Development and Validation of a HPLC–MS–MS Method for Quantification of Metronidazole in Human Plasma

Marina Silva, Simone Schramm, Eunice Kano, Eunice Koono, Valentina Porta, and **Cristina Serra**
USP, Pharmacy

Abstract

The objective of this study was to describe an analytical validation of a simple, rapid, sensitive, and selective liquid chromatography (LC)-tandem mass spectrometry (MS–MS) method for quantification of metronidazole in human plasma samples from bioavailability studies. The plasma samples were pre-treated by liquid–liquid extraction (LLE) with ethyl acetate. Metronidazole and the internal standard (IS) zidovudine were analyzed by combined reversed-phase LC–MS–MS with positive ion electrospray ionization. Multiple reactions monitoring (MRM) of the transitions were used as follows: *m/z* 171.97 > 127.97 for metronidazole and *m/z* 268.08 > 126.96 for IS. The main chromatography conditions used were a Varian C_{18} Microssorb model (150 mm × 4.6 mm i.d., 5 µm particle size) column, and the mobile phase was composed of acetonitrile and 10 mM ammonium acetate (80:20, v/v) and 0.1% formic acid. Flow rate used was 1.0 mL/min with splitter. The method was linear over a concentration range of 0.05–8.00 µg/mL. Acceptable precision and accuracy were obtained for concentrations over the standard curve range. The developed method was validated and successfully applied to a pilot bioequivalence study of metronidazole in healthy volunteers.

Introduction

Metronidazole is the reference agent of the nitroimidazole antibiotic family (1,2) and it is widely used in the therapy of several infesting diseases, such as amoebiasis, trichomoniasis, lambliasis, and anaerobic infections (2,3).

The drug is one of the most promising agents in combination antimicrobial therapies used in the eradication of helicobacter pylori, a recognized cause of gastritis and duodenal ulcers (2,4,5). It is most frequently used in association with others antibiotics because of the resistance of this organism (6).

After oral administration, metronidazole is well absorbed, and serum concentrations reach high levels with tissues concentrations being generally similar to or slightly lower (1). It can be eliminated with $t_{½}$ (elimination half-life) between 6–12 h. Bioavailability after oral administration is almost complete (95%); rectal administration also yields sufficient and reproducible high extents of availability (3,7). The drug is intensively metabolized by the hepatic microsomal cytochrome P-450-dependent mono-oxygenase system to form two biologically active main metabolites (3,8).

*Author to whom correspondence should be sent: email marinadefreitas@usp.br.

Extraction and clean-up of drugs from biological fluids are usually the first and most difficult step in bioanalysis due to the need to selectively remove interferant, such as proteins, without significant analyte loss (9).

There are some chromatographic techniques previously published for quantification of metronidazole in human plasma, but they present some disadvantages for its application in bioequivalence studies, such as multiple-steps procedures for metronidazole extraction from biological samples with extensive chromatographic run times (13), and expensive solid-phase procedures for samples preparation (6), or protein precipitation (2,4). Other methods describe liquid–liquid extraction with large volumes of solvent (1,7,9–11) or double liquid–liquid extraction, both methods which result in a large consumption of solvents (3) and longer time for sample analyses. The method presented in this study, however, has a short run time and a simple liquid–liquid extraction procedure, which is an important issue in bioequivalence studies considering the large amount of samples that have to be analyzed in this kind of test (12,13). The method proposed by Wibawa et al. uses small volumes of plasma sample and solvent, but it has an extensive run time (5).

Metronidazole quantification is accomplished mostly by the use of high-performance liquid chromatography (HPLC) with UV detection (2–6,14–16) usually set at 313–318 nm, mass spectrometry (MS/MS) detection, or diode array detection set at 313 nm (2) and at 315 nm (9). Only one published work presents a method using gas-liquid chromatography with mass spectrometry (MS–MS) (7).

In the present study a simple and rapid HPLC method with MS–MS detection with a short run time and an adequate concentration range for quantification of metronidazole in human plasma from bioequivalence studies is described.

Experimental

Materials

Metronidazole and zidovudine (internal standard, IS) reference standards were kindly supplied by FUNED (Fundação Ezequiel Dias, Belo Horizonte, Brazil). HPLC-grade acetonitrile, ethyl acetate, methanol, analytical-grade formic acid, and ammonium acetate were purchased from Merck (São Paulo, Brazil). Water (18.2 MΩ) used in the mobile phase was freshly prepared from Milli-Q Academic (Millipore, Bedford, MA). Blank plasma was obtained from healthy volunteers.

Reproduction (photocopying) of editorial content of this journal is prohibited without publisher's permission.

Apparatus and chromatographic conditions

Analyses were performed on a Shimadzu Scientific Instruments (Kyoto, Japan) liquid chromatographic system composed of a LC-10ADVP pump accompanied with an SIL-10ADVP auto-sampler, fitted with a 50-µL loop, and a SCL-10AVP controller unit. The analytical column was a Varian C_{18} Microssorb (150 mm × 4.6 mm i.d., 5 µm particle size) protected with a Phenomenex AJO-4287 C_{18} guard cartridge (5 mm × 4.6 mm i.d., 5 µm particle size) (Torrance, CA). This HPLC system was coupled with an ESI interface with a Micromass Quattro triple quadrupole mass spectrometer (Milford, MA) equipped with an electrospray ion source.

The isocratic HPLC mobile phase was composed by acetonitile and 10 mM ammonium acetate (80:20, v/v) and 0.1% formic acid; it was prepared daily, filtered through a 0.22-µm filter, and degassed ultrasonically for 20 min before use. Flow rate was 1.0 mL/min, and a splitter was used. The column was maintained at room temperature (22°C). The injection volume was 50 µL, and the total run time was 5.0 min.

The mass spectrometer was operated in the positive ion detection mode. Tuning parameters were optimized for both analytes by infusing a solution containing 1 µg/mL of metronidazole and the IS at a flow rate of 20 µL/min via an external syringe pump directly connected to the mass spectrometer. The source temperature was set at 100ºC, and desolvation temperature was 300°C. Optimized cone voltage values were 25 V for metronidazole and 30 V for IS, while capillary voltage was set at 3.0 kV. The multiplier was set at 650 V, and argon was used as the collision gas. Quantification was performed using multiple reaction monitoring (MRM) of the transitions *m/z* 171.97 > 127.97 for metronidazole and *m/z* 268.08 > 126.96 for IS with a dwell time of 0.5 s per transition. Collision energies of 15 eV and 20 eV were used for metronidazole and the IS, respectively. Data were acquired using MassLynx 4.0 software.

Standard solution preparation

A metronidazole stock solution with final concentration of 5000 µg/mL was prepared by dissolving 500 mg of reference standard in 100 mL of methanol. A metronidazole intermediate solution of 500 µg/mL in methanol was prepared by dilution of the metronidazole stock solution. A zidovudine stock solution (100 µg/mL) was prepared by dilution of 10 mg of reference standard in water–methanol (90:10, v/v). The metronidazole standard solutions were prepared by dilution of stock solutions with water–methanol (90:10, v/v) to obtain the following concentrations: 0.5, 1.5, 5.0, 10.0, 20.0, 40.0, 60.0, and 80.0 µg/mL. Stock/intermediate and standard solutions of both compounds were stored at –20°C and 4°C, respectively. All stock and standard solutions were stable for six and two months, respectively.

Calibration standard and quality control samples preparation

Preparation of calibration standard plasma samples (0.05, 0.15, 0.5, 1.0, 2.0, 4.0, 6.0, and 8.0 µg/mL) was accomplished daily by adding known amounts (25 µL) of metronidazole standard solutions and 50 µL of IS stock solution (100 µg/mL) in 8-mL glass tubes and adding 250 µL of drug-free plasma. Quality control plasma samples (0.15, 3.0, and 7.0 µg/mL) were prepared in 25-mL volumetric flasks by spiking drug-free plasma with known amounts (175–375 µL) of metronidazole solutions (10 and 500 µg/mL), aliquoted, and stored at –20°C.

Metronidazole extraction procedure

All frozen human plasma samples were thawed at ambient temperature. Fifty microliters of IS solution (100 µg/mL) in water–methanol (90:10, v/v), and 250 µL of plasma were added in glass 8-mL tubes and vortexed for 30 s. After this procedure, ethyl acetate (3.0 mL) was added to all tubes, and extraction was performed by vortex mixing for 60 s, followed by centrifugation for 10 min at 3500 rpm. Aqueous layers were frozen, and the upper organic phases were transferred to 8-mL conical glass tubes and evaporated to dryness at 40°C under a nitrogen stream. The extracts were reconstituted with 300 µL of mobile phase and 50 µL were injected into the chromatographic system.

Bionalytical method validation

The specificity of the method was investigated by analyzing six drug-free plasma samples obtained from healthy human volunteers who did not take metronidazole (four normal plasma samples, one hemolzyed plasma sample and one lipemic plasma sample) for interference of endogenous compounds.

The standard curve was obtained through analysis of calibration standard plasma samples and plotting of peak area ratio of metronidazole and zidovudine versus the corresponding metronidazole concentrations (0.05, 0.15, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0 µg/mL). The linearity of the standard curve was evaluated using least-squares linear regression analysis. The lower limit of quantification (LLOQ) was the smallest analytical concentration which could be measured with accuracy and precision still better than 20% (0.05 µg/mL).

The analytical recovery was determined by comparing the response of pre-treated quality control plasma samples with the response of identical standards prepared in the mobile phase, which did not undergo sample pre-treatment.

To evaluate the inter-assay precision and accuracy, quality control plasma samples were analyzed together with one independent calibration standard curve for three days, while intra-assay precision and accuracy were evaluated through analysis of quality control plasma samples in replicate of six ion the same day. Inter-assay and intra-assay precision were expressed as relative standard deviation (RSD%). The accuracy was expressed as the percent ratio between the experimental concentration and the nominal concentration for each sample

Stability of metronidazole in spiked plasma samples was determined in triplicate at concentrations of quality control plasma samples after three freezing-and-thaw cycles. Stability of spiked, processed plasma samples during storage in the auto sampler for 24 h and 48 h at room temperature was evaluated. Additionally, the stability was evaluated during the period in which the drug was in plasma samples before the samples had been analyzed.

Application

A bioequivalence study was carried out to verify the actual working conditions in volunteers' plasma samples. It also served to evaluate the magnitude of metronidazole concentrations in human plasma after administration of metronidazole single dose of 250 mg.

The study was approved by the ethical committee from College of Pharmaceutical Sciences - University of São Paulo (Brazil). The study was performed according to the rules of Good Clinical Practice. Twenty-two healthy volunteers, 11 females and 11 males, participated in the pilot study. Subjects were selected after a clinical screening procedure including a physical examination and laboratory tests. All subjects avoided using other drugs for at least one week prior to the study and until after its completion. They also abstained from alcoholic beverages and xanthine-containing foods and beverages 48 h prior to each dosing until the collection of the last blood sample. The study was an open, randomized, two-period crossover trial with a one week wash-out period.

Subjects were admitted into hospital at 7:00 p.m. the day before the study and fasted 10 h before each drug administration.

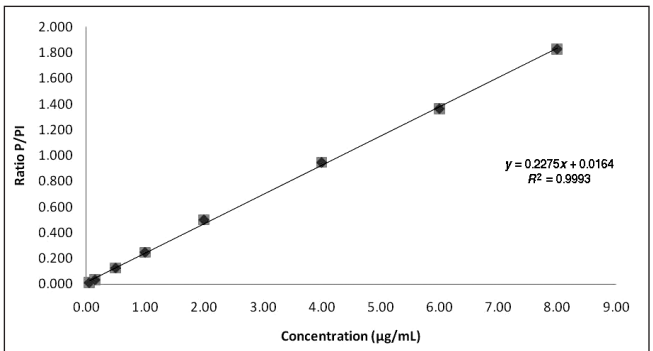

Figure 1. Calibration curve for the analytical method for metronidazole quantification in human plasma in the range of 0.05 µg/mL to 8 µg/mL 246 × 150 mm.

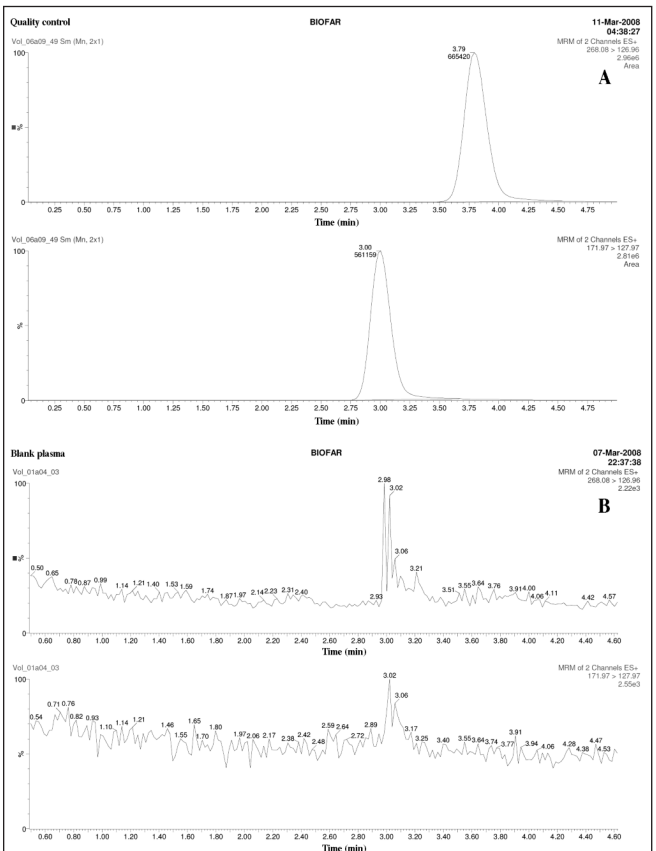

Figure 2. Representative chromatogram of blank plasma (A); Representative chromatogram of the medium quality control (QC) sample (3,00 6 g/mL) containg containg metronidazole and zidovudine (IS) 133 × 171mm (B).

A single dose (250 mg metronidazole) consisting of reference or test according to the randomization plan was given to each subject in a fasting state for each treatment period. Fasting continued for a further 4 h after drug administration. The drug was administered with 200 mL of water. Subjects were provide with standard meals 4 h (lunch), 7 h (snack), and 10 h (dinner) after drug administration in each treatment.

Eight milliliters of heparinized venous blood samples were collected by means of an indwelling venous canula of the cubital vein on profile days according to the time schedule, which included a sample just prior to dosing and then at 0.33, 0.67, 1.0, 1.33, 1.67, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12.0, 24.0, 36.0, 48.0 h after drug administration. Any deviation from the stated sampling times was recorded. Plasma was immediately separated by centrifugation at 3500 rpm for 15 min, then was transferred to properly labeled tubes, and stored at –20ºC until the HPLC analysis.

Results and Discussion

Bionalytical method validation

The HPLC–MS–MS method for metronidazole quantification was linear in the range of 0.05–8 mg/mL as shown in Figure 1. The method showed specificity because metronidazole and zidovudine were well-resolved and no interfering peaks from endogenous components of normal, hemolised, and lipemic plasma were observed as it appears in Figure 2. Retention times were 3.00 min for metronidazole and 3.81 min for zidovudine.

Accuracy was between 97.95%–103.85%, and precision, measured in RSD%, was always lower than 10.65%, depicting the high precision of the method (Table I). The mean recovery was 82.09% for metronidazole and 82.00% for IS (Table II). The processed samples were considered stable at room temperature at least for 48 h as shown in Table III. Metronidazole concentrations were not altered after three freeze-thaw cycles (Table IV) and plasma samples were stable for at least 46 days when stored at –20°C (Table V).

Table I. Precision and Accuracy Intra-assay and Inter-assay of the Method Metronidazole in Human Plasma (*n* = 6)

Metronidazole conc. (µg/mL)	Precision (%)		Accuracy (%)	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
0.15	7.20	10.65	103.39	97.85
3.00	3.25	6.10	103.85	99.94
7.00	1.84	6.04	101.16	98.47

Table II. Recovery of Metronidazole and Zidovudine (IS) After the Extraction Procedure (*n* = 6)

Metronidazole conc. (µg/mL)	% Recovery	
	Metronidazole	IS
0.15	89.04	88.91
3.00	74.04	77.72
7.00	83.20	79.36
Mean	82.09	82.00
SD	7.56	6.04
RSD%	9.21	7.37

Application

The validated method has been successfully used to quantify metronidazole concentrations in human plasma samples after the administration of two products in a bioequivalence study. Representative metronidazole plasmatic concentration versus time profile for 22 healthy volunteers after administration of a single dose of metronidazole test and reference product is presented in Figure 3.

Conclusion

In conclusion, a simple, sensitive, precise, and accurate reversed-phase LC–MS–MS method has been described for the quantification of metronidazole in human plasma. It was successfully applied to a bioequivalence study of two drug products containing metronidazole.

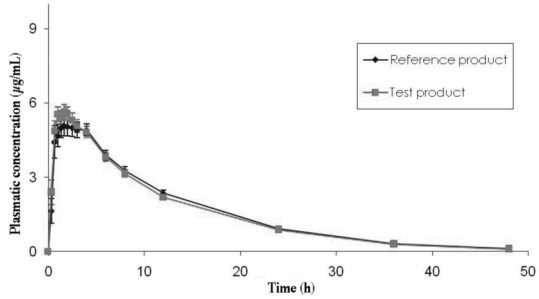

Figure 3. Plasma concentration-time profile of a study with 22 healthy volunteers after oral single dose administration of metronidazole 250 mg 245 × 142 mm.

Table III. Metronidazole Stability of Processed Plasma Samples for 48 h (*n* = 3)

	Plasma samples (fresh)			Plasma samples (after 48 h)			
Metronidazole conc. (µg/mL)	Conc. (µg/mL)	Precision (%)	Accuracy (%)	Conc. (µg/mL)	Precision (%)	Accuracy (%)	RSD (%)
0.15	0.14	5.05	93.71	0.15	6.42	99.11	5.76
3.00	3.44	3.28	98.20	3.52	1.44	100.58	2.42
7.00	6.92	0.78	98.86	6.55	0.43	93.55	–5.38

Table IV. Metronidazole Stability in Human Plasma After 3 Freeze–Thaw Cycles (*n* = 3)

	Plasma samples (fresh)			Plasma samples (after three F–T cycles)			
Metronidazole conc. (µg/mL)	Conc. (µg/mL)	Precision (%)	Accuracy (%)	Conc. (µg/mL)	Precision (%)	Accuracy (%)	RSD (%)
0.15	0.14	5.05	93.71	0.14	3.49	93.89	0.19
3.00	3.44	3.28	98.20	3.45	2.10	98.70	0.50
7.00	6.92	0.78	98.86	6.09	2.93	86.93	–12.07

Table V. Metronidazole Stability in Human Plasma Stored at –20°C for 46 days (*n* = 3)

	Plasma samples (fresh)			Plasma samples (after 46 days at –20°C)			
Metronidazole conc. (µg/mL)	Conc. (µg/mL)	Precision (%)	Accuracy (%)	Conc. (µg/mL)	Precision (%)	Accuracy (%)	RSD (%)
0.15	0.14	5.05	93.71	0.14	4.34	91.10	–2.79
3.00	3.44	3.28	98.20	3.75	5.51	107.15	9.12
7.00	6.92	0.78	98.86	7.23	2.37	103.33	4.51

Acknowledgements

Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

1. C. Sagan, A. Salvador, D. Dubreuil, P.P. Poulet, D.Duffaut and I. Brumpt. Simultaneous determination of metronidazole and spiramycin I in human plasma, saliva and gingival crevicular fulid by LC–MS/MS. *J. Pharm. Biomed. Anal.* **38:** 298–306 (2005).
2. A. Menelau, A.A. Somogyi, M.L. Barclay, and F. Bochner. Simultaneous quantification of amoxicillin and metronidazole in plasma using high-performance liquid chromatography with photodiode array detection. *J. Chromatogr. B* **731:** 261–266 (1999).
3. C. Hoffmann, N. Focke, G. Franke, M. Zschiesche, and W. Siegmund. Comparative bioavailability of metronidazole formulations (Vagimid) after oral and vaginal administration. *Int. J. Clin. Pharmacol. Ther.* **33:** 232–239 (1995).
4. P.T. Pollak. A liquid chromatography assay for the study of serum and gastric juice metronidazole concentrations in the treatment of Helicobacter pylori. *Ther. Drug Monitoring.* **18:** 678–687 (1996).
5. J.I.D. Wibawa, P.N. Shaw, and D.A. Barett. Quantification of metronidazole in small-volume biological samples using narrow-bore high-performance liquid chromatography. *J. Chromatogr. B* **761:** 213–219 (2001).
6. P.K.F. Yeung, R. Little, Y.Q. Jiang, S.J. Buckley, P.T. Pollak, H. Kapoor, and S.J.O. Veldhuyzen van Zanten. A simple high performance liquid chromatography assay for simultaneous determination of omprazole and metronidazole in human plasma and gastric fluid. *J. Pharm. Biomed. Anal.* **17:** 1393–1398 (1998).
7. J.G. Hardman; L.E. Limbird, and A.G. Gilman. As bases farmacológicas da terapêutica. 10 ed. Rio de Janeiro, RJ, 2001, pp. 1506.
8. K.K. Midha, I.J. McGilveray and J.K. Cooper. Determination of therapeutic levels of metronidazole in plasma by gas-liquid Chromatography. *J. Chromatogr.* **87:** 491–497(1973).
9. T.G. Nascimento, E.J. Oliveira, and R.O. Macedo. Simultaneous determination of ranitine and metronidazole in human plasma using high performance liquid chromatography with diode array detection. *J. Pharm. Biomed. Anal.* **37:** 777–783 (2005).
10. N.M. Idkaidek and N.M. Najib. Enhancement of oral absorption of metronidazole suspensions in humans. *Eur. J. Pharm. Ther.* **50:** 213–216 (2000).
11. S. Fraselle, V. Derop, J-M. Degroodt, and J.V. Loco. Validation of a method for detection and confirmation of nitroimidazoles and the corresponding hydroxyl metabolites in pig plasma by high performace liquid-chromatography–tandem mass spectrometry. *Anal. Chim. Acta.* **586:** 383–393 (2007).
12. V. Porta, S.G. Schramm, E.K. Kano, E.E. Koono, Y.P. Armando, K. Fukuda, and C.H.R. Serra. HPLC-UV determination of metformin in human plasma for application in pharmacokinetics and bioequivalence studies. *J. Pharm. Biomed. Anal.* **46:** 143–147 (2008).
13. Y.P. Armando, S.G. Schramm, M.F. Silva, E.K. Kano, E.E.M. Koono, V. Porta, and C.H.R. Serra. Bioequivalence assay between orally disintegrating and conventional tablet formulations in healthy volunteers. *Int. J. Pharm.* **366:** 149–153 (2009).
14. M.J. Galmier, A.M. Frasey, M. Bastide, E. Beyssac, J. Petit, J.M. Aiache, and C. Lartigue-Mattei. Simple and sensitive method for determination of metroni-dazole in human serum by high-performance liquid chromatography. *J. Chromatogr. B* **720:** 239–243 (1998).
15. M.J. Jessa, D.A. Barrett, P.N. Shaw, and R.C. Spiller. Rapid and selective high-performance liquid chromatographic method for the determination of metronidazole and its active metabolite in human plasma, saliva and gastric juice. *J. Chromatogr. B* **677:** 374–379 (1996).
16. J. Spénard, C. Aumais, J. Massicotte, J.S. Brunet, C. Tremblay, M. Grace, and M. Lefebvre. Effects of food and formulation on the relative bioavailability of bismuth biskalcitrate, metronidazole, and tetracycline given for Helicobacter pylori eradication. *British J. Clin. Pharm.* **60(4):** 374–377 (2005).

Manuscript received November 2, 2008;
Revision received April 15, 2009.