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Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 1460–1464
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Determination of clarithromycin in human plasma by liquid chromatography–electrospray ionization tandem mass spectrometry

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Received 13 July 2006; received in revised form 18 October 2006; accepted 21 November 2006
Available online 15 December 2006

Abstract

A rapid and sensitive method has been developed for the determination of clarithromycin in human plasma with liquid chromatography–tandem mass spectrometry. Clarithromycin and the internal standard, telmisartan were precipitated from the matrix (50 μl) with 200 μl acetonitrile and separated by HPLC using formic acid:10 mM ammonium acetate:methanol (1:99:400, v/v/v) as the mobile phase. The assay based on detection by electrospray positive ionization mass spectrometry in the multiple-reaction monitoring mode was finished within 2.4 min. Linearity was over the concentration range 10–5000 ng/ml with a limit of detection of 0.50 ng/ml. Intra- and inter-day precision measured as relative standard deviation were <3.73% and <9.93%, respectively. The method was applied in a bioequivalence study of two tablet formulations of clarithromycin.

Keywords: Clarithromycin; LC–MS/MS

1. Introduction

Clarithromycin, 6-*o*-methylerythromycin, a semi-synthetic 14-membered macrolide antibiotic, is active against Gram-positive and some Gram-negative bacteria and particularly useful in the treatment of *Mycoplasmas*, *Haemophilus influenzae*, *Chlamydia* species and *Rickettsia* [1,2].

Various analytical methods have been developed to determine clarithromycin in formulations and biological samples, such as high-performance liquid chromatography (HPLC) with fluorescent [3], ultraviolet [4,5], electrochemical [6–17], amperometric [18] and mass spectrometric detection [19–24]. The fluorescence detection method [3] needs derivatization before the analysis. The HPLC-UV assay reported recently [5] with the lower limit of quantification (LLOQ) of 31.25 ng/ml, which is much lower than another one [4] (LLOQ 1.56 μg/ml), was validated. But the long chromatographic run time (8.4 min) and complex sample preparation are inappropriate in clinical studies with large numbers of samples. Methods of electrochemical and amperometric detection also suffer from time-consuming problems in both sample preparation steps and the chromatography. The liquid chromatography with tandem mass spectrometric detection (LC–MS/MS) methods achieve sufficient sensitivities in short chromatographic run times (e.g. 3 min) but the liquid–liquid extraction sample preparations need relative large amount of labor and organic solvents which may pollute the environment to achieve the expected sample amount (about 230 samples per day).

This paper describes the development and validation of an improved method for the quantification of clarithromycin in human plasma using LC–MS/MS. The sample preparation method was protein precipitation from a small volume of sample (50 μl) using 200 μl acetonitrile. The chromatographic run time was short: 2.4 min. This method has been applied to a bioequivalence study of two oral tablet formulations of clarithromycin in 20 healthy volunteers.

2. Experimental

2.1. Materials and reagents

Clarithromycin (99.5%) and telmisartan (99.0%) (see Fig. 1 for structures) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). Acetonitrile and methanol were HPLC-grade. All other chemicals were of analytical grade and used without further

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doi:10.1016/j.jpba.2006.11.025

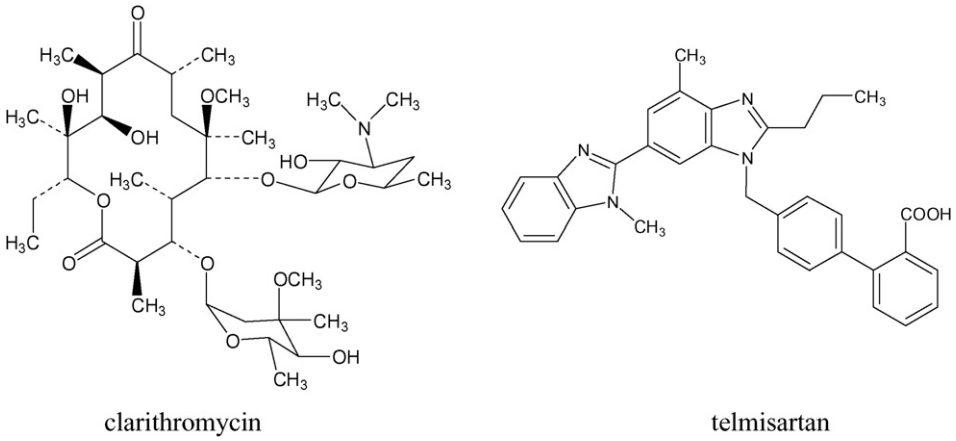

Fig. 1. Structures of clarithromycin and telmisartan.

purification. Distilled water, prepared from demineralized water, was used throughout the study. Blank human plasma from six different drug-free volunteers was obtained from Changchun Blood Donor Service (Changchun, China).

2.2. Calibration standards and quality controls

Stock solutions of clarithromycin and telmisartan (1.0 mg/ml, respectively) were prepared in methanol:water (50:50, v/v). Clarithromycin standard solutions with concentrations of 10, 30, 100, 300, 1000, 2000 and 5000 ng/ml were prepared by dilutions of aliquots of the stock solution with methanol:water (50:50, v/v). Low, medium and high concentration quality control (QC) solutions (30, 300, 4000 ng/ml) were prepared in a similar way. A working internal standard (I.S.) solution (telmisartan, 250 ng/ml) was also prepared in methanol:water (50:50, v/v). All the solutions were stored at 4 °C.

2.3. LC–MS/MS

The LC–MS/MS system consisted of an Agilent 1100 series (Agilent Technologies, Palo Alto, CA, USA) binary pump, an autosampler connected to a Zorbax extend C_{18} column (5 μm, 150 mm × 4.6 mm I.D. from Agilent Technologies) and an Applied Biosystems Sciex Q-trap™ mass spectrometer (Concord, Ontario, Canada) using electrospray ionization (ESI). The mobile phase was formic acid:10 mM ammonium acetate:methanol (1:99:400, v/v/v) delivered at 1.0 ml/min under ambient temperature. An approximately 1:1 split of the column eluent was included so that only 0.50 ml/min entered the mass spectrometer.

The detector was operated at unit resolution in the multiple-reaction monitoring (MRM) mode using the transitions of the protonated molecular ions of clarithromycin at *m/z* 749.6 → 158.1 and telmisartan at *m/z* 515.1 → 276.1. MS parameters were optimized by syringe pump infusing of a solution containing analyte and I.S. in mobile phase. Optimized parameters were as follows: curtain gas, gas 1 and gas 2 (nitrogen) 15, 50 and 55 units, respectively; dwell time 200 ms; source temperature 500 °C; ion spray voltage 1800 V. Declustering potential and collision energy were 60 V and 39 eV for clarithromycin and 90 V and 60 eV for telmisartan, respectively. The instrument was interfaced to a computer running Applied Biosystems Analyst version 1.3.2 software.

2.4. Sample preparation

To 50 μl human plasma were added 50 μl I.S. solution, 50 μl methanol:water (50:50, v/v) or a standard or QC solution of clarithromycin and 200 μl acetonitrile in a 1.5 ml Eppendorf tube. The mixture was vortex-mixed for 30 s and centrifuged at 12,000 × *g* for 10 min, the supernatant was transferred to an autosampler vial and 5 μl was injected into the LC–MS/MS system.

2.5. Assay validation

Three independent calibration curves and six replicates of QC samples (30, 300, 4000 ng/ml, respectively) were analyzed on three different days. Linearity was analyzed by weighted linear regression ($1/x^2$) of analyte-internal standard peak area ratios. Accuracy and precision were based on assay of six replicates of QC samples analyzed on three different days. The LLOQ was the concentration below which the inter-day coefficient of variation (CV) exceeded 20%. The limit of detection (LOD) was determined as the concentration with signal-to-noise ratio of 3. Recoveries of clarithromycin were determined by comparing peak areas of QC samples with those of corresponding concentration QC solutions dissolved in the supernatant of the processed blank plasma. Stability tests including three freeze–thaw cycles, storage for 1 month at −20 °C and at room temperature for 24 h were evaluated by QC samples.

The matrix effects were evaluated by comparing the peak areas of clarithromycin in QC samples (30, 300 and 4000 ng/ml) with those of the standard solutions, which were prepared in the same way as QC samples except water substituted for drug free plasma.

2.6. Bioequivalence study

The method was applied to evaluate the bioequivalence of two tablet formulations of clarithromycin in 20 healthy adult male

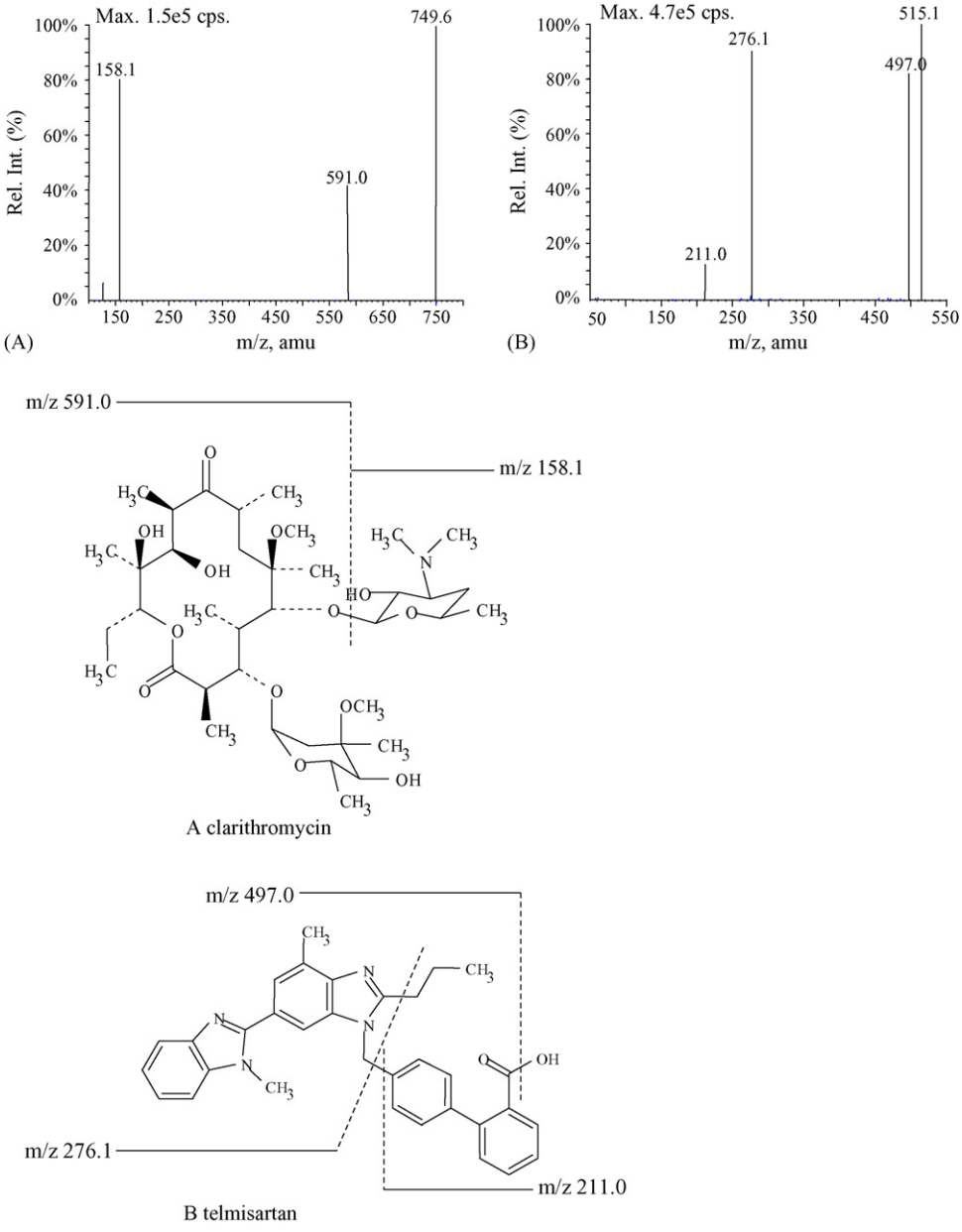

Fig. 2. Full-scan product ion spectra and fragmentation pathways of (A) clarithromycin and (B) telmisartan.

volunteers who received a single dose (500 mg clarithromycin) in a two-period randomized crossover design with a 1-week washout period between doses.

Venous blood samples were collected into heparinized tubes at the following times: immediately before administration, 0.25, 0.50, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10, 12 and 24 h after dosing. Plasma samples were obtained by centrifugation of the whole blood at 3000 × *g* for 10 min and stored at −20 °C. Bioequivalence of the two formulations was assessed according to US-FDA methodology [25].

Table 1
Precision and accuracy for the determination of clarithromycin in human plasma

Nominal concentration (ng/ml)	Calculated concentration (mean ± S.D.) (ng/ml)	Intra-day R.S.D. (%)	Inter-day R.S.D. (%)	Relative error (%)
30	31.3 ± 1.42	3.73	8.37	4.48
300	301 ± 13.8	3.27	9.93	0.46
4000	3900 ± 147	3.22	6.65	−2.51

Six replicates per day, on 3 different days.

3. Results and discussion

3.1. Mass spectrometry

Both analyte and I.S. responded best to the protonated molecular ions $[M+H]^+$. The full-scan product ion spectra and fragmentation pathways of clarithromycin and telmisartan are shown in Fig. 2. The fragment ions at *m/z* 158.1, 591.0 and 276.1, 497.0 were chosen for MRM acquisition of clarithromycin and I.S., respectively. Finally, two ion-pairs (*m/z* 749.6 → 158.1, 515.1 → 276.1) were used for quantification.

3.2. Chromatography

Sample pretreatment was performed by single protein precipitation using 200 μl acetonitrile, based on advisement as follows: the strong enough response to be diluted by acetonitrile; much less amount of organic solvents contacted by assay operators than liquid–liquid extraction; a small volume of plasma (50 μl) which can reduce the collected blood amount and alleviate the pain of the volunteers; more economical than SPE and a relatively simple procedure. Hence, no additional attempts were made to evaluate other procedures.

The LC eluent was diverted to the mass spectrometer at 1.30 min after injection to avoid hydrophilic impurities flowing into the instrument. In the final procedure, only a small fraction (5.0 μl) of the sample after preparation was injected on the column to maintain high efficiency and resolution.

The inclusion of 10 mM ammonium acetate instead of pure water in the mobile phase resulted in an optimal signal response, and peak shape was improved because of the adjustment of formic acid. Under the optimum conditions, analyte and I.S. were free of interference from endogenous substances and gave retention times of 1.76 and 1.84 min, respectively.

3.3. Assay validation

Representative chromatograms of blank plasma, the standard sample at LLOQ (10 ng/ml) and a study sample containing a low concentration of clarithromycin are shown in Fig. 3.

The calibration curves were linear in the concentration range 10–5000 ng/ml ($r^2 > 0.9954$) with the LOD of 0.50 ng/ml. Intra-

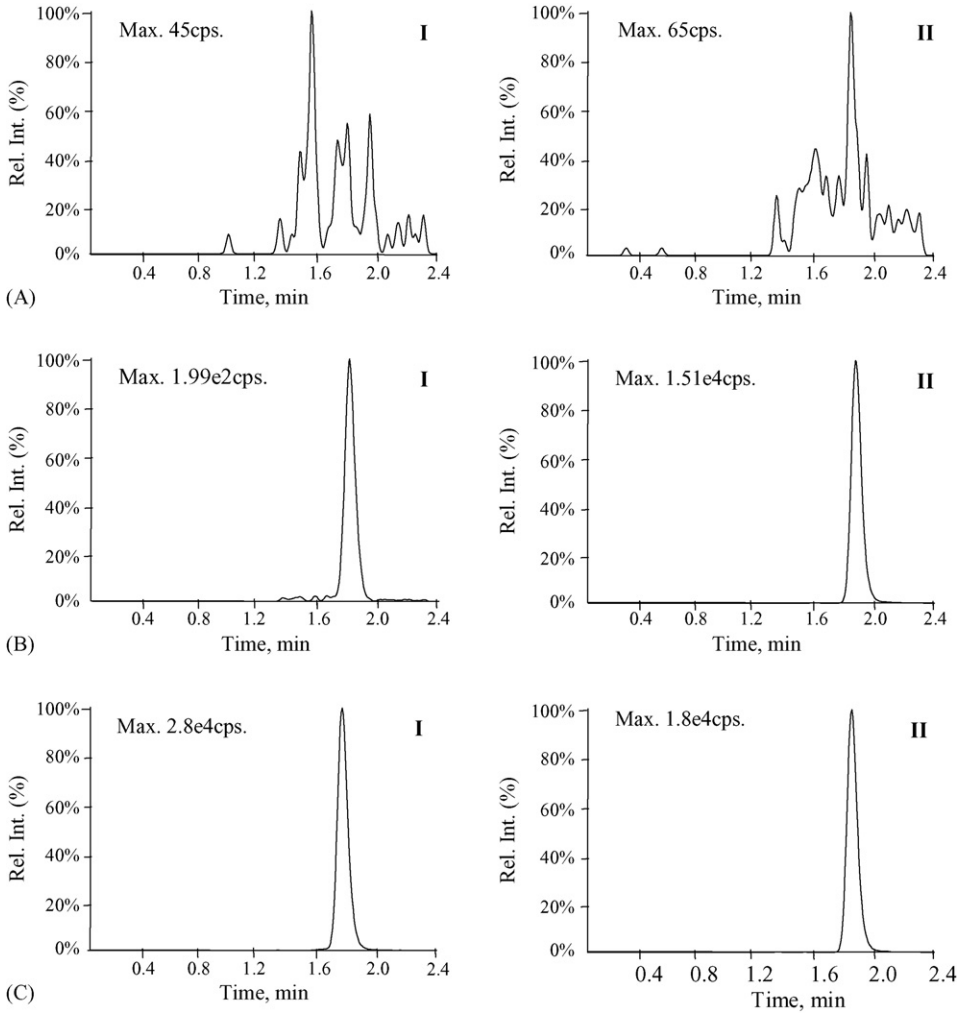

Fig. 3. Representative MRM chromatograms of (A) blank plasma, (B) the standard sample at LLOQ (10 ng/ml) and (C) a plasma sample from a volunteer 2 h after an oral administration of 500 mg clarithromycin. Peak I, clarithromycin; peak II, telmisartan.

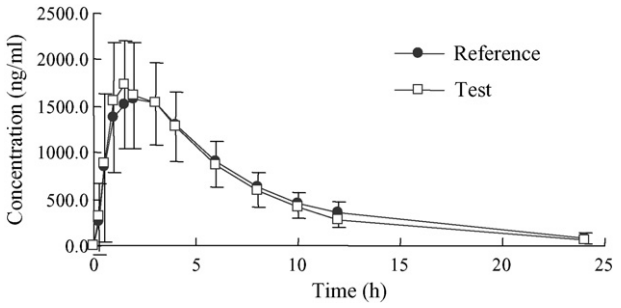

Fig. 4. Plasma concentration vs. time curve for two clarithromycin tablet formulations in healthy volunteers ($n = 20$). Data are mean ± S.D.

Table 2
Pharmacokinetic parameters of clarithromycin in test and reference formulations (mean ± S.D., $n = 20$)

Parameter	Clarithromycin	
	Test formulation	Reference formulation
C_{max} (ng/ml)	1861.75 ± 643.55	1781.05 ± 614.28
T_{max} (h)	1.7 ± 0.6	2.0 ± 0.9
$T_{1/2}$ (h)	5.32 ± 2.86	5.49 ± 2.63
AUC_{0-t} (ng h/ml)	12762.58 ± 3425.56	13443.13 ± 3099.79
$AUC_{0-\infty}$ (ng h/ml)	13297.85 ± 3246.72	14264.68 ± 3194.26

and inter-day precisions were 3.22–3.73% and 6.65–9.93%, respectively, and relative error was below 4.48% (Table 1).

Absolute recoveries of clarithromycin at concentrations of 30, 300 and 4000 ng/ml were 97.0 ± 7.19%, 95.4 ± 3.94% and 96.5 ± 6.00%, respectively. Clarithromycin was stable under all the storage conditions evaluated with mean recoveries of 92.3–109.0% of the nominal concentrations.

Matrix effects were minimal based on concentrations being 108.9–111.0% of nominal concentrations for clarithromycin. The matrix effects values above 100% were caused by the final lower supernatant volume of QC samples relative to the corresponding aqueous samples. This volume difference occurs when the acetonitrile is added to the plasma causing plasma proteins to precipitate, thus the supernatant volume is smaller than when no precipitation occurs [26].

3.4. Pharmacokinetic study

The mean plasma concentration versus time curve obtained after a single oral dose (500 mg clarithromycin) of two tablet formulations is shown in Fig. 4. The main pharmacokinetic parameters of clarithromycin, similar to those reported in the literature [5,27], are shown in Table 2. The 90% confidence intervals of C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ (after log-transformed) were 85.17–124.98%, 80.52–108.69% and 80.06–107.85%, respectively. The results indicated that the valued data were within the bioequivalence acceptance range of 80–125% required by the US-FDA [25]. Based on these, the two tablet formulations were found to be bioequivalent.

4. Conclusion

A selective, sensitive and rapid LC–MS/MS method is reported for the determination of clarithromycin in human plasma. The sensitivity is sufficient to determine the drug in human plasma after oral administration. The method allows high sample throughput (more than 200 samples per day) due to the short run time and relatively simple sample preparation procedure.

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