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Determination of minocycline in human plasma by high-performance liquid chromatography coupled to tandem mass spectrometry: application to bioequivalence study

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Abstract

Minocycline was determined in human plasma by HPLC–MS–MS using clarithromycin as an internal standard. The method is fast (single liquid extraction and run time of <3 min) and sensitive (5 ng/ml) and it was employed in a bioequivalence study of two 100 mg tablet formulations in 24 healthy volunteers. The 90% confidence interval of the individual ratio geometric mean for both $AUC_{(0-96\ h)}$ and C_{max} were 99.2–111.1% and 95.6–117.5%, respectively. Thus, Minoderm was considered bioequivalent to Minomax according to both the rate and extent of absorption. No food interaction was observed with either formulation.

Keywords: Bioequivalence study; Minocycline

1. Introduction

Minocycline (4*S*,4a*S*,5a*R*,12a*S*)-4,7-bis(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,10,12,12a-tetrahydroxy-1,11-dioxo-2-naphthacenecarboxamide (MN), is a semi-synthetic tetracycline antibiotic. Owning to its high penetration characteristics throughout the body, MN can be used in the treatment of a wide variety of extracellular and intracellular pathogens [1]. The absorption of MN is believed to be affected by simultaneous administration with food, since reductions in both maximum concentration reached (C_{max}) and area under the curve (AUC) were reported [2,3]. Several methods based on high-performance liquid chromatography (HPLC) have been reported for the determination of MN in human serum and plasma [4–8], but all these methods have inconveniences for routine analysis. For instance, some employ large sample volumes [4,5,8], have long extraction procedures (solid-phase extractions [4,6,8] or liquid–liquid reextractions [5]) and long retention time [7,8]. The most sensitive method had a limit of quantification (LOQ) of 30 ng/ml [5]. Quantification of drugs in biological matrices by liquid chromatography–tandem mass spectrometry (LC–MS–MS) is becoming more usual, due to the improved sensitivity and specificity of this technique [9,10].

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This paper describes a fast, sensitive and specific LC–MS–MS method for determination of MN using clarithromycin (CR) as an internal standard (I.S.) in human plasma. This LC–MS–MS method was applied in a bioequivalence study of two 100 mg tablet formulations of MN and in a food–drug interaction study in 24 healthy volunteers.

2. Experimental

2.1. Chemicals

Minocycline hydrochloride was provided by Laboratórios Stiefel (Guarulhos, SP, Brazil), lot No. 1644/99. Clarithromycin was provided by Medley Indústria Farmacêutica (Campinas, SP, Brazil), lot No. BA019298. HPLC-grade solvents and analytical-grade reagents were purchased from Mallinckrodt (Paris, KY, USA) and Nuclear (São Paulo, Brazil). Trifluoroacetic acid (TFA) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma (Saint Louis, MO, USA) and Mallinckrodt, respectively. Plasma samples were stored at −20°C after collection and necessary processing.

2.2. Calibration standards and quality controls

Stock solutions of MN and CR were prepared at 1 mg/ml in 50% aqueous methanol. A standard work solution of MN at 10 μg/ml in mobile phase was used for spiking pooled blank plasma at concentrations of 5, 10, 20, 50, 100, 200, 500, 1000 and 2000 ng/ml. Calibration standard curves were performed in duplicate for each batch. Quality controls were fixed at 20, 100 and 1000 ng/ml and prepared in the same blank plasma.

2.3. Sample preparation

A 200-μl volume of plasma samples (spiked or from volunteers) was added to 200 μl of EDTA buffer/internal standard solution (10 m*M* EDTA/0.2 μg/ml of CR). The tubes were vortex-mixed for approximately 10 s. Diethyl ether–dichloromethane (70:30, v/v, 4 ml) was added and the samples vortex-mixed for 35 s. The upper organic layer was removed and transferred to new tubes. The solvent was removed using a flow of N_2 at 37°C. The samples were reconstituted with 200 μl of mobile phase and transferred to microvials, capped and placed in a Shimadzu Avp10 autosampler.

2.4. Liquid chromatography and mass spectrometry

An aliquot (40 μl) of the plasma extract was injected into a Zorbax RX-C8 15 cm×4.6 mm column (Hewlett-Packard, USA) using a Shimadzu AVP LC system (Shimadzu, Japan). Separation and elution were achieved using acetonitrile (ACN)–water–TFA (80.0:19.9:0.1) as the mobile phase, at a flow-rate of 1 ml/min. The oven temperature was 40°C and the time between injections was 3 min.

Mass spectrometric detection was performed using a MicroMass (Manchester, UK) Quattro LC triple quadrupole mass spectrometer, equipped with an electrospray (ES) source. The temperature of the probe and source block was 350 and 120°C, respectively. Nitrogen was used as nebuliser (75 l/h) and desolvation gas (621 l/h). The electrospray source was operated in the positive ionization mode (ES+), and multiple reaction monitoring mode (MRM), m/z 457.9→441.0 and m/z 748.4→157.8, were used for quantification of MN and CR, respectively. The dwell time, the cone voltage, the collision energy and collision gas pressure (argon) were 0.1 s, 25 V, 20 eV and $1.7 \cdot 10^{-3}$ mbar, respectively for MN, and 0.1 s, 30 V, 25 eV and $1.7 \cdot 10^{-3}$ mbar for CR, respectively. Data were acquired by MassLynx software (version 3.2, MicroMass) and calibration curves for the analyte were constructed using the calibration samples using MN to I.S. peak area ratios (PARs) via a weighted ($1/x$) least-squares linear regression. Unknown sample PARs were then interpolated from the calibration curve to provide concentrations of MN.

2.5. Bioequivalence and food–drug interaction

The method was applied to evaluate, on human volunteers, the performance of one MN tablet formulation (Minoderm, tablets, 100 mg, batch No. CT010/99, Stiefel, Brazil) against one standard MN tablet formulation (Minomax, tablets, 100 mg, batch No. 12227, Wyeth, Brazil) taken with or without food. The comparison was done through the quantification of MN in plasma. The bioequivalence

between both formulations and both regimens (fasting or after food intake) were assessed by calculating individual C_{max}, $AUC_{(0-96\ h)}$, $AUC_{(0-\infty)}$ and $C_{max}/AUC_{(0-96\ h)}$ ratios (test/reference) together with their mean and 90% confidence intervals (CIs) after log transformation of the data (additive model [11]). The inclusion of the 90% CI for the ratio in the 80 to 125% range was analyzed by a parametric [analysis of variance (ANOVA)] method.

Twenty-four healthy volunteers of both sexes (12 females and 12 males) who were between the ages of 19 and 39 years (mean±SD, 23.3±4.2 years), of heights between 155.0 and 181.0 cm (mean±SD, 166.7±6.9 cm) and who weighed between 50.1 and 77.8 kg (mean±SD, 65.3±8.1 kg) and within 15% of their ideal body weight were enrolled in the study. All subjects gave written informed consent, and the Ceará Federal University Hospital Ethics Committee of Clinical Investigation approved the clinical protocol.

The volunteers were free from significant cardiac, hepatic, renal, pulmonary, neurological, gastrointestinal and hematological disease, as assessed by physical investigation, EGC and the following laboratory tests: blood pressure, urea, creatinine, AST, ALT, alkaline phosphatase, γ-GT, total bilirubin, uric acid, total cholesterol, triglycerides, albumin and total protein, hemoglobin, hematocrit, total and differential white cell counts, erythrocyte sedimentation rate, and routine urinalysis. All subjects were negative for HIV, HBV and HCV.

The study was conducted in an open, randomized, four-period crossover fashion with a 3-week washout period between doses. During each period, the volunteers were hospitalized at 11:00 p.m., having already had a normal evening meal, and after an overnight fast they received at 7:00 a.m. a single 100 mg dose of the appropriate MN formulation along with 200 ml of tap water. For those subjects on confinements whose drug–food interaction was to be tested, a breakfast with orange juice (200 ml), strawberry yogurt (200 ml), cream cracker biscuits (4 units) and cheese (1 unit) were received 15 min before dose. No food was allowed during 5 h following drug administration, after which a standard lunch was consumed and an evening meal was provided 12 h after dosing. No other food was permitted during the ''in-house'' period. Liquid consumption was permitted ad libitium after lunch but xanthine-containing drinks, including tea, coffee and cola, were avoided.

Blood samples (10 ml) from a suitable antecubital vein were collected into EDTA-containing tubes before and 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0, 16.0, 24.0, 48.0, 72.0 and 96.0 h after the administration of each dose of MN. The blood samples were centrifuged at 2500 *g* for 10 min at room temperature and the plasma decanted and stored at −20°C until assayed. All samples from a single volunteer were analyzed on the same day to avoid inter-assay variation.

3. Results and discussion

3.1. Method development

Full scan (MS1) mass spectra of MN and CR showed the protoned molecular ion $[M+H]^+$ at m/z 458 and 748, respectively. The most abundant ion in the product ion spectra (MS–MS) was at m/z 441 for MN and m/z 158 for CR (Fig. 1). The loss of 17 u (NH_3) for MN has been previously observed [12]. The MS system was set as follows: m/z 458 for MN and m/z 748 for CR as precursor ions and m/z 441 for MN and 158 for CR as product ions in the MRM mode. No peak was observed in the mass chromatogram of blank human plasma under the conditions mentioned and the retention times for MN and CR were 1.8 and 2.1 min, respectively (Fig. 2). The recoveries of MN based on PARs of extracted plasma/mobile phase, both previously spiked at final concentrations of 20, 100 and 1000 ng/ml were 101.4±9.0, 77.9±8.3 and 79.2±4.0% (mean±RSD, $n=5$), respectively. For the I.S. (200 ng/ml) the recovery was 82.9±5.6% (mean±RSD, $n=5$).

3.2. Assay performance

Linearity, precision and accuracy assess the performance of method. Precision was determined as the percent relative standard deviation, RSD (%)= $100\cdot(SD/M)$ and accuracy as the percent relative error, RE (%)=$(E-T)\cdot(100/T)$, were M is the mean, SD is the standard deviation, T is theoretical

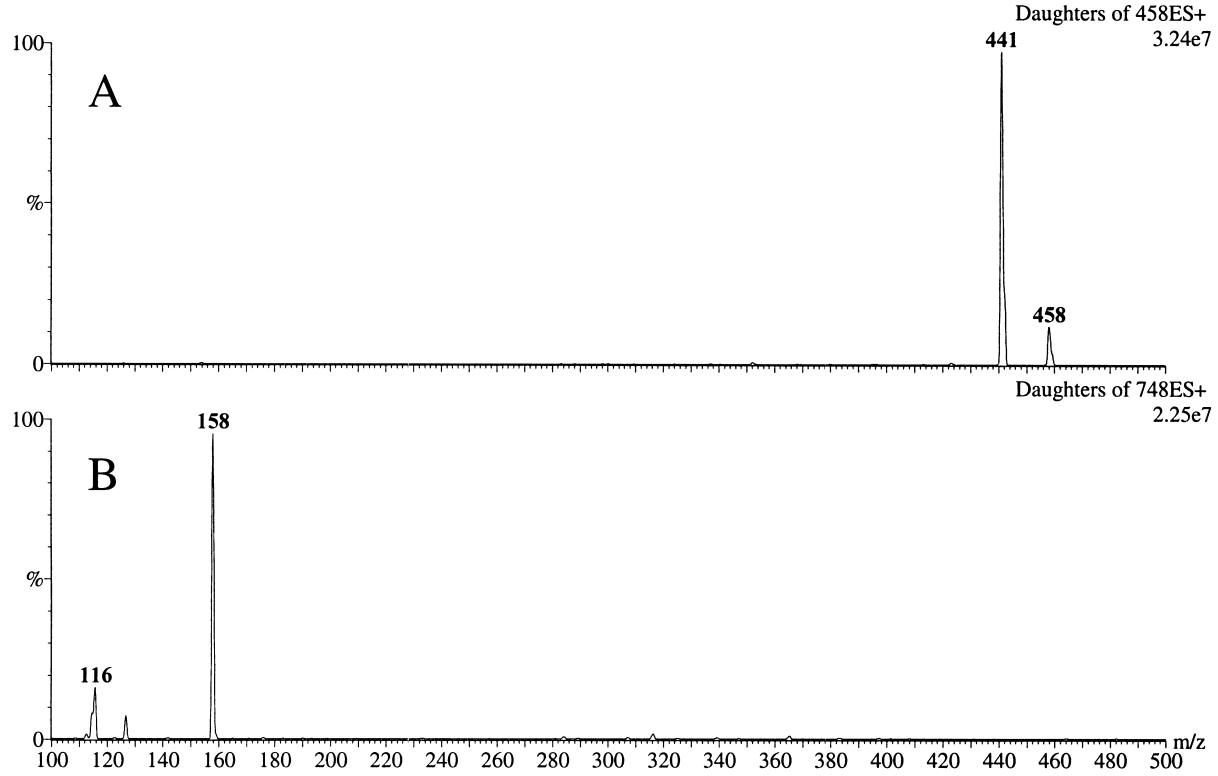

Fig. 1. Product ion spectrum of (A) minocycline and (B) clarithromycin.

concentration and E is the experimentally determined concentration.

The linearity of the MN calibration curve was proven for the range from 5 to 2000 ng/ml. The between-run accuracy and precision are shown in Table 1. Between-run precision was estimated as 4.0–16.7% and accuracy as 3.0–19.0% over the range. The coefficient of correlation for all measured curves was at least 0.995 and its mean was greater then 0.999. The lower LOQ was 5 ng/ml and defined as the lowest concentration at which both precision and accuracy were less than or equal to 20%.

The within-run accuracy was ±9.2 (M= 21.8±SD=0.9, n=9), 9.7 (109.7±6.7, 9) and 11.8% (1118.0±72.0, 9) for QCA, QCB and QCC, respectively, and the within-run precision was ≤12.0 (21.2±2.5, 9), 11.3 (97.3±11.0, 9) and 12.2% (1018.1±123.7, 9), respectively. The between-run accuracy was ±2.3, 2.8 and 2.8% and between-run precision was ≤6.9, 7.4 and 8.1% for QCA (20.5±1.4, 98), QCB (102.8±7.6, 98) and QCC (1028.3±83.1, 98), respectively.

3.3. Bioequivalence and food–drug interaction

Both MN formulations were well tolerated at the dose administered. One volunteer during the second confinement complained of severe headache, whose symptom relation to the drug is unknown (reference formulation with food). No significant changes in the biochemical parameters were observed. Pharmacokinetic parameters are shown in Table 2 and means of plasma concentrations of minocycline are shown in Fig. 3. The maximum concentration reached (C_{max}) and the areas under the curve ($AUC_{0-96\ h}$) were compared. The geometric mean and 90% confidence intervals of Minoderm/Minomax; Minoderm+food/Minomax+food; Mino-

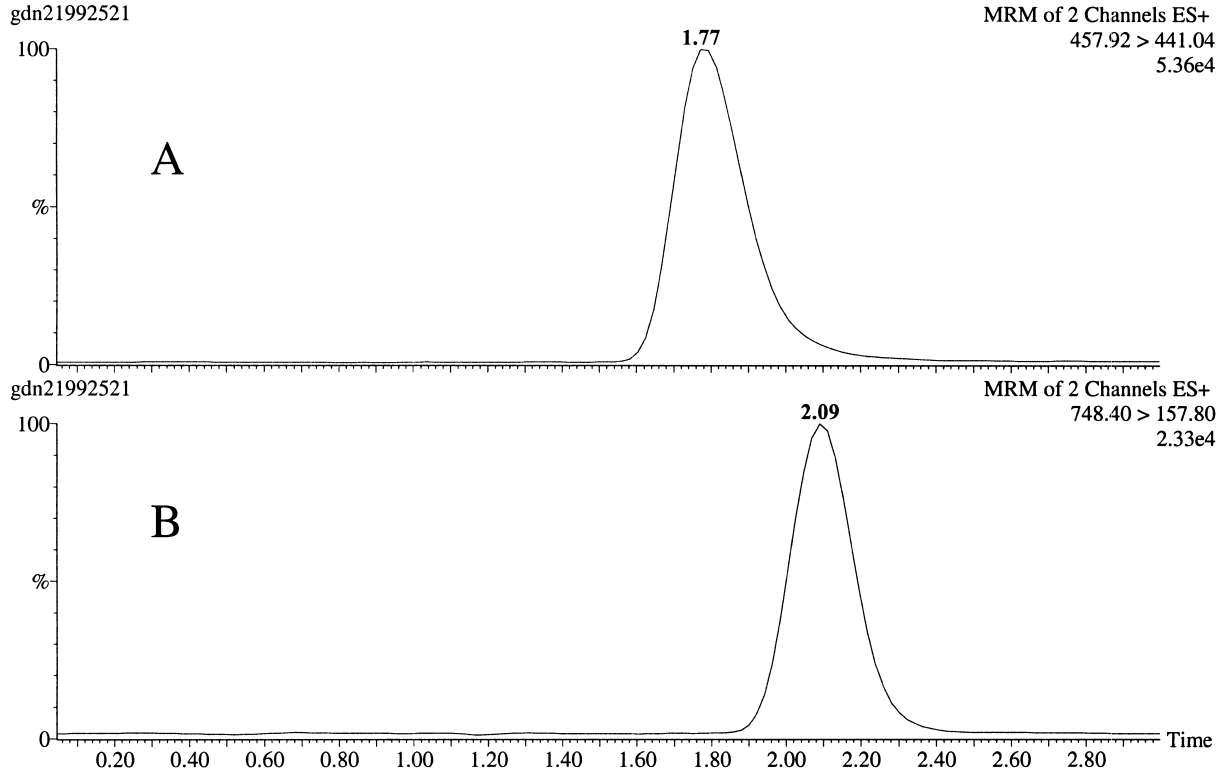

Fig. 2. MRM chromatogram of collected plasma 3 h after oral administration of a single 100 mg minocycline tablet: (A) minocycline and (B) clarithromycin.

derm+food/Minoderm and Minomax+food/Minomax ratios are summarized in Table 3.

Since the 90% CI for both C_{max} and $AUC_{(0-96\ h)}$ ratio (test/reference, fasting or after food intake) were inside the 80–125% interval proposed by the US Food and Drug Administration [13,14], it is concluded that Minoderm 100 mg tablets are bioequivalent to Minomax 100 mg tablets for both the rate and the extent of absorption, under both fasting conditions and after food intake.

Table 1
Calibration curve quality report

Standard calibration	5	10	20	50	100	200	500	1000	2000	*r*
n	21[a]	22	22	22	20[a]	22	22	22	22	0.9995
Mean	5.95	10.84	21.09	48.52	94.55	192.62	479.67	968.13	2070.14	
SD	0.99	1.40	3.09	7.14	13.91	24.26	51.64	94.65	82.13	
RSD (%)	16.73	12.91	14.68	14.71	14.70	12.59	10.76	9.78	3.97	
RE (%)	−18.92	−8.42	−5.44	2.96	5.45	3.69	4.07	3.19	−3.51	

[a] Three points of calibration curve, one at 5 ng/ml (batch 02) and two at 100 ng/ml (batches 03 and 09), were rejected due to high deviation of nominal concentration. Calibration curves of these batches were performed as without duplicate at the mentioned concentrations.

Table 2
Pharmacokinetic parameters from bioequivalence study of minocycline in 24 human volunteers

	Minoderm		Minomax		Minoderm+food		Minomax+food	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
$AUC_{all\ (0-96\ h)}$ (ng h/ml)	17 132	5133	17 128	6210	15 249	4974	15 864	5965
$AUC_{(0-\infty)}$ (ng h/ml)	17 416	5221	17 389	6347	15 518	5115	16 193	6157
$AUC_{(0-96\ h)/(0-\infty)}$ (%)	98.4	–	98.5	–	98.3	–	98.0	–
C_{max} (ng/ml)	1183	324	1130	380	950	298	1032	299
T_{max} (h) (range)	2.0	(1.0–6.0)	2.0	(1.5–6.0)	2.0	(1.5–8.0)	2.0	(1.5–8.0)
$T_{1/2}$ (h) (range)	15.3	(10.8–21.6)	16.0	(11.0–22.4)	15.5	(9.7–24.1)	17.1	(11.6–32.7)
K_e (1/h) (range)	0.05	(0.03–0.06)	0.04	(0.03–0.06)	0.05	(0.03–0.07)	0.04	(0.02–0.06)

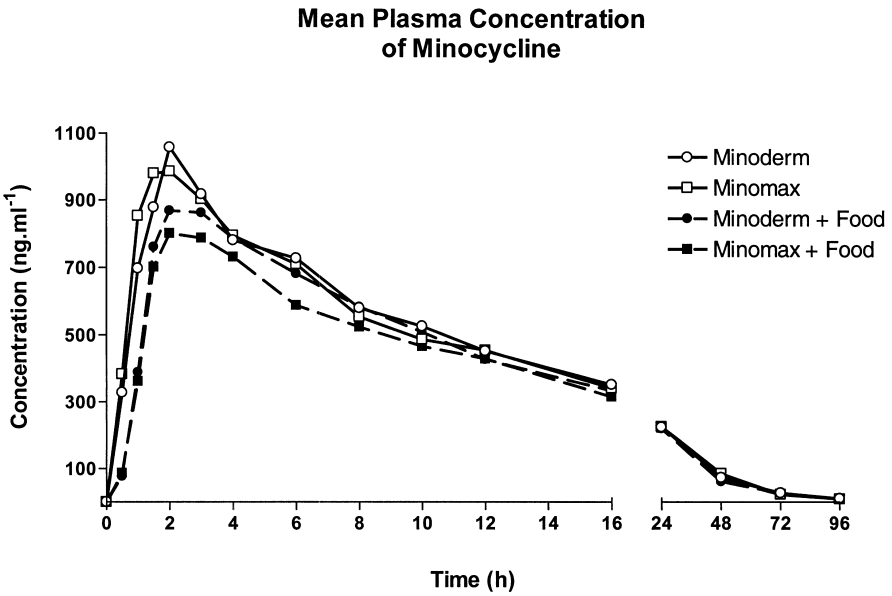

Fig. 3. Mean plasma concentration of minocycline in 24 human volunteers follow oral administration of a single 100 mg tablet.

Leyden [2] and Meyer [3] have reported that meals reduce the bioavailability of minocycline, but their studies were performed with a small group of healthy volunteers and did not estimate the 90% CI for individual $AUC_{(0-96\ h)}$ and C_{max} ratios. Indeed, food interaction studies should be analyzed statistically, similarly to bioequivalence studies [13,14]. Our study reveals that no food interaction took place,

Table 3
Geometric mean and 90% confidence intervals

	C_{max}	AUC
Minoderm/Minomax	106.0 (90% CI=95.6–117.5)	101.2 (90% CI=92.2–111.1)
Minoderm+food/Minomax+food	91.2 (90% CI=82.8–100.5)	97.2 (90% CI=92.2–102.5)
Minoderm+food/Minoderm	80.0 (90% CI=70.9–90.4)	89.2 (90% CI=82.7–96.3)
Minomax+food/Minomax	92.8 (90% CI=84.9–101.5)	93.1 (90% CI=87.3–99.2)

since the ratios for AUC (food/fasting) were included in the 80–125% interval proposed for bioequivalence (Table 3).

4. Conclusion

A first LC–MS–MS method for the determination of MN in human plasma was developed and monitored during a bioequivalence study. This method was shown to be fast, simple and sensitive with acceptable accuracy and precision. These results indicated that the method is rugged and suitable for routine analysis of MN in human plasma.

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