chromatograph.

2.5. Calibration

An amount of 100 μl from each working solutions was evaporated in disposable glass tubes (16 mm × 100 mm) under a gentle stream of nitrogen at 50 °C. The residue was reconstituted in 1 ml drug-free human serum. The samples were then submitted to the procedures of extraction, derivatization and chromatographic analysis described above. Calibration curves (unweighted regression line) were obtained by linear least-squares regression analysis plotting of peak-area ratios (azithromycin/I.S.) versus the azithromycin concentrations.

2.6. Performance assessment

The recovery of azithromycin from serum was determined at concentration ranges of 10, 100 and 500 ng/ml by comparing peak areas obtained after derivatization of azithromycin extracted from serum with peak areas obtained after derivatization of the same amounts of unextracted azithromycin solutions in acetonitrile. The recovery of I.S. from serum was determined at a concentration of 25 ng/ml by the same method. Within-day variation was measured by assessing the different controls in replicates of six. Between-days variation was based on repeated analysis of the same concentration controls in ten analytical run performed on different days. The specificity of the method was investigated by the analysis of 12 human blank serum samples from different volunteers. These samples were pretreated according to the sample preparation procedure except from the addition of the I.S. The selectivity of the assay was evaluated by derivatization and analysis of a group of potentially co-administrated drugs with the azithromycin. The limit of detection was defined as the concentration of drug giving a signal to noise ratio of 4:1. The lower limit of quantification was defined as the lowest serum concentration of azithromycin quantified with a coefficient of variation of less than 20% (range recommended by the Conference Report on Bioanalytical Methods Validation [15]).

3. Results and discussion

3.1. Specificity and selectivity

Representative chromatograms of human blank serum and human blank serum spiked with azithromycin (10 ng/ml) and the I.S. are shown in Fig. 1A and B, respectively.

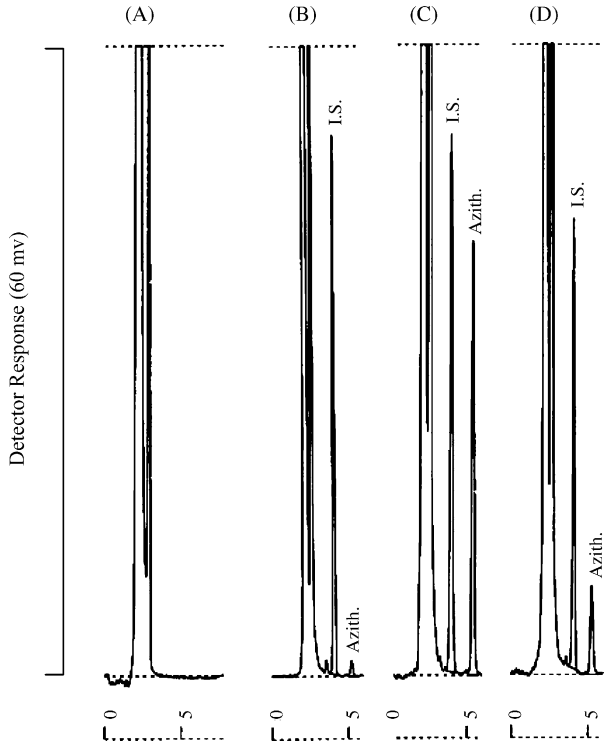

Fig. 1. Typical chromatograms obtained from an extract of (A) human blank serum (B) human blank serum spiked with 10 ng/ml azithromycin and clarithromycin as the I.S., (C) serum samples from a volunteer 6 and 48 h after a single oral dose of 2 × 250 mg drug containing 220 and 72 ng/ml of azithromycin, respectively.

Azithromycin and the I.S. were well resolved with good symmetry with respective retention times of 5.3 and 3.9 min, respectively. Endogenous components chromatographed within 2 min and no endogenous peaks from serum were found to interfere with the elution of the drug or I.S. Fig. 1C and D show the chromatograms of serum samples obtained at 6 and 48 h after a single oral dose of 2 × 250 mg azithromycin from a healthy volunteer. The results of the selectivity study showed that the following drugs were not detected with the described analytical method: acetaminophen, theophylline, naproxen, cefalexin, acyclovir, lamivudine, codeine, caffeine, diclofenac, diazepam, nalidixic acid, ciprofloxacin, fluconazole, ketoconazole, sulfametoxazole, trimethoprim, and ceftriaxone. Gentamicin, etidronate, topiramate and erythromycin react with the FMOC-Cl but did not give interference with the analysis of the I.S. or azithromycin.

3.2. Recovery, accuracy and precision

The mean recoveries of azithromycin and I.S. from serum were found to be 101 ± 3% and 99 ± 5%, respectively. The within-day and between-days accuracy and precision values of the assay method are presented in Table 1. The coefficient variation values of both within day and between days were all less than 13.5% whereas accuracy never deviated from 100% by more than 8%.

Table 1
Precision and accuracy results of the validation

Known concentration (ng/ml)	Concentration found (mean ± S.D.)	Coefficient of variation (%)	Accuracy (%)
Within-day (n = 6)			
10	10.8 ± 1.13	11.9	107.8
25	25.9 ± 1.5	5.9	103.7
50	51.1 ± 2.3	4.4	102.2
100	101.3 ± 3.0	3	101.3
200	202.5 ± 5.4	2.7	101.3
500	501.7 ± 8.7	1.7	100.3
Between-day (n = 10)			
10	10.7 ± 1.4	13.3	107.2
25	25.6 ± 1.9	7.3	102.3
50	50.9 ± 2.9	5.6	101.8
100	101.3 ± 3.2	3.2	101.3
200	202.8 ± 6.2	3.1	101.4
500	503 ± 9.8	2	100.7

Accuracy has been calculated as a percentage of the real concentration.

3.3. Limit of quantification, linearity and stability

The limit of detection was approximately 3 ng/ml and LOQ was 10 ng/ml. The standard calibration curves were linear over the concentration ranges of 10–500 ng/ml. The correlation coefficients for calibration curves were equal to or better than 0.9980. Intra-assay reproducibility was determined for calibration curves prepared the same day in replicate (n = 6) using the same stock solutions. The intra-day average slope of the fitted straight lines was 3.7017 ± 0.095 ng/ml (CV = 2.57%) and the mean intercept of the calibration curves was 7.2166 ± 0.1530 (CV = 2.13%). The corresponding mean (±S.D.) coefficient of the linear regression analysis was 0.9985 ± 0.008 (CV = 0.112%). For calibration curves prepared on different days (n = 10), the mean ± S.D. of results were as follows: slope = 3.6844 ± 0.101 ng/ml (CV = 3.12), coefficient of the linear regression analysis = 0.9982 ± 0.006 (CV = 0.118%) and intercept = 6.84 ± 0.2411. Stock solutions of azithromycin and clarithromycin were stable for at least 60 days when stored at 4 °C and the derivatized solutions were found to be stable (>95%) for 24 h. After 60 days the concentration of azithromycin in serum stored at −80 °C were found to be 102% from the initial value.

Various extraction procedures including protein precipitation methods and liquid-liquid extraction were investigated for extraction of azithromycin from serum. Direct protein precipitation with acetonitrile, trichloroacetic acid or perchloric acid gave low recovery with presence of endogenous impurities in the chromatograms. Comparison of efficiency of different extracting solvents including ethyl acetate, hexane, diethyl ether, dichloromethane and chloroform showed that hexan and diethyl ether gave the best recoveries for azithromycin and the I.S. However, due to co-extracted impurities with diethyl ether, *n*-hexan was used as the extracting solvent.

A number of drugs with secondary or primary amines or hydroxyl groups (e.g. erythromycin, spiramycin, etidronate,

Table 2
Mean (S.D.) pharmacokinetic parameters of azithromycin for different azithromycin preparations (1: Pfizer, 2: Chimodaruo, 3: Tehran Shimi and 4: Loghman pharmaceutical companies) in 12 human volunteers after administration of a single 2 × 250 mg oral dose

Parameter	Preparations			
	1	2	3	4
T_{max} (h)	2.63 (0.52)	2.67 (0.47)	2.7 (0.55)	2.71 (0.51)
C_{max} (ng/ml)	394.2 (133.9)	348.3 (118.9)	346.2 (107.8)	348 (112.0)
AUC_{0-48} (ngh/ml)	1754 (733.2)	1540 (514.2)	1574 (488.0)	1780 (571.9)
$AUC_{0-\infty}$ (ngh/ml)	2159 (854.7)	1911 (623.6)	2024 (569.0)	2220 (795.6)
$T_{1/2}$ (h)	10.9 (2.1)	11.4 (2.4)	11.7 (3.1)	10.5 (3.6)

T_{max}: time to maximum concentration, C_{max}: maximum concentration, AUC: area under the concentration time curve, $T_{1/2}$: elimination half life.

alendronate, gentamicin, amikacin, clarithromycin) were tested and clarithromycin was selected as internal standard because of its suitable retention time. Different analytical columns (C8, CN, phenyl and TMS) were tested and considering the resolution of the drug from both endogenous peaks and the I.S the Shimpack CLC-phenyl was selected.

Quantitative determination of macrolides in human serum using FMOC-Cl as fluorescent labeling agent has been reported by Torano and Guchelaar [14]. In their method however, LOQ is not enough for bioequivalence studies and long retention time of analysis is time consuming.

Although fluorescence response can be increased by increment of the derivatization reagent however, in our study maximal yields of the derivatives for different concentrations of azithromycin were observed using 160 μl FMOC-Cl (500 μg/ml in acetonitrile). A solution of FMOC-Cl with concentration of 2.5 mg/ml has been used in the method of Torano and Guchelaar [14]. In their method the excess of FMOC-Cl is eluted in the first part of chromatogram which leads to significant band-broadening and interfere with the peak of the I.S. and further separation of reagent excess and the I.S has been achieved at the expense of longer analysis time and reduction of the sensitivity. In our method however, lower amounts of the derivatization reagents was applied and the reagent excess was eliminated by addition of an amino acid such as glycine and direct injection of reaction products without further separation of reagent excess and the I.S. (FMOC-glycine being eluted in the first part of the chromatogram). In their method diethyl ether has been used as extracting solvent, however, cleaner chromatograms with the same recoveries for both the drug and I.S. are achieved by *n*-hexan in our method. Column temperature in our method was 62 °C (recommended range of temperature by column manufacture is up to 70 °C), this temperature reduces the time of analysis, improves resolution and reduces the column back pressure.

4. Application of the method

The developed method has been applied in a randomized crossover bioequivalence study in which the concentrations of azithromycin was measured in serum samples from 12 healthy volunteers after single oral doses (2 × 250 mg) of four different azithromycin preparations [Tehran Shimi, Chimidaru, Loghman (Tehran, Iran) and Zithromax (Pfizer, USA)]. The drugs were administrated under fasting conditions and blood samples were collected at suitable intervals up to 48 h after drug administration. Typical serum concentration–time profiles for two preparations are presented in Fig. 2. Pharmacokinetic parameters obtained from all preparations are summarized in Table 2.

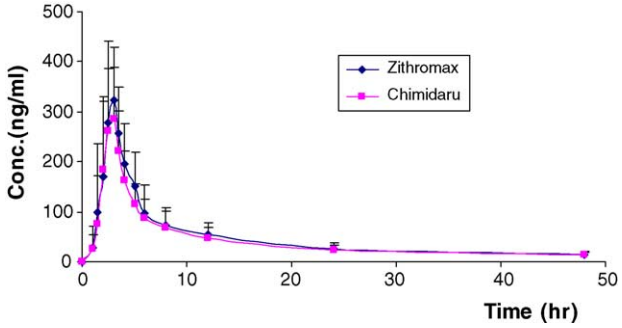

Fig. 2. Mean serum concentrations vs. time profiles of azithromycin for two azithromycin preparations in 12 human volunteers after administration of a single 2 × 250 mg oral dose.

5. Conclusion

In conclusion a sensitive, rapid and specific reverse-phase HPLC method with fluorescence detection has been described for the determination of azithromycin in serum. In this method which has been demonstrated to be suitable for use in pharmacokinetic studies of azithromycin, comparing to the previously published method the speed and limit of quantification have been improved and less time is needed for analysis of the drug in human serum.

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