Tropical Journal of Pharmaceutical Research October 2012; 11 (5): 799-805

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v11i5.14

Research Article

New Analytical Method for the Determination of Metronidazole in Human Plasma: Application to Bioequivalence Study

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Abstract

Purpose: *To develop new sensitive, accurate, rapid and reproducible high performance liquid chromatography (HPLC) method to determine metronidazole levels in human plasma and to apply the method in a bioequivalence study.*

Methods: *Metronidazole was extracted from human plasma through one step of protein precipitation by methanol using carbamazepine as internal standard (IS). After centrifugation of the plasma sample, the supernatant layer was separated and injected into HPLC system using Eclipse XDB-phenyl column. The mobile phase consisted of phosphate buffer (pH 4.5): acetonitrile (95:5, v/v). The UV detector was set at 320 nm. The bioavailability of the test metronidazole product (Brand A) was compared to a commercial metronidazole brand as reference product in 24 healthy volunteers who received a single dose equivalent to 500 mg of the test and reference products in a randomized balanced two-way cross-over design separated by two-week wash-out period.*

Results: *Mean standard calibration curves of metronidazole over the concentration range of 0.05 – 30 µg/ml were linear. No significant differences were found based on analysis of variance of the pharmacokinetics parameters required for the assessment of bioequivalence of test and reference formulations. The mean value and 90 %CI of test/reference ratios for the derived parameters were: C_{max}, 9.64 vs. 8.38 (0.93 – 1.10), AUC_{0-24}, 124.6 vs.122.3 µg.h/mL (0.973 – 1.051) and $AUC_{0-\infty}$, 140.9 vs. 128.4 h/mL (1.15 – 1.23).*

Conclusion: *The test metronidazole product was bioequivalent to the reference. The method is suitable for bioequivalence and pharmacokinetic studies in humans with a low limit of quantification of 0.05 µg/ml.*

Keywords: *Metronidazole, Bioequivalence, HPLC, Pharmacokinetics, Human plasma*

Received: 17 December 2011 *Revised accepted: 6 July 2012*

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INTRODUCTION

Metronidazole is a prototype of the nitroimidazole class of antimicrobials, it has

been evaluated in the treatment of diverse anaerobic and gastrointestinal tract infections. Moreovere, metronidazole has often been studied for antibacterial activity against gram-negative aerobes and some gram-positive bacteria, including *Bacteriodes fragilis* that produces β-lactamases [1]. In healthy humans, metronidazole is absorbed rapidly and completely from the gastrointestinal tract and is metabolized in the liver by an oxidative pathway [2]. Liver is the main site of metabolism by side chain oxidation and glucuronide conjugation. A major portion of the dose of the drug is excreted in urine, largely as metabolites [3].
Metronidazole is one of the most widely used antibacterial compounds in the treatment of some types of periodontal disease such as aggressive periodontitis. Several methods have been reported for the measurement of metronidazole in biological fluids using mass-spectrometry [4-6] or high performance liquid chromatography [7-10].

The purpose of this study was to develop a rapid, low-cost method for extraction of metronidazole from plasma and apply it to a simple, accurate and sensitive chromato-graphic determination of metronidazole bioavaialbility of generic and reference products of the drug..

EXPERIMENTAL

Chemicals

Sodium acetate (ADWIC, Egypt), glacial acetic acid, phosphoric acid, acetonitrile and methanol were obtained from Lab-Scan, Ireland. These reagents were of analytical grade except acetonitrile which was of HPLC grade. Metronidazole and the internal standard (carbamazepine) were obtained from Sigma (UK), The test product was metronidazole 125 mg/5 ml suspension (Brand A) while the reference product was Flagyl® 125 mg/5 ml suspension (Aventis Pharma).

Sample preparation

A stock solution of metronidazole (1 mg/ml) and solution of carbamazepine as internal standard (200 µg/ml) were prepared in acetonitrile. Serial dilutions of the metronidazole standards (0.05 - 30 µg/ml) were prepared in acetonitrile as well as spiked in plasma. All the solutions were stored at 4 °C. Metronidazole extraction involved protein precepitation by methanol. Methanol (500 µl) was added to a glass tube containing 200 µl of plasma sample and 25 µl of internal standard (I.S.). After mixing for 10 s on a vortex mixer and centrifugation for 10 min at 3500 rpm at 4 °C, 50 µl of the supernatant was injected directly into the HPLC instrument.

Chromatographic system

The high pressure liquid chromatography (HPLC) apparatus used comprised of Waters pump controlled by Waters 610 controller, Waters 717 autosampler injector and Waters 486 UV detector. Separation was performed using an analytical Eclipse XDB-phenyl (250 x 4.6 mm), 5 µm particle size column (Agilent). For data acquisition and integration, Waters millennium software operated by Pentium III (450 MHz) processor (Compaq, UK) was used.

The mobile phase consisted of 0.05M sodium acetate: acetonitrile: glacial acetic acid (75:25:1, v/v/v) with the pH adjusted to 4.0 with phosphoric acid.

Method validation

Specificity and selectivity

The HPLC assay validation was carried out according to guidelines recommended by United States Food and Drug Administration (2001) [11]. The specificity and selectivity of the assay were investigated by comparing the retention times of metronidazole detected in the assay of drug-free plasma with those from injection of drug extracted from spiked plasma.

Limit of detection

Calibration curves were constructed using series of standard metronidazole in the range of 0.05- 30 µg/ml. The lower limit of detection was defined as the concentration at which the signal is to-noise ratio was more than 3 times in response (peak area). Similarly, the lower limit of quantification was defined as the concentration at which the signal-to-noise ratio was not less than 5 times in response (peak area). Both parameters were calculated using the calibration curve containing the least concentration of analyte.

Recovery

Extraction recovery was assessed by comparing the response for the same concentration of metronidazole dissolved in acetonitrile and injected directly to HPLC with those injected after extraction from spiked plasma using the extraction method indicated above.

Accuracy, percision and linearity

Intra-day precision and accuracy were assessed by analyzing six replicates of plasma standard calibration curves during a single day. Mean, standard deviation and coefficient of variation values were calculated for each concentration of the standard calibration curves. The accuracy of the estimates for each concentration was determined as the ratio of the mean concentration found after analysis to the nominal theoretical concentration, and expressed as a percentage. Inter-day precision and accuracy were evaluated for 18 plasma standard calibration curves run on 3 separate days (6 assays/day). This was expressed as coefficient of variation.
Moreover, seeded control duplicate samples (0.5, 1.5, 3.0) µg/ml were included in every analytical run of volunteers samples and injected just before and after each of these samples

Stability study

The stability of metronidazole in plasma was evaluated as short term stability or bench top stability (for 6 hour at room temperature), long-term stability (6 weeks at -80 ºC) and freeze-thaw stability (Three cycles). For thaw and freeze stability, replicates (n=6) of the low-QC and high-QC samples were analyzed after 3 freeze/thaw cycles. Samples were stored at approximately -80°C for at least 12 hours between thaw cycles and thawed under ambient conditions and mixed gently. On the day of preparation and after the three freeze/thaw cycles the samples were analyzed against a standard curve and a blank.

The acceptance criteria for the validated method were based on precision, accuracy, sensitivity, specificity and stability. An upper and lower limit of 10 % of the original concentration was established as acceptance.

Bioequivalence study

Subjects

Twenty four healthy adult male volunteers were included in this study. Their ages ranged from 21 to 43 years and their weight between 62 and 87 kg. On the basis of medical history, clinical examination and laboratory investigations (virus scanning, haematology, blood biochemistry and urine analysis), none of the participants revealed any medical abnormality. In addition, the subjects had no history of hospitalization or involvement in any clinical trials within the last 12 weeks. Informed written consent was obtained from the volunteers and the protocol of the study was approved by the Ethical Committee for Bioavailability Studies at National Organization for Drug Control and Research, Egypt. (approved no. 0509). The study was conducted in accordance with Good Clinical Practice guidelines and the Revised Declaration of Helsinki [12].

Drug administration and blood sampling

Each subject received the two products on two treatment days, separated by a two-week wash-out period. Each volunteer's blood (5 ml) was withdrawn via an indwelling cannula in the forearm at 0.0 (pre-dose), 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0 and 24 hours. The cannula was kept clean of blood by flushing with heparinized saline after each sample collection. About 0.2 ml of blood was discarded from the cannula before each sample. The blood samples were transferred to the heparinized tubes and centrifuged for 5 min at 3000 rpm. Plasma was separated with polypropylene disposable tips, transferred to Eppendorf tubes and then stored in a laboratory deep freezer at -80 °C for sebsequent determination of metronidazole plasma levels as described above.

Pharmacokinetic analysis

Pharmacokinetic parameters of metronidazole were obtained using standard non-compartmental methods. The maximal plasma concentration (C_{max}) and the time to the peak plasma concentration (t_{max}) of metronidazole were derived directly from the data while the area under the plasma concentration-time curve (AUC) was calculated by the linear trapezoidal rule. The pharmacokinetic calculations were performed using the software, Kinetica™ 2000 (ThermoFisher Scientific, US).

Statistical analysis

All results were expressed as mean ± SD. Coefficient of variation (CV) for the obtained pharmacokinetic parameters as well as statistical analysis were performed by two-way analysis of variance (ANOVA). AUC_{0-24}, $AUC_{0-\infty}$ and C_{max} values were logarithmically transformed prior to data analysis. On the other hand, the t_{max} values of the two preparations were analyzed using Wilcoxon Signed Rank Test for paired samples. For the parametric analysis of bioequivalence for log-transformed data, the acceptance ranges were set at 80.0 - 125.0 % for both AUC_{0-t} and C_{max}. Statistical analysis was performed using Kinetica™ 2000 (Thermo Scientific, USA).

RESULTS

Selectivity and specificity

The current method showed excellent chromatographic specificity with no endogenous plasma interference at the retention times of metronidazole and carbamazepine (IS). Metronidazole and carbamazepine were well resolved with retention times of 4.06 and 11.52 min, respectively.

Linearity

The calibration curve was linear over the range of 0.05 – 30.0 µg/ml in human plasma. The regression coefficient of the curve, constructed from the mean standard curves analyzed in intra-day precision study (n = 3), was 0.999 and 0.998 for metronidazole dissolved in acetonitrile and that extracted from spiked plasma, respectively (Fig. 1). Low limit of detection (LLOQ) was 0.05 µg/ml and was at a signal to noise ratio of 5 times.

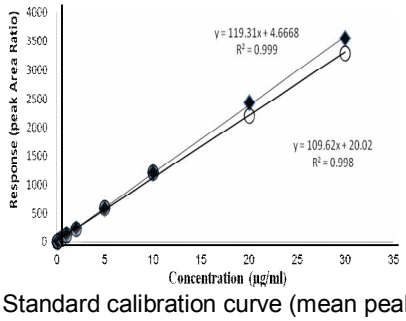

Fig 1: Standard calibration curve (mean peak area vs. concentration in µg/ml) for metronidazole dissolved in mobile phase (♦) **and** extracted from plasma (o)

Table 1: Intra-day and inter-day reproducibility of standard plasma calibration curve of metronidazole obtained by HPLC

Theoretical conc. (µg/ml)	Intra-day			Inter-day		
	Mean ± SD	Accuracy (%)	CV%	Mean ± SD	Accuracy (%)	CV%
0.05	0.053 ± 0.002	106.00	3.77	0.054 ± 0.005	108.00	9.26
0.10	0.104 ± 0.001	104.00	0.96	0.104 ± 0.004	104.42	3.85
0.50	0.532 ± 0.011	106.40	2.07	0.532 ± 0.014	106.15	2.63
1.00	1.0193 ± 0.039	101.93	3.83	1.013 ± 0.031	101.28	3.60
2.00	2.0085 ± 0.075	100.43	3.73	1.999 ± 0.078	99.96	3.90
5.00	4.982 ± 0.237	99.64	4.76	4.997 ± 0.091	99.94	8.21
10.00	9.951 ± 0.237	99.51	2.38	9.921 ± 0.165	99.22	1.66
20.00	19.970 ± 0.40	99.85	2.00	20.110 ± 0.285	100.55	1.42
30.00	29.947 ± 0.182	99.82	3.77	30.103 ± 0.239	100.34	0.79

Table 2: Peak area of metronidazole dissolved in mobile phase and extracted from plasma

Conc. (µg/ml)	Mobile phase *			Plasma **			Recovery (%) ***
	Mean	*SD*	*CV%*	*Mean*	*SD*	*CV%*	
0.05	8386.17	404.78	4.827	6308.33	525.04	8.323	75.223
0.1	13001.67	434.83	3.344	9422.47	915.53	9.716	72.471
0.50	62761.83	1429.22	2.277	55310.3	1716.25	3.103	88.127
1.00	127121.33	4155.65	3.269	115341	6329.06	5.487	90.733
2.00	238267.17	7843.43	3.292	224694	11827.4	5.264	94.304
5.00	586335.50	24933.06	4.252	602883	44043.2	7.305	102.822
10.00	1210552.00	56451.08	4.663	1207019	106465	8.821	99.708
20.00	2429618.00	95142.00	3.916	2205425	204503	9.278	90.773
30.00	3556699.83	38368.06	1.079	3278891	308711	9.415	92.189

** Metronidazole dissolved in mobile phase injected directly; ** Metronidazole spiked in plasma injected after liquid-liquid extraction; *** Absolute Recovery = 89.6*

The mean of coefficient of variation (CV%) data are presented in Table 1.

Accuracy, precision, recovery and quality control

The assay showed good intra-day and inter-day precision, and accuracy over the entire calibration range. The precision of the developed method (CV %) varied from 0.96 to 4.76 % and from 0.79 to 9.26 % for intra-assay and inter-assay respectively. The mean accuracy of the developed method ranged between 99.51 to 106.40% for intra-assay and from 99.22 to 108.00% inter-assay, (Table 1). The absolute recovery of metronidazole was 89.6 % (Table 2). The variation between runs (CV %) for the quality control samples was < 10 %.

The mean plasma drug concentration vs. time curves following oral administration of metronidazole suspension are presented in Figure 2 and Table 3. Maximum plasma concentrations of metronidazole was 9.64 ± 2.61 µg/ml and 8.38 ± 2.52 µg/ml for test and reference products, respectively, while the time to reach these maximum concentrations was about 2.75 and 3.0 h, respectively. Total area under the curve ($AUC_{0-\infty}$) was 140.9 ± 50.7 and 128.4 ± 29.7 µg.h/ml for test and reference tablets, respectively, while mean residence time was 16.3 ± 7.6 and 14.1 ± 2.7 h, respectively. Half-life ($t_{1/2}$) was 12.45 ± 5.36 and 10.89 ± 1.95 for test and reference

Table 3: Pharmacokinetic parameters of metronidazole in healthy volunteers

Parameter	Test	Reference	90% CI

	Mean	**Range**	**Mean**	**Range**	
C_{max} (µg/ml)	9.64 ± 2.61	5.77 -19.09	8.38 ± 2.52	4.22 - 12.94	0.927 – 1.100
t_{max} (h)	2.75 ± 1.06	2.00 - 6.00	3.00 ± 1.17	2.0 - 8.0	
AUC_{0-24} (µg.h/ml)	124.6 ± 32.35	59.5 – 186.6	122.3 ± 28.8	78.2 – 179.5	0.973 – 1.051
AUC_{0-inf} (µg.h/ml)	140.9 ± 50.67	82.0 – 300.7	128.4 ± 29.65	79.6 – 189.0	1.147 – 1.233
$t_{1/2}$ (h)	12.45 ± 5.36	6.74 – 30.56	10.89 ± 1.95	6.64 – 14.68	1.234 – 1.425
MRT (h)	16.33 ± 7.64	9.99 – 44.37	14.05 ± 2.68	8.31 – 17.83	0.429 – 1.036

products, respectively. There was no significant difference between the test and reference products in respect of the pharmacokinetic parameters required for assessment of drug bioequivalence (C_{max}, AUC and t_{max}), and this was within the bioequivalence acceptance range of 0.80 - 1.25 for log transformed data.

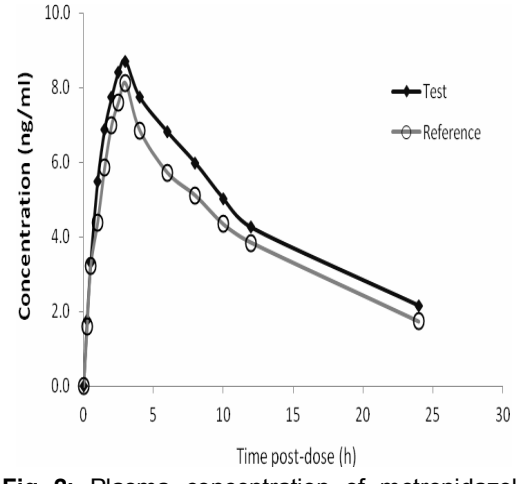

Fig 2: Plasma concentration of metronidazole following administration of test metronidazole uspension and reference product (Flagyl®) to healthy volunteers.

DISCUSSION

A simple, sensitive, cost-effective, and reliable HPLC method for determination of metronidazole in human plasma over the concentration range 0.05–30 µg/mL has been developed and validated. Several methods for the determination of metronidazole in biological fluids has previously been published. Some of these methods required the use of relatively large volume of plasma or extraction solvent [7,8]. Other more complicated extraction procedures, such as solid-phase extraction, have also been reported [5,13]. Solid-phase extraction procedures require solid-phase extraction cartridges, which increase the cost of the analysis. Some recent studies reported the determination of metronidazole by LC-MS-MS detection (4,6] but this method is not economical for routine use in pharmaco-kinetic studies since numerous samples must be analysed.

The method applied here was accurate, precise, specific, sensitive and capable of determining concentrations of metronidazole in a small volume of human plasma (200 µl) and entails reduced utilization of organic solvent. This assay method was also rapid (one step only), and the preparation of 96 samples took less than 90 min from the initial extraction step to final placement of samples in the HPLC auto-sampler vials. Morover, the protein precipitation was carried out using methanol rather than the perchloric acid used in another method [8]; perchloric acid an affect the life span of the separation column.

CONCLUSION

We can conclude that metronidazole 125 mg/5 ml suspension (Brand A) is bioequivalent to the commercial standard, Flagyl® 125mg/5ml suspension (Aventis Pharma). The present method was useful for the determination of the bioequivalence of metronidazole products, and would,also be useful for routine drug monitoring and other bioavailability studies with the desired precision and accuracy along with high-throughput.

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