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Determination of clarithromycin in human serum by high-performance liquid chromatography after pre-column derivatization with 9-fluorenylmethyl chloroformate: Application to a bioequivalence study

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Abstract

A sensitive liquid chromatographic method for the analysis of clarithromycin, a macrolide antibiotic, in human serum using pre-column derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl) is described. The method involved liquid–liquid extraction of the drug and an internal standard (amantadine) followed by pre-column derivatization of the analytes with FMOC-Cl. A mixture of 0.05 M phosphate buffer containing triethylamine (2 mL L^{-1}; pH 3.8) and methanol (17:83, v/v) was used as mobile phase and chromatographic separation was achieved on a Shimpack CLC-ODS column. The eluate was monitored by a fluorescence detector with respective excitation and emission wavelengths of 265 and 315 nm. The analytical method was linear over the concentration range of 0.025–10 μg mL^{-1} of clarithromycin in human serum with a limit of quantification of 0.025 μg mL^{-1}. The assay is sensitive enough to measure drug levels obtained in human single dose studies. In the present method, sensitivity and run time of analysis have been improved, and successfully applied in a bioequivalence study of three different clarithromycin preparations in 12 healthy volunteers.

Keywords: Clarithromycin; Reverse phase chromatography; Bioequivalence study; Macrolide antibiotics

1. Introduction

Clarithromycin is a macrolide antibiotic with improved acid stability, better oral absorption, lower frequency of gastrointestinal intolerance, longer half life and more antibacterial activity against some pathogens compared with erythromycin [1]. Due to weak UV absorbance of macrolide antibiotics, it is difficult to develop a simple method for analysis of the drugs in biological fluids using conventional UV detection, thus, high-performance liquid chromatography (HPLC) with electrochemical detection (ED) is the standard method for analysis of the drugs in biological matrix. Several HPLC methods have been reported for analysis of clarithromycin in human serum using EC [2–9], MS [10], UV [11] and fluorescence detections [12]. Determination of the drug in serum samples using HPLC with UV detection at 205 nm has been recently reported by Amini and Ahmadiani [11] and although, limit of quantification (LOQ) of 31.25 ng mL^{-1} has been obtained using injection volume of 100 μL, however, due to weak UV absorbance of the drug the system should be set at highest sensitivity. Furthermore, high background noises are provided at this wavelength which makes difficult to obtain a stable baseline. Also, two steps sample preparation including liquid–liquid extraction followed by back extraction into diluted acid and analytical running time of 11 min have been reported in their method. Different sensitivities (10.03 μg mL^{-1} [2], 0.5 μg mL^{-1} [3], 0.03 μg mL^{-1} [4], 0.1 μg mL^{-1} [6], 0.01 μg mL^{-1} [8], 0.15 μg mL^{-1} [9]) have been reported using HPLC-ED methods. A very sensitive method (LOQ 2.95 ng mL^{-1}) for analysis of the drug in human plasma by HPLC–MS has been published [10]. Quantitative analysis of macrolide antibiotics including erythromycin, roxithromycin, azithromycin and clarithromycin in human serum using pre-column derivatization with 9-fluorenylmethyl chloroformate (FMOC-Cl) and fluorescence detection has been

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reported by Torano and Guchelaar [12]. In their method, however, the sensitivity of analysis is not enough for human pharmacokinetic studies following a single dose administration of the drug (LOQ 0.20 μg mL^{-1} using 50 μL injections). Furthermore, long time is needed for sample preparation as well as for reaction of the macrolides with FMOC-Cl (40 min). Based on those observations the present paper describes an improved method for analysis of clarithromycin in human serum using FMOC-Cl as labeling agent and fluorescence detection. This method was applied for quantification of the drug in a bioequivalence study following oral administration of three different clarithromycin preparations in 12 healthy volunteers. It should be emphasized that, less time is needed for sample preparation and derivatization reaction, whereas sensitivity has been improved, and the running analysis time was reduced.

2. Experimental

2.1. Chemicals

Clarithromycin was from Abbott Pharmaceutical Company (Kent, UK). Amantadine (I.S.) and FMOC-Cl were from Sigma (Sigma, St. Louis, MO, USA). HPLC grade methanol, monobasic sodium phosphate, phosphoric acid, anhydrous sodium sulfate, triethylamine and dichloromethane were purchased from Merck (Darmstand, Germany) All reagents were of the maximum available purity and were used without further purification. Water was glass-double distilled and further purified for HPLC with a Maxima purification system (USF ELGA, England).

2.2. Equipment

The HPLC system used consisted of two pumps of Shimadzu LC-10A solvent delivery system, a system controller (SCL 10AD), a spectroflurometric detector (RF-551) operated at an excitation and emission wavelengths of 265 and 315 nm, respectively, a column oven (CTO-10A), a degasser (DGU-3A) and a data processor (C-R4A) all from Shimadzu, Kyoto, Japan. The analytical column was a Shimpack CLC-ODS (Shimadzu, Kyoto, Japan), 150 mm × 4.6 mm i.d., 5 μm particle size which was protected by a Shim-pack G-ODS guard column (1 cm × 4.0 mm i.d., 5 μm particle size). A mixture of 0.05 M sodium phosphate buffer containing triethylamine (2 mL L^{-1}; pH 3.8) and methanol (17:83, v/v) was used as the mobile phase. The column oven temperature was set at 58 °C and the mobile phase was filtered, degassed and pumped at a flow rate of 2.0 mL min^{-1}.

2.3. Solutions

Stock solutions of clarithromycin (1000 μg mL^{-1}) and the I.S. (600 μg mL^{-1}) were prepared by dissolving the drugs in acetonitrile and distilled water, respectively. The clarithromycin stock solution was further diluted with acetonitrile to obtain the different working solutions ranging from 0.25 to 100 μg mL^{-1}. The I.S. stock solution was diluted with distilled water to obtain working solution of 6 μg mL^{-1}. A 1000 μg mL^{-1} solution of FMOC-Cl was prepared in acetonitrile. All solutions were stored at 4 °C and were stable for at least 4 weeks except clarithromycin stock solution which was prepared freshly before the analysis.

2.4. Extraction procedure and derivatization

Serum samples were stored at −40 °C until assay. Frozen serum samples were thawed in water at 37 °C. Aliquots of blank, calibration standard or unknown human serum samples (1 mL) were pipetted into 100 mm × 16 mm disposable glass tubes, containing 100 μL of working internal standard solution. The samples were mixed with 200 μL of a phosphate buffer (0.05 M; pH 3) and extracted with 5 mL of dichloromethane as extracting solvent. After vortex mixing for 30 s and centrifugation (5 min at 6000 × *g*) the organic phase was removed and evaporated to dryness under stream of nitrogen at 50 °C. The residue was reconstituted in 100 μL of the FMOC-Cl solution. Following addition of 25 μL phosphate buffer (0.05 M; pH 8.5) and brief mixing, the samples were kept at 60 °C for 15 min and then a volume of 20 μL of the reaction mixture was injected onto the HPLC system.

2.5. Preparation of calibration curve standards

Samples for calibration curves were prepared within the concentration range of 0.025–10 μg mL^{-1}. In disposable glass tubes (100 mm × 16 mm), after evaporation of 100 μL from each working solutions of the drug, under a gentle stream of nitrogen at 50 °C, the residues were reconstituted in 1 mL of drug-free human serum and mixed for 10 s on a vortex mixer and the samples were subjected to extraction, derivatization and analysis as described above.

2.6. Method validation

Assay linearity was evaluated with nine calibration standards in duplicate using blank serum samples obtained from healthy volunteers. Calibration curves (weighted regression line) were obtained by linear least-squares regression analysis of plots of peak–area ratio of clarithromycin to I.S. versus drug concentrations. Quality control samples used in method validation were prepared with the drug working solutions to make low (0.025 μg mL^{-1}), medium (0.5 μg mL^{-1}) and high (5 μg mL^{-1}) concentrations. Within and between day variations were determined by repeated analysis ($n = 6$) of different concentrations of the drug in a single analytical run and in 10 analytical runs performed on different days, respectively, using the same stock solutions and serum batches. The specificity of the method was examined by presence of disturbing endogenous peaks in twelve human serum samples from different volunteers. These samples were pretreated according to the sample preparation procedure except from the addition of the I.S. The absolute recoveries of clarithromycin at the above mentioned concentrations as well as the I.S. at applied concentration were calculated in replicates ($n = 5$) by comparing the respective peak areas obtained by derivatization of the extracted samples from serum, with those obtained after derivatization of the same amounts of unextracted

solutions in acetonitrile. The limits of detection (LOD) and quantification were defined as the concentration of the drug giving a signal-to-noise ratio of 3:1 and 10:1, respectively, and stability of solutions of clarithromycin and the I.S. was studied over a period of 4 weeks by comparing of the peak areas at different times. Stability of the drug in serum samples was examined by comparing of the determined concentration in different times up to 30 days maintenance of the samples at −40 °C and following three thaw–freeze cycles.

2.7. Application of the method

The present method was applied in a three-way randomized crossover bioequivalence study of different clarithromycin preparations. Twelve male healthy volunteers aged 28.5 ± 2.5 years and weighing 69.3 ± 6.1 kg with normal biochemical parameters were enrolled in this study. All subjects received a single oral dose of 500 mg clarithromycin from either Tehran Chimi (Tehran, Iran), Chimidaruo (Tehran, Iran) or Abbott (Kent, UK) pharmaceutical companies on 3 working days separated by a wash-out period of 2 weeks. All the subjects were asked to refrain from food or water consumption for 3 h after drug administration. Blood sampling were carried out at suitable intervals up to 24 h and pharmacokinetic parameters including maximum concentration (C_{max}), area under the concentration time curve from 0 to time of last sampling (AUC0–t) and area under the concentration time curve from 0 to infinity (AUC0–∞) were compared. Bioequivalence between the preparations was determined by calculating 90% confidence intervals for the ratio of C_{max}, AUC0–t, and AUC0–∞ values for different products, using logarithmic transformed data. One-way analysis of variance (ANOVA) was used to statistical comparison of the data.

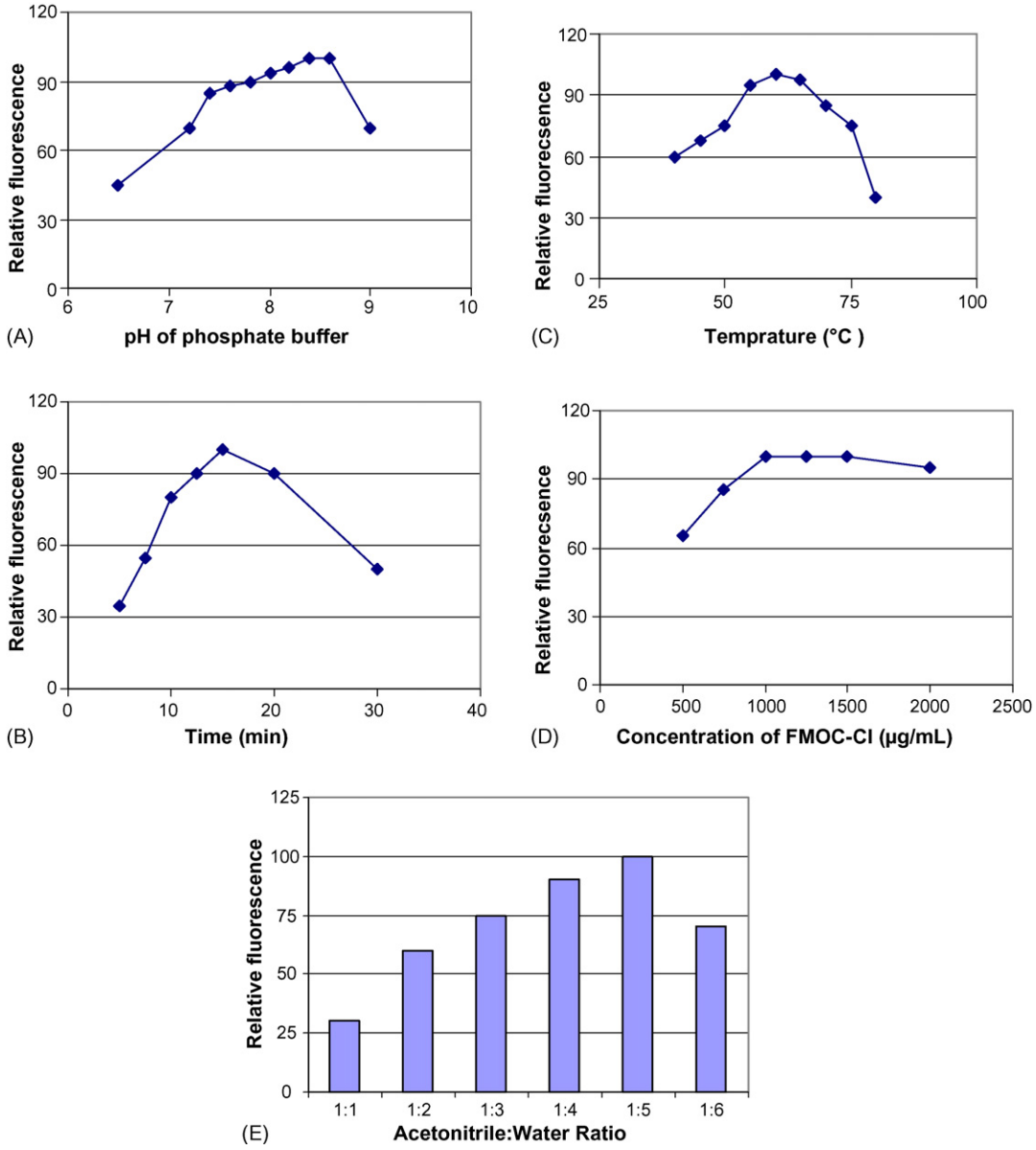

Fig. 1. Effects of (A) pH of phosphate buffer, (B) time of derivatization, (C) temperature of reaction, (D) concentration of the labeling agent and (E) polarity of the medium on the reaction of clarithromycin with FMOC-Cl.

3. Results

The reaction of clarithromycin and the I.S. with FMOC-Cl appeared to be highly dependent on pH of buffer solution (Fig. 1A), time (Fig. 1B), temperature (Fig. 1C), concentration of the labeling agent (Fig. 1D) and polarity of the medium (Fig. 1E). Based on these results, the optimal conditions were found to be: a buffer solution with pH of 8.5 consisting of water–acetonitrile (1:5, v/v), a reaction temperature of 60 °C for 15 min and the reagent concentration of 1000 μg mL^{-1}.

Typical chromatograms of human blank serum and human blank serum spiked with clarithromycin (0.05 μg mL^{-1}) and the I.S. are shown in Fig. 2A and B, respectively. Clarithromycin and the I.S. were eluted with respective retention times of 6.6 and 5.5 min. No endogenous substance from the plasma components was eluted in the retention times of clarithromycin or the I.S. Fig. 2C and D show the chromatograms of serum samples obtained at 5 and 24 h after a single oral dose of 500 mg clarithromycin from a healthy volunteer. The following drugs were tested for selectivity study and none of them were interfered with analysis of the drug using the described method: acetaminophen, amoxicillin, cefalexin, cefradine, ceftriaxone, theophylline, acyclovir, codeine, caffeine, diclofenac, diazepam, nalidixic acid, ciprofloxacin, fluconazole, ketoconazole, gentamicin, etidronate, alendronate, baclofen, topiramate, erythromycin, propranolol and azithromycin.

LOD was approximately 0.01 μg mL^{-1} and LOQ was 0.025 μg mL^{-1}. The proposed HPLC method was linear over the concentration range of 0.025–10 μg mL^{-1}. The correlation coefficients (r) for calibration curves were equal to or better than 0.9982. Intra-assay reproducibility was determined for calibration curves prepared in the same day in replicate

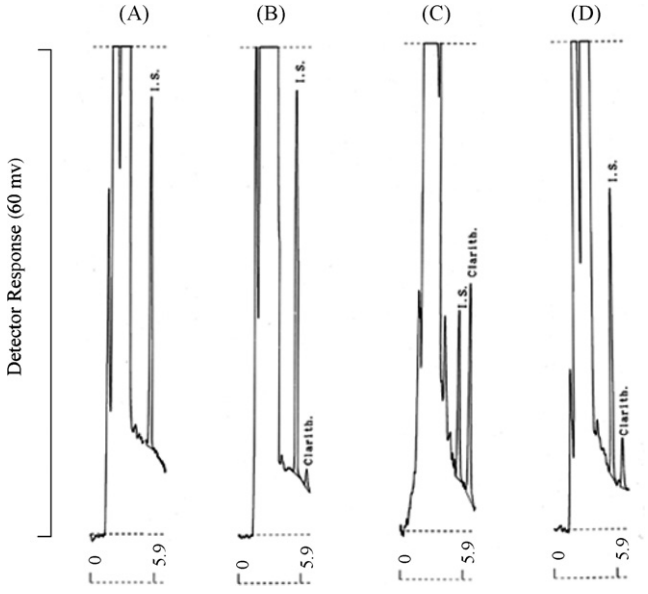

Fig. 2. Typical chromatograms obtained from an extract of (A) human blank serum spiked with amantadine as the I.S., (B) human blank serum spiked with 0.05 μg mL^{-1} clarithromycin and the I.S. and (C and D) serum samples from a volunteer 5 and 24 h after a single oral dose of 500 mg drug containing 3.3 and 0.38 μg mL^{-1} of clarithromycin, respectively.

Table 1
Inter- and intra-day precision and accuracy for determination of clarithromycin in human serum by the HPLC method

Known concentration (μg mL^{-1})	Concentration found (mean ± S.D.)	Coefficient of variation (%)	Accuracy (% mean deviation)
Within-day (n = 6)			
0.025	0.024 ± 0.004	16.6	96.7
0.5	0.49 ± 0.039	7.9	98
2.0	1.99 ± 0.07	3.6	99.3
5.0	4.97 ± 0.12	2.4	99.3
10	10.02 ± 0.20	2.0	99.8
Known concentration (μg mL^{-1})	Concentration found (mean ± S.D.)	Coefficient of variation (%)	Accuracy (%)
Between-day (n = 10)			
0.025	0.023 ± 0.004	16.0	94.7
0.5	0.5 ± 0.036	7.3	99.7
2.0	1.97 ± 0.06	3.0	98.2
5.0	4.97 ± 0.16	3.2	99.4
10	10.1 ± 0.23	2.6	98.9

Accuracy has been calculated as a mean deviation from the nominal values.

(n = 6) using the same stock solutions. The intra-day average slope of the fitted straight lines was 0.0354 ± 0.0014 μg mL^{-1} (C.V. = 3.8%) and the mean intercept of the calibration curves was 3.28 ± 0.16 (C.V. = 4.2%). The corresponding mean (±S.D.) coefficient of the linear regression analysis was 0.9966 ± 0.007 (C.V. = 0.7%). For calibration curves prepared on different days (n = 10), the mean ± S.D. of results were as follows: slope = 0.0362 ± 0.0016 μg mL^{-1} (C.V. = 4.8), coefficient of the linear regression analysis = 0.9956 ± 0.008 (C.V. = 0.8%) and intercept = 3.11 ± 0.15 (C.V. = 3.7%).

Stock solutions of clarithromycin and the I.S. were stable for 4 and 60 days, respectively, when stored at 4 °C and the derivatized solutions were found to be stable (>95%) for 12 h. After 60 days, the concentrations of clarithromycin in serum stored at −40 °C were found to be 101% from the initial values. The mean recoveries of clarithromycin and I.S. from serum were 93 ± 4 and 90 ± 3%, 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 16.6% whereas accuracy never deviated from 100% by more than 7.3%.

4. Discussion

FMOC-Cl reacts with primary and secondary amines in alkaline conditions and less readily with hydroxyl groups (Fig. 3). Analysis of macrolide antibiotics in human serum using FMOC-Cl as labeling agent has been previously reported [12] and derivatization of azithromycin by this reagent using off-line [13] and on-line [14] methods had been performed in our laboratory. Also Tris(2,2′-bipyridine)ruthenium(II) as labeling agent, has been used for analysis of macrolide antibiotic, erythromycin A by HPLC using electrogenerated chemiluminescence detection [15]. In the our previously published method for analysis of azithromycin [13], derivatization of the drug was easily

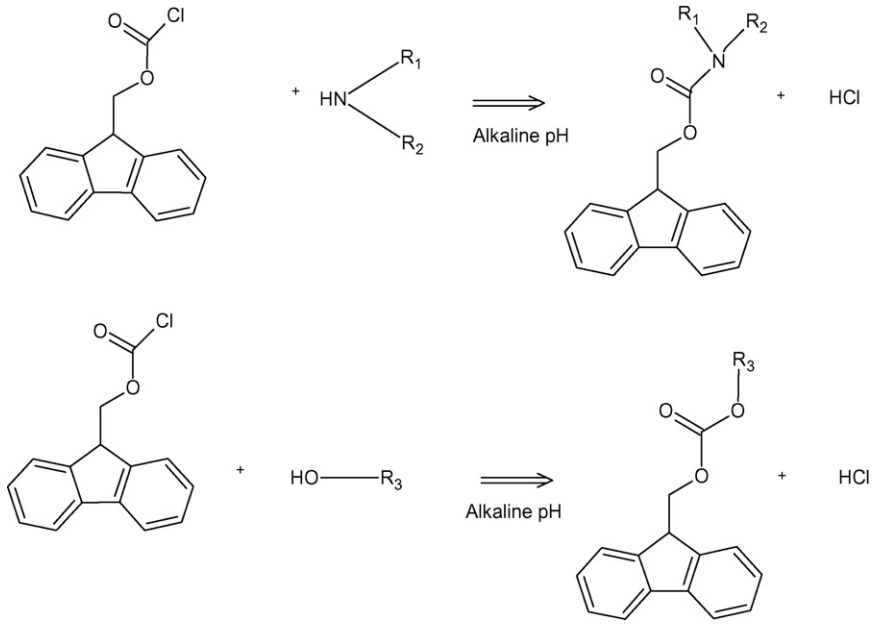

Fig. 3. Derivatization reaction of FMOC-Cl with an amine and an alcohol.

performed using FMOC-Cl at concentration of 500 μg mL^{-1}, temperature of 40 °C and reaction time of 40 min. However, under these conditions, low serum levels of clarithromycin which are usually found in single dose pharmacokinetic studies cannot be detected. Thus, higher concentrations of FMOC-Cl, as well as increasing temperature and time of reaction were examined. Concentrations of the labeling agent between the ranges of 500 and 5000 μg mL^{-1} were tested and although the yield of reaction was increased at higher concentrations of FMOC-Cl however, significant band-broadening was seen for a large excess of the reagent which interfered with analysis of the low amounts of clarithromycin. The temperature of reaction between the range of 40 and 80 °C and reaction time of 10 and 60 min were studied. The reactions of FMOC-Cl have been found to take place within an aqueous-organic phase system and due to the insolubility of FMOC-Cl and derivatization products in water, it is necessary to apply a reaction solution with high content of acetonitrile (80%, v/v), however, the organic phase is evaporated if high temperature reaction (more than 64 °C) or long incubation time is used. Hence, a solution of the labeling agent with concentration of 1000 μg mL^{-1}, temperature medium of 60 °C, and incubation time of 15 min were selected. In our previously published methods for analysis of azithromycin, using an aqueous solution of the glycine, the excess of FMOC-Cl was removed. Sufficient proportion of the organic phase is needed for reaction of glycine with the reagent, however, in the present method due to application of higher reaction temperature and partial evaporation of the organic solvent, excess of FMOC-Cl does not react with glycine. Furthermore, due to the insolubility of FMOC-Cl and derivatization products in water, the clarithromycin peak is significantly reduced following addition of the aqueous solution of glycine to the reaction mixture. Comparing of the chemical structures of clarithromycin and azithromycin (Fig. 4A and B, respectively) shows that the hydroxyl group in the position of six of pentadecan has been substituted by methoxy group in the clarithromycin (arrow in Fig. 4B). As there are more hydroxyl groups in the azithromycin, it seems reaction between the reagent and azithromycin proceeds more efficiently in comparison to clarithromycin. Thus, LOQ of 0.025 μg mL^{-1} was obtained for clarithromycin in the present study, while this value was 0.01 μg mL^{-1} for azithromycin in our previously published method [13]. (Also the LOQ of 0.092 and 0.2 μg mL^{-1} have been reported in method described by Torano and Guchelaar [12] for azithromycin and clarithromycin, respectively.)

A number of drugs with secondary or primary amines or hydroxyl groups (e.g. erythromycin, betalactam antibiotics, etidronate, alendronate, aminoglycosides, topiramate, gabapentin and amantadine) were tested and amantadine (Fig. 4C) was selected as internal standard because of its suitable retention time. A mobile phase with at least 2 mL L^{-1} of triethylamine was necessary to separate the peaks of the drug and endogenous substance as well as excess of the reagent which were eluted at the first parts of chromatogram. Unlike the I.S., retention behavior of the drug was pH-dependent and its retention time was found to increase proportionally with the pH of the mobile phase. Thus, pH of 4.3 was selected for the mobile phase.

5. Application of the method

The developed method has been applied in a randomized crossover three-ways bioequivalence study of three different clarithromycin preparations. Typical serum concentration–time profiles for the preparations are presented in Fig. 5 and pharmacokinetic parameters are summarized in Table 2.

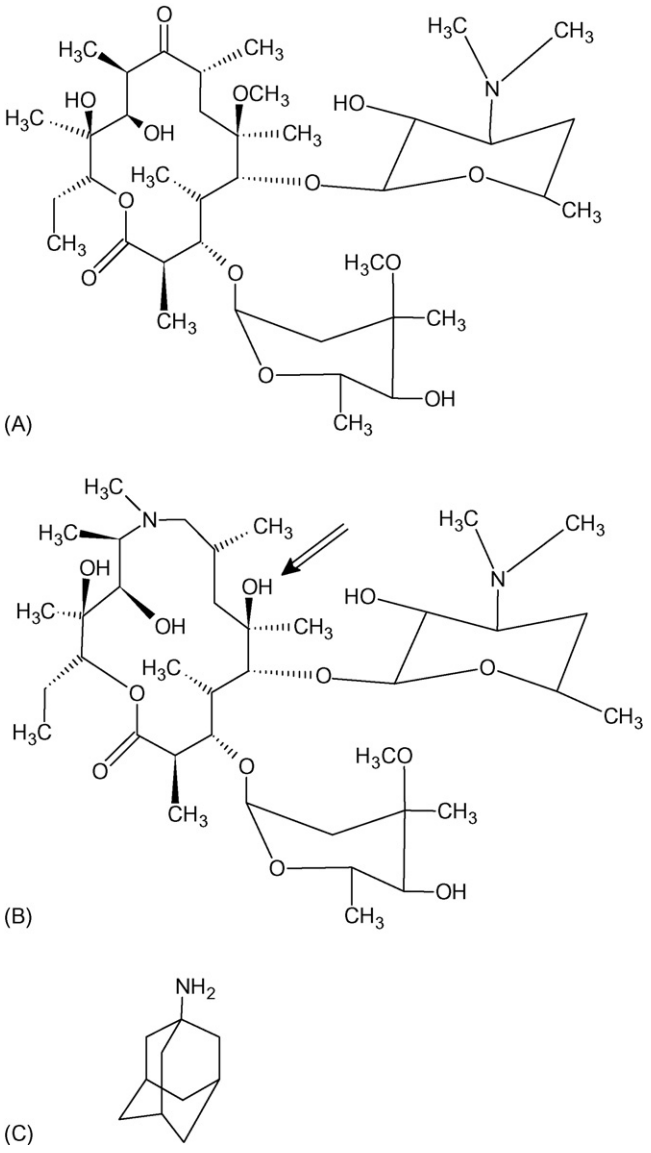

Fig. 4. Chemical structures of (A) clarithromycin, (B) azithromycin and (C) amantadine.

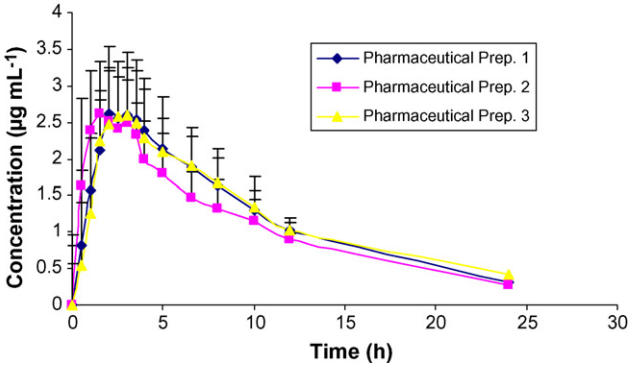

Fig. 5. Mean serum concentrations vs. time profiles of clarithromycin for three clarithromycin preparations in 12 human volunteers after administration of a single 500 mg oral dose.

Table 2
Mean (S.D.) pharmacokinetic parameters of clarithromycin for different clarithromycin preparations (pharmaceutical prep. 1 = Abbott, pharmaceutical prep. 2 = Chimodaruo and pharmaceutical prep. 3 = Tehran Shimi) in 12 human volunteers after administration of a single 500 mg oral dose

Parameter	Prep.		
	1	2	3
T_{max} (h)	2.7 (1.1)[a]	1.92 (0.9)	3.0 (1.1)
C_{max} (μg mL^{-1})	3.19 (0.50)	3.04 (0.69)	2.88 (0.62)
AUC0–24 (μg h mL^{-1})	27.49 (6.03)	24.93 (0.58)	26.37 (0.83)
AUC0–∞ (μg h mL^{-1})	31.07 (0.96)	27.37 (0.64)	29.80 (0.91)
$T_{1/2}$ (h)	6.9 (2.6)	6.6 (1.7)	7.2 (3.3)

T_{max}, time to maximum concentration; C_{max}, maximum concentration; AUC, area under the concentration–time curve; $T_{1/2}$, elimination half life.
[a] Values in the parentheses are S.D. of the amounts in 12 healthy volunteers.

6. Conclusion

In conclusion, a sensitive and specific method has been described for the determination of clarithromycin in serum. This method has demonstrated to be suitable for its use in pharmacokinetic studies of clarithromycin. In comparison to the previously published method, analysis time and LOQ were improved, and less time is needed for derivatization of the drug in human serum.

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