

A Sensitive Liquid Chromatographic Method for the Analysis of Clarithromycin with Pre-Column Derivatization: Application to a Bioequivalence Study

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Abstract

Objective(s)

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.

Materials and Methods

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; 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.

Results

The analytical method was linear over the concentration range of 0.025-10 µg/ml of clarithromycin in human serum with a limit of quantification of 0.025 µg/ml. The assay is sensitive enough to measure drug levels obtained in human single dose studies.

Conclusion

In the present method, sensitivity and the running time of analysis have been improved and successfully applied in a bioequivalence study of three different clarithromycin preparations in 12 healthy volunteers.

Keywords: Bioequivalence, Clarithromycin, High-performance liquid chromatography, Macrolide antibiotics

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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 has been obtained, using injection volume of 100 μ l, 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 obtaining a stable baseline difficult. 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 μ g/ml (2), 0.5 μ g/ml (3), 0.03 μ g/ml (4), 0.1 μ g/ml (6), 0.01 μ g/ml (8), 0.15 μ g/ml (9)) have been reported, using HPLC-ED methods. A very sensitive method (LOQ 2.95 ng/ml) 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 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 μ g/ml, using 50 μ l injections). Furthermore, long time is needed for sample preparation and for reaction of the macrolides with FMOC-Cl (40 min). Based on these above-mentioned studies, the present study 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.

Materials and Methods

Chemicals

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

Equipment

The HPLC system used in our study, consisted of two pumps of Shimadzu LC-10A solvent delivery system, a system controller (SCL 10AD), a spectrofluorometric 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 \times 4.6 mm id, 5 μ m particle size which was protected by a Shim-pack G-ODS guard column (1 cm \times 4.0 mm id, 5 μ m particle size). A mixture of 0.05 M sodium phosphate buffer

containing triethylamine (2 ml/l; 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.

Solutions

Stock solutions of clarithromycin (1000 µg/ml) and the IS (600 µg/ml) 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. The IS stock solution was diluted with distilled water to obtain working solution of 6 µg/ml. A 1000 µg/ml solution of FMOC-Cl was prepared in acetonitrile. All solutions stored at 4 °C and were stable for at least 4 weeks, except clarithromycin stock solution which was prepared freshly before the analysis.

Extraction procedure and derivatization

Serum samples stored at -40 °C until the time of 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 sec 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 into the HPLC system.

Preparation of calibration curve standards

Samples for calibration curves were prepared within the concentration range of 0.025-10 µg/ml. 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 sec on a vortex mixer. The samples were subjected to extraction, derivatization and analysis as described above.

Method validation

Assay linearity was evaluated with nine calibration standards in duplicate, using blank serum samples obtained from healthy volunteers. Calibration curves (weighed regression line) were obtained by linear least-squares regression analysis of plots of peak-area ratio of clarithromycin to IS versus drug concentrations. Quality control samples used in method validation were prepared with the drug working solutions to make low (0.025 µg/ml), medium (0.5 µg/ml) and high (5 µg/ml) 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 for the addition of the IS. The absolute recoveries of clarithromycin at the above mentioned concentrations as well as the IS 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. The stability of solutions of clarithromycin and the IS was studied over a period of 4 weeks by comparing the peak areas at different times. The stability of the drug in serum samples was examined by comparing the determined concentration in different times up to 30 days maintenance of the samples at -40 °C and following three thaw-freeze cycles.

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 hr after drug administration. Blood samplings were carried out at suitable intervals up to 24 hr and pharmacokinetic parameters including maximum concentration (C_{max}), area under the concentration time curve from 0 to the time of last sampling (AUC_{0-t}) and area under the concentration time curve from 0 to infinity ($AUC_{0-\infty}$) were compared. Bioequivalence between the preparations was determined by calculating 90% confidence intervals for the ratio of C_{max} , AUC_{0-t} , and $AUC_{0-\infty}$ values for different products, using logarithmic transformed data. One-way analysis of variance (ANOVA) was used for the statistical analysis. Human study was approved by a local ethics committee.

Results

The reaction of clarithromycin and the IS with FMO-CI appeared to be highly dependent on pH of buffer solution (Figure 1A), time (Figure 1B), temperature (Figure 1C), concentration of the labeling agent (Figure 1D), polarity of the medium (Figure 1E) and the percentage of recovery (Table 1).

Table 1. Polarity of the medium on the recovery of clarithromycin reacted with FMO-CI.

Acetonitrile: Water Ratio	Recovery Drug(μ g)	Recovery (%)
1:1	301.0	30.1
1:2	594.3	59.43
1:3	754.7	75.47
1:4	896.2	89.62
1:5	1000	100
1:6	698.1	69.81

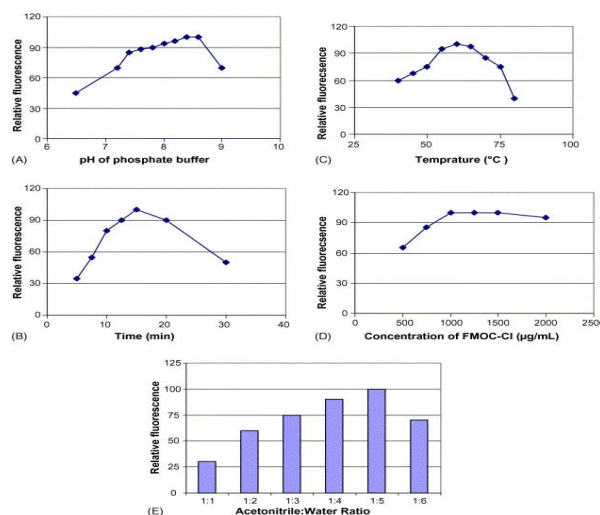


Figure 1. Effects of (A) pH of phosphate buffer, (B) time of derivatization, (C) temperature of reaction, (D) concentration of the labeling agent and (E) polarity of the medium on the reaction of clarithromycin with FMO-CI.

The separation constant (K =drug concentration in organic phase/ drug concentration in aqueous phase) was 0.43. Based on the above-mentioned 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 μ g/ml.

Typical chromatograms of human blank serum and human blank serum spiked with clarithromycin (0.05 μ g/ml) and the IS are shown in Figure 2A and B respectively. Clarithromycin and the IS 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 IS. Figure 2C and D show the chromatograms of serum samples obtained at 5 and 24 hr 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.

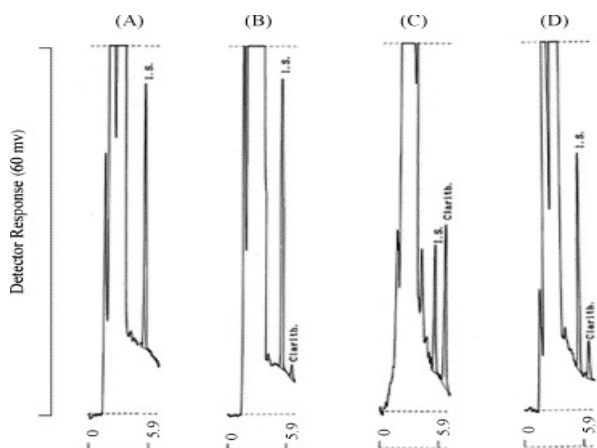


Figure 2. Typical chromatograms obtained from an extract of (A) human blank serum spiked with amantadine as the IS, (B) human blank serum spiked with 0.05 µg/ml clarithromycin and the IS and (C and D) serum samples from a volunteer 5 and 24 hr after a single oral dose of 500 mg drug containing 3.3 and 0.38 µg/ml of clarithromycin respectively.

LOD was approximately 0.01 µg/ml and LOQ was 0.025 µg/ml. The proposed HPLC method was linear over the concentration range of 0.025-10 µg/ml. The correlation coefficients (*r*) for calibration curves were equal to or more than 0.9982. Intra-assay reproducibility was determined for calibration curves prepared in the same day in replicate (*n*=6), using the same stock solutions.

The intra-day average slope of the fitted straight lines was 0.0354 ± 0.0014 µg/ml (CV=3.8%) and the mean intercept of the calibration curves was 3.28 ± 0.16 (CV=4.2%). The corresponding mean (\pm SD) coefficient of the linear regression analysis was 0.9966 ± 0.007 (CV=0.7%). For calibration curves prepared on different days (*n*=10), the mean \pm SD of results were as follows: slope = 0.0362 ± 0.0016 µg/ml (CV=4.8), coefficient of the linear regression analysis = 0.9956 ± 0.008 (CV=0.8%) and intercept = 3.11 ± 0.15 (CV=3.7%).

Stock solutions of clarithromycin and the IS 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 hr. After 60 days, the concentrations of clarithromycin in serum stored at -40 °C were found to be 101% of the initial values. The mean recoveries of clarithromycin and IS from serum were 93 ± 4 and $90 \pm 3\%$ respectively.

The within day and between days accuracy and precision values of the assay method are presented in Table 2. 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%.

Table 2. Inter- and intra-day precision and accuracy for determination of clarithromycin in human serum by the HPLC method.

Known concentration (µg/ml)	Concentration found (mean \pm SD)	Coefficient of variation (%)	Accuracy (%)
Within-day (n=6)			
0.025	0.024 \pm 0.004	16.6	96.7
0.5	0.49 \pm 0.039	7.9	98
2	1.99 \pm 0.07	3.6	99.3
5	4.97 \pm 0.12	2.4	99.3
10	10.02 \pm 0.20	2.0	99.8
Known concentration (µg/m)	Concentration found (mean \pm SD)	Coefficient of variation (%)	Accuracy (%)
Between-day (n=10)			
0.025	0.023 \pm 0.004	16.0	94.7
0.5	0.5 \pm 0.036	7.3	99.7
2	1.97 \pm 0.06	3.0	98.2
5	4.97 \pm 0.16	3.2	99.4
10	10.1 \pm 0.23	2.6	98.9

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

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 Figure 3 and pharmacokinetic parameters are summarized in Table 3.

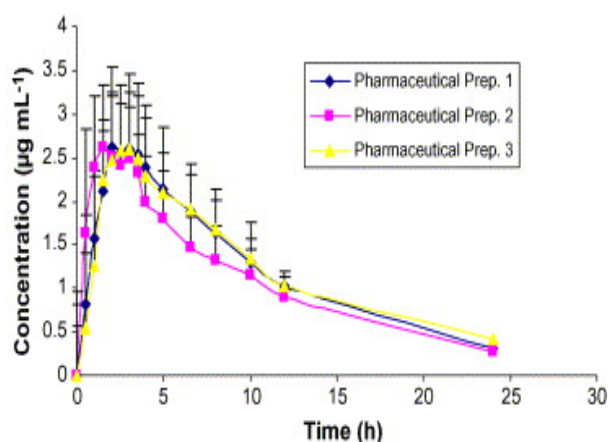


Figure 3. Mean serum concentrations vs. time profiles of clarithromycin for three clarithromycin preparations in 12 human volunteers after administration of a single 500 mg oral dose in 12 human volunteers after administration of a single 500 mg oral dose.

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

Parameter	Prep.		
	1	2	3
Tmax (hr)	2.7 (1.1) ^a	1.92 (0.9)	3.0 (1.1)
Cmax (µg/ml)	3.19 (0.50)	3.04 (0.69)	2.88 (0.62)
AUC ₀₋₂₄ (µg hr/ml)	27.49 (6.03)	24.93 (0.58)	26.37 (0.83)
AUC _{0-∞} (µg hr/ml)	31.07 (0.96)	27.37 (0.64)	29.80 (0.91)
T1/2 (hr)	6.9 (2.6)	6.6 (1.7)	7.2 (3.3)

Tmax, time to maximum concentration; Cmax, maximum concentration; AUC, area under the concentration-time curve; T1/2, elimination half life.

^a Values in the parentheses are SD of the amounts in 12 healthy volunteers.

Discussion

FMOC-Cl reacts with primary and secondary amines in alkaline conditions and less readily with hydroxyl groups (Figure 4). 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 the analysis of macrolide antibiotic, erythromycin A by HPLC, using electrogenerated chemiluminescence detection (15).

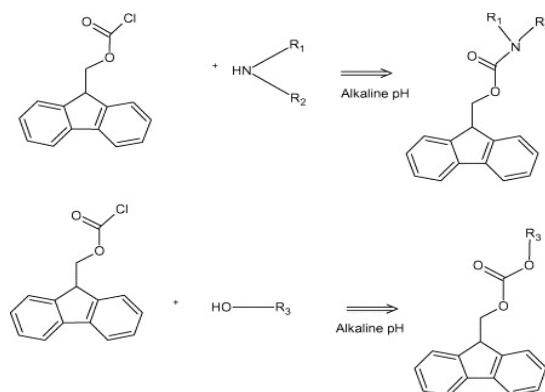


Figure 4. Derivatization reaction of FMOC-Cl with an amine and an alcohol.

In our previously published method for the analysis of azithromycin (13), derivatization of the drug was easily performed, using FMOC-Cl at concentration of 500 µg/ml, 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, can not 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 were tested and although the yield of reaction was increased at higher concentrations of FMOC-Cl, 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, temperature medium of 60 °C, and incubation time of 15 min were selected. In our previously published methods for the 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 the 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 the chemical structures of clarithromycin and azithromycin (Figure 5A 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 Figure 5B). As there are more hydroxyl groups in the azithromycin, it seems that reaction between the reagent and azithromycin proceeds more efficiently in comparison to clarithromycin. Thus, LOQ of 0.025 µg/ml was obtained for clarithromycin in the present study, while this value was 0.01 µg/ml for azithromycin in our previously published method (13). Also, the LOQ of 0.092 and 0.2 µg/ml have been reported in the method described by Torano and Guchelaar (12) for azithromycin and clarithromycin respectively.

A number of drugs with secondary or primary amines or hydroxyl groups (eg erythromycin, beta-lactam antibiotics, etidronate, alendronate, aminoglycosides, topiramate, gabapentin and amantadine) were tested and amantadine (Figure 5C) was selected as internal standard, because of its suitable retention time. A mobile phase with at least 2 ml/l 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 IS, 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.

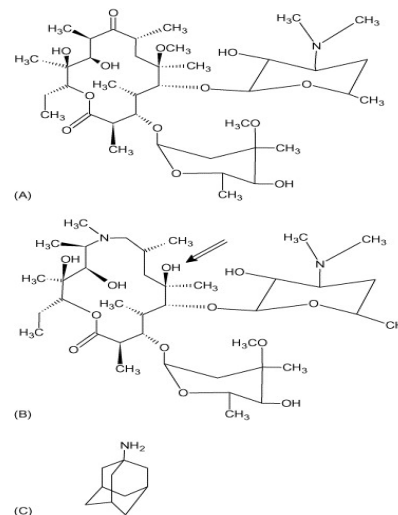


Figure 5. Chemical structures of (A) clarithromycin, (B) azithromycin and (C) amantadine.

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.

Acknowledgments

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References

1. Chambers HF. Chloramphenicol, Tetracyclines, Macrolides, Clindamycin, and Streptogramins. In: Katzung BG. editor. Basic and clinical pharmacology. New York, NY: McGraw-Hill; 2004.p.760.
2. Chu SY, Sennello LT, Sonders RC. Simultaneous determination of clarithromycin and 14(R)-hydroxylclarithromycin in plasma and urine using high-performance liquid chromatography with electrochemical detection. J Chromatogr 1991; 57:199.
3. Hedenmo M, Eriksson BM. Liquid chromatographic determination of the macrolide antibiotics roxithromycin and clarithromycin in plasma by automated solid-phase extraction and electrochemical detection. J Chromatogr A 1995; 692:161.

4. Taninaka PC, Ohtani H, Hanada E, Kotaki H, Sato H, Iga T. Determination of erythromycin, clarithromycin, roxithromycin, and azithromycin in plasma by high-performance liquid chromatography with amperometric detection. *J Chromatogr B* 2000; 738:405.
5. Niopas I, Daftsios AC. Determination of clarithromycin in human plasma by HPLC with electrochemical detection: validation and application in pharmacokinetic study. *Biomed Chromatogr* 2001; 15:507.
6. Choi SJ, Kim SB, Lee H-Y, Na DH, Soon YS, Lee SS, *et al*. Column-switching high-performance liquid chromatographic determination of clarithromycin in human plasma with electrochemical detection. *Talanta* 2001; 54:377.
7. Pappa-Louisi A, Papageorgiou A, Zitrou A, Sotiropoulos S, Georgarakis E, Zougrou F. Study on the electrochemical detection of the macrolide antibiotics clarithromycin and roxithromycin in reversed-phase high-performance liquid chromatography. *J Chromatogr B* 2001; 755: 57.
8. 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.
9. Wibawa JID, Shaw PN, Barrett DA. Quantification of clarithromycin, its 14-hydroxy and decladinose metabolites in rat plasma, gastric juice and gastric tissue using high-performance liquid chromatography with electrochemical detection. *J Chromatogr B* 2003; 783:359.
10. Van Rooyen GF, Smit MJ, De Jager AD, Hundt HKL, Swart KJ, Hundt AF. Sensitive liquid chromatography–tandem mass spectrometry method for the determination of clarithromycin in human plasma. *J Chromatogr B* 2002; 768:223.
11. Amini H, Ahmadiani A. Sensitive determination of clarithromycin in human plasma by high-performance liquid chromatography with spectrophotometric detection. *J Chromatogr B* 2005; 817:193.
12. Torano JS, Guchelaar HJ. Quantitative determination of the macrolide antibiotics erythromycin, roxithromycin, azithromycin and clarithromycin in human serum by high-performance liquid chromatography using pre-column derivatization with 9-fluorenylmethyloxycarbonyl chloride and fluorescence detection. *J Chromatogr B* 1998; 720: 89.
13. Bahrami Gh, Mirzaeei Sh, 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.
14. Bahrami Gh, 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.
15. Hori T, Hashimoto H, Konishi M. Determination of erythromycin A in rat plasma and urine by high-performance liquid chromatography with chemiluminescence detection using Tris (2,2- bipyridine) ruthenium (II). *Biomed Chromatogr* 2006; 20:17.