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Sensitive determination of clarithromycin in human plasma by high-performance liquid chromatography with spectrophotometric detection

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Abstract

A rapid, selective and sensitive high-performance liquid chromatographic method with spectrophotometric detection was developed for the determination of clarithromycin in human plasma. Liquid–liquid extraction of clarithromycin and norverapamil (as internal standard) from plasma samples was performed with *n*-hexane/1-butanol (98:2, v/v) in alkaline condition followed by back-extraction into diluted acetic acid. Chromatography was carried out using a CN column (250 mm × 4.6 mm, 5 μm) under isocratic elution with acetonitrile–50 mM aqueous sodium dihydrogen phosphate (32:68, v/v), pH 4.5. Detection was made at 205 nm and analyses were run at a flow-rate of 1.0 ml/min at 40 °C. The analysis time was less than 11 min. The method was specific and sensitive with a quantification limit of 31.25 ng/ml and a detection limit of 10 ng/ml in plasma. The mean absolute recovery of clarithromycin from plasma was 95.9%, while the intra- and inter-day coefficient of variation and percent error values of the assay method were all less than 9.5%. Linearity was assessed in the range of 31.25–2000 ng/ml in plasma with a correlation coefficient of greater than 0.999. The method was used to analyze several hundred human plasma samples for bioavailability studies.

Keywords: Clarithromycin; Ultraviolet detection

1. Introduction

Clarithromycin, 6-*O*-methylerythromycin (Fig. 1A), is a semi-synthetic macrolide antibiotic with a broad antibacterial spectrum. It has a good stability in gastric acid, a better bioavailability and a more favorable pharmacokinetic profile than erythromycin [1].

High-performance liquid chromatography (HPLC) with electrochemical detection has been widely used for the determination of clarithromycin in plasma [2–8]. Other chromatographic methods include HPLC with mass-spectrometry [9], or pre-column fluorescence derivatization [10]. Although these methods offer selective detection of clarithromycin, they are much more complex than the conventional ultraviolet (UV) detection.

Clarithromycin has only a weak UV absorbance in the low wavelength range (<220 nm) as it lacks a suitable chromophore. Its very non-specific UV absorption, where substantial UV absorption occurs, makes it difficult to develop a specific, selective and sensitive UV method, particularly when using complex matrices such as biological fluids [11]. HPLC–UV method has been reported for plasma determination of other related macrolides such as erythromycin [12], roxithromycin [13] and oleandomycin [14] with limit of quantification (LOQ) of 0.25 μg/ml. In our knowledge, there is only one published HPLC–UV method for clarithromycin assay in gastric juice with LOQ of 1.56 μg/ml [15]. However, much higher sensitive HPLC–UV method is required for plasma clarithromycin assay, which has a maximum plasma

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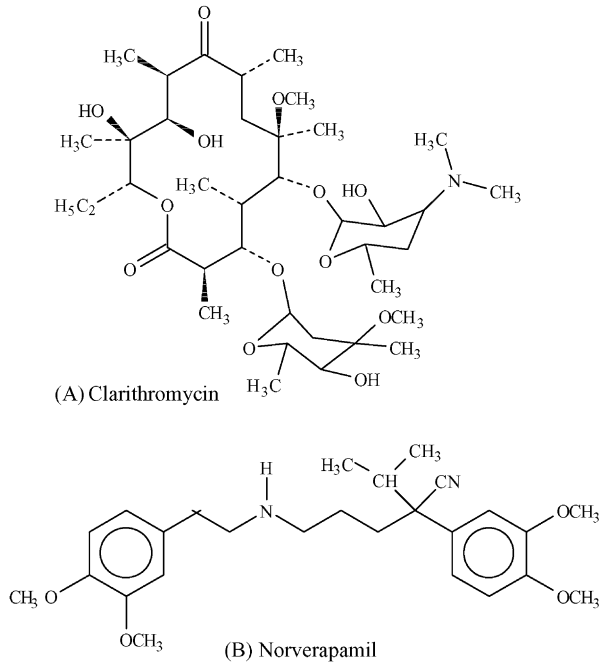

Fig. 1. Chemical structure of clarithromycin (A) and the internal standard norverapamil (B).

concentration of 0.78 ± 0.25 μg/ml following an oral dose of 250 mg [8].

The assay specificity is not only depends on selective detection, but also it is largely affected by the sample clean-up procedure and chromatographic separation. Using a suitable extraction procedure and mobile phase, we present herein for the first time, a sensitive and selective HPLC–UV method for the clarithromycin assay in plasma. The present method was found reliable and applied for a bioavailability study of clarithromycin tablets in healthy volunteers.

2. Experimental

2.1. Reagents

Clarithromycin (purity, 98.4%) was prepared from Ranbaxy (New Delhi, India). Norverapamil (internal standard, Fig. 1B) was obtained from Fermion (Orion Corporation, Espoo, Finland). Analytical grade phosphoric acid, acetic acid and HPLC grade methanol were purchased from E. Merck (Darmstadt, Germany). Analytical grade 1-butanol (*n*-butyl alcohol) was from BDH Chemicals (England). HPLC grade acetonitrile was purchased from Carlo Erba Reagenti (Italy). All other reagents were of analytical grade.

2.2. Instrumentation

The analyses were performed on a Shimadzu chromatographic system (Kyoto, Japan) equipped with an LC-6A solvent delivery pump, SPD-10AVP ultraviolet detector (operated at 205 nm), and C-R8A integrator. The samples were applied by a Rheodyne 7725 loop injector with an effective volume of 100 μl. A Shimpack CLC-CN column (250 mm × 4.6 mm i.d.; 5 μm particle size) was used for the chromatographic separation. The mobile phase comprised of acetonitrile–50 mM aqueous sodium dihydrogen phosphate (32:68, v/v), adjusted to pH 4.5 with concentrated phosphoric acid and 4 M sodium hydroxide. Analyses were run at flow-rate of 1 ml/min at 40 °C.

2.3. Standard solutions

Standard solutions of clarithromycin and norverapamil were prepared by dissolving 5 mg of each in 50 ml of acetonitrile and stored at −20 °C. The internal standard stock solution was diluted in acetonitrile to produce a final concentration of 0.5 μg/ml.

2.4. Calibration curves and quantitation

The concentration of the clarithromycin was calculated using peak height ratios of sample to internal standard peak height. The evaluation by peak height was more precise at low concentrations, as compared to the peak area method.

Calibration standards and quality control standards were prepared in human plasma by spiking a pool of plasma to a known concentration and then serially diluting it with blank plasma to attain the desired concentration range (31.25–2000 ng/ml). The concentrations of individual standards were 31.25, 62.5, 125, 250, 500, 1000 and 2000 ng/ml. The prepared calibration standards and quality control standards (1 ml) were pipetted into 4 ml polypropylene tubes and stored at −20 °C pending analysis.

2.5. Extraction procedure

Extraction was performed by adding 20 μl of the internal standard (10 ng of norverapamil), 20 μl of 1 M NaOH and 2.5 ml of *n*-hexane/1-butanol (98:2, v/v) to 1 ml of plasma in 4 ml polypropylene tube (10 mm × 70 mm) and shaking for 2 min. After centrifugation at 12 000 × *g* for 3 min, the whole organic layer was separated and transferred into another 4 ml tube. Then, 50 μl of 0.1% acetic acid was added. The mixture was vortex-mixed for 2 min and then, some of the upper organic phase was discarded and the remaining mixture (about 1 ml) was transferred into a 1.5 ml microcentrifuge tube. After centrifugation at 11 300 × *g* for 2 min, the upper organic phase was discarded completely. Finally, a volume of 40 μl of aqueous phase was injected into the chromatograph.

2.6. Assay validation

The specificity of the method was evaluated by comparing the chromatograms obtained from the samples containing clarithromycin and internal standards with those obtained from blank samples. Besides calibration standards, additional

standards were prepared for the determination of intra-day ($n = 3$) and inter-day ($n = 3$) of the assay accuracy and precision. The absolute recoveries ($n = 3$) was calculated by comparing peak heights obtained from prepared sample extracts with those found by direct injection of drug solution made in 0.1% acetic acid at the same concentration.

The LOQ was estimated by analyzing clarithromycin at low concentrations of the calibration curves. The LOQ was defined as a concentration level where accuracy and precision were still better than 10%. To determine the limit of detection (LOD), lower plasma concentrations than the lower end of the calibration curves were used. The LOD was then defined as the concentration which caused a signal three times the noise (S/N = 3/1).

2.7. Application

The assay was used for a comparative bioavailibility study of three tablets preparations containing 250 mg clarithromycin. The reference product was Klacid (from Abbott, UK) and two test products with different formulations were from Arya pharmaceutical Co. (Tehran, Iran).

Fourteen healthy volunteers participated in the study. The study was conducted using a three-way crossover design, as a single dose, randomized trial. The three formulations were administrated on three treatment days, separated by a washout period of 7 days, to fasted subjects who received one of the study medications. Food and drinks were not allowed until 3 h after ingestion of the tablet. Multiple blood samples (5 ml) were collected before and 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10, 12 and 24 h post-dosing. The plasma was immediately separated by centrifugation and frozen at −20 °C until analysis.

3. Results and discussion

3.1. UV detection

Significant UV absorption for clarithromycin was only obtained at wavelengths below 215 nm. Spectrophotometric detection was employed at 205 nm, since in this wavelength the clarithromycin gave two and three times higher peaks in comparison to 210 and 215 nm, respectively. Although detection could be performed at lower wavelengths than 205 nm, the resulting increases in noise and drift levels make it difficult to obtain a stable baseline. The performance of the detector instrument at low wavelengths was also important, as the older model of the detector (i.e. SPD-6A) had more noise and drift levels at these wavelengths. A mobile phase containing acetonitrile was the first choice at 205 nm, since it had lower UV cut-off than methanol.

3.2. Sample preparation

Methods reported previously for the assay of clarithromycin have mainly used sample preparation procedure involving a single step liquid–liquid extraction into a variety of organic solvents under basic condition. First, we tried the same extraction methods under UV detection, but we observed numerous interferences in the chromatograms, which made the separation impossible. Therefore, we tried back-extraction of clarithromycin into an acidic aqueous solution. Although in comparison with other macrolides, clarithromycin has somehow better acid stability, back-extraction of clarithromycin into acid has not been tried by previous investigators. In fact, clarithromycin is rapidly degradated in 0.1% hydrochloric acid [15], which could lead to a incorrect conclusion that back-extraction of clarithromycin into acid is not possible.

However, we observed that clarithromycin is sufficiently stable in mildly acidic condition such as 0.1% acetic acid and could be effectively back-extracted from a suitable organic solvent into it. More importantly, back-extraction into acetic acid also provided a clear chromatogram under UV detection at 205 nm. The extractability of both clarithromycin and internal standard from plasma was tested in recovery experiments using various solvent extraction system, different *n*-hexane–1-butanol mixtures and different ratios of extraction solvent to sample volume. The maximum extraction recovery ($95.9 \pm 5.5\%$ and $95.2 \pm 4.8\%$ for clarithromycin and internal standard, respectively) and the clearest chromatograms were obtained by using *n*-hexane/1-butanol (98:2, v/v). Other extraction solvents either failed to allow back-extraction or produced many interfering peaks. Acetic acid concentrations higher than 0.1% do not improve the extraction procedure and could decrease the stability of clarithromycin.

3.3. Selection of internal standard

The internal standard, norverapamil (the *N*-demethylated metabolite of verapamil) was selected after extensive investigation. Both clarithromycin and norverapamil have a pK_a of about 9 for their basic group, and therefore, could be present predominately as the ionized species in an acidic aqueous solution. The norverapamil showed highly reproducible recovery like clarithromycin with the applied extraction method. Roxithromycin, which is usually used as internal standard for clarithromycin was not available in our study. However, azithromycin was tested and found not stable enough in 0.1% acetic acid.

3.4. Acid stability of clarithromycin and internal standard

The stability of clarithromycin and norverapail in 0.1% acetic acid were evaluated at different concentrations during storage at room temperature after 0, 1, 3 and 7 days. It was found that clarithromycin and norverapamil were stable (less than 5% loss) for at least 3 and 7 days, respectively. The clarithromycin showed some instability (about 30% loss) after 7 days storage. Considering usual short-time delay between

sample extraction and injection, it was concluded that both compounds are sufficiently stable in diluted acetic acid.

3.5. Chromatographic separation

The separation of clarithromycin under varying mobile phase composition and column temperature was investigated. It has been reported that temperature affect the peak area and shape of the clarithromycin in addition to its retention time [11]. We observed that the HPLC column should be maintained at high temperature (e.g. 60 °C), only if the mobile phase contains high amounts of an ion-pair or a silanol blocking agents like triethylamine. Otherwise, the plate numbers of the column for clarithromycin is highly reduced. Knowing this limitation, the addition of an ion-pair or triethylamine to the mobile phase was avoided in this study. However, temperature increases and decreases clarithromycin and internal standard retention times, respectively, which helps adjusting their distance from each other, and therefore, was used in our study.

A simple buffered acetonitrile mobile phase was found appropriate for the separation. Initially we were using a mobile phase containing 20 mM buffer. In that condition, several major interfering peaks were observed following diluted acetic acid injection. These interfering peaks were disappeared after using 50 mM buffer in the mobile phase. It was also observed that acidic mobile phases produce more stable baseline following injection of diluted acetic acid solution.

3.6. Assay validation

Representative chromatograms of drug-free plasma, plasma spiked with clarithromycin and two samples from a volunteer collected after oral dosing with clarithromycin are shown in Fig. 2. The retention times for clarithromycin and the internal standard were 8.4 and 10.3 min, respectively. No interfering peaks from the endogenous plasma components were observed in the retention time of clarithromycin or internal standard. In addition, no late-eluting peak was observed and new injection could be done in every 11 min. Several drugs including azithromycin, omeprazole, amoxicillin, ranitidine, metformin, acyclovir, ciprofloxacin, diazepam, moclobemide, prazosin, terazosin, loratadine, codeine and morphine were tested and none of them interfered.

The calibration curves were linear over the concentration range of 31.25–2000 ng/ml in human plasma, with a correlation coefficient greater than 0.999. The limit of quantification was 31.25 ng/ml and the limit of detection was about 10 ng/ml. The method LOQ is close to that of some electrochemical methods [4,5] and higher than some [2,3,6] with an LOQ of 100 ng/ml, but much more sensitive than the fluorescence method [10] with an LOQ of 200 ng/ml.

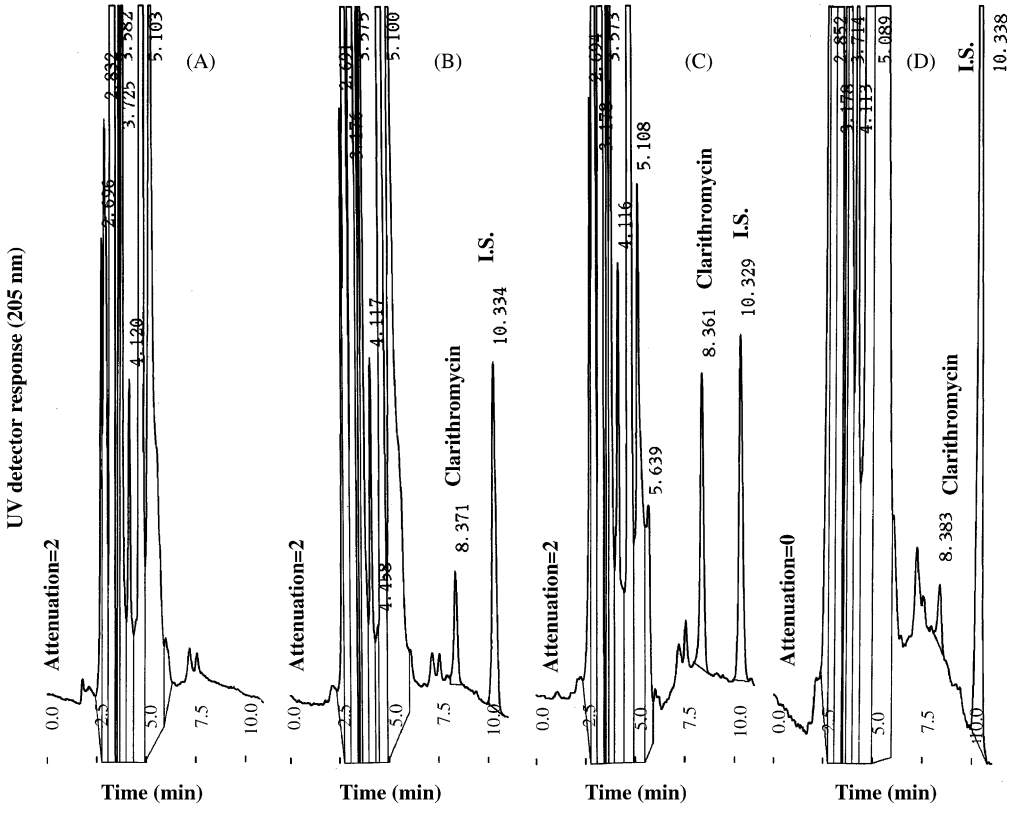

Fig. 2. Chromatogram of: (A) a blank plasma; (B) plasma spiked with 250 ng/ml clarithromycin, a volunteer samples 1.5 h (C) and 24 h (D) after oral administration of 250 mg of clarithromycin. The plasma clarithromycin concentrations were determined to be 616.8 and 36.4 ng/ml, respectively. The last chromatogram (D) is shown at attenuation of zero (highest sensitivity of the detector).

Table 1
The intra- and inter-day precision and accuracy, and recovery data for the measurement of clarithromycin in human plasma ($n = 3$)

Nominal concentration (ng/ml)	Recovery (%)	Intra-day			Inter-day		
		Mean ± S.D.	Precision (%)	Accuracy (%)	Mean ± S.D.	Precision (%)	Accuracy (%)
31.25	104 ± 8	32.6 ± 2.4	7.4	4.5	34.2 ± 2.5	7.3	9.5
125	94.7 ± 6.1	125.6 ± 7.9	6	0.5	120.6 ± 6.6	5.4	−3.5
500	92.7 ± 4.1	492.2 ± 18.3	3.7	−1.6	482.8 ± 13.3	2.8	−3.4
1000	92.3 ± 4.3	986.9 ± 19.6	2	−1.3	1027.3 ± 29.5	2.9	2.7

The results of the method intra- and inter-day accuracy and precision are presented in Table 1 and are all less than 9.5%.

3.7. Pharmacokinetic results

The proposed method was applied to the determination of clarithromycin in plasma samples for the purpose of the bioequivalence study. The plasma clarithromycin profiles for volunteers after taking products are shown in Fig. 3. The mean (±S.D.) of maximum plasma concentration (C_{max}), time to reach C_{max} (T_{max}), $AUC_{0-\infty}$ and plasma half-life ($t_{1/2}$) of clarithromycin after administration of the reference product (Klacid) were 1067 ± 198.2 ng/ml, 1.64 ± 0.49 h, 6341.9 ± 1620.9 ng h/ml and 4.31 ± 0.87 h, respectively. The two test products had similar pharmacokinetics to reference products. The extrapolated fraction of the $AUC_{0-\infty}$ accounted only for 6–7%, which indicates a suitability of the analytical method for pharmacokinetic studies.

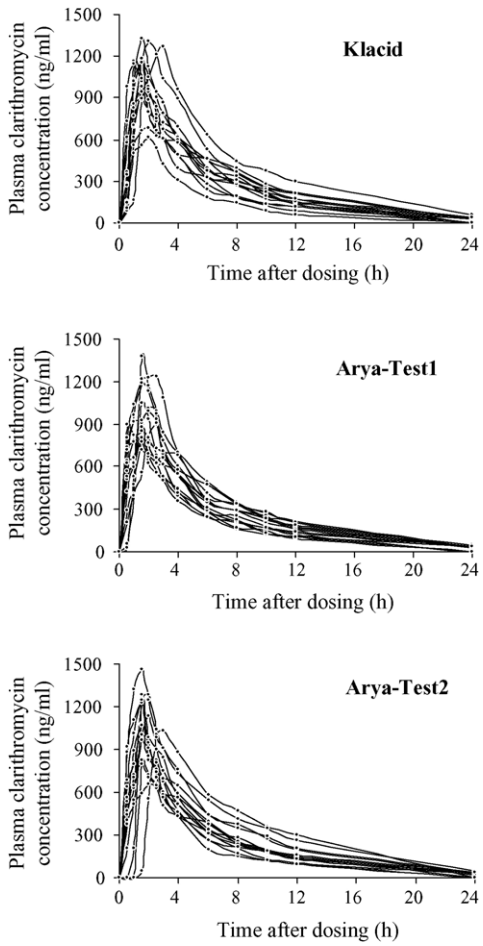

Fig. 3. Plasma concentration–time profiles of clarithromycin in 14 healthy volunteers following a 250 mg oral dose of three different formulations of clarithromycin (Klacid vs. two test formulations from Arya Pharmaceutical Co.) in a three-way crossover study.

4. Conclusion

For the first time, a highly selective and sensitive UV method for the quantification of clarithromycin in human plasma has been developed and validated. The simple extraction procedure is based on liquid–liquid extraction followed by back-extraction into diluted acid. The method is time-saving and economical, and provides the best alternative for other assay methods such as electrochemical or mass-spectrometry, which are quite complicated in comparison with UV detection. The sensitivity of the assay is sufficient to follow the pharmacokinetics of clarithromycin even after a low oral dose administration of clarithromycin.

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