A RAPID AND SENSITIVE HPLC METHOD FOR THE ANALYSIS OF METRONIDAZOLE IN HUMAN PLASMA: APPLICATION TO SINGLE DOSE PHARMACOKINETIC AND BIOEQUIVALENCE STUDIES

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ABSTRACT

A sensitive, accurate and rapid reverse phase HPLC method was developed to quantitate plasma levels of metronidazole in order to conduct a comparative bioavailability studies. The drug and internal standard were added to plasma samples, vortexed and then zinc sulfate solution was added in order to precipitate the plasma proteins. Samples were centrifuged at 3000 rpm for 10 min. The supernatant layer was separated and analyzed on a phenyl (300 × 4.6mm) column, with 5% acetonitrile in 0.1 M KH_2PO_4 buffer (pH = 4.5) at 324 nm. The standard curve covering 0.15 – 30 µg/ml concentration range, was linear (r^2 = 0.9999), relative errors were within 2.48 to 9.15 % and the CV% ranged from 2.999 to 10.796. The method is suitable for bioavailability, pharmacokinetic, and bioequivalent studies in human.

The in-vivo study was carried out in 12 healthy volunteers according to a single dose, two-sequence, cross over randomized design. The bioavailability was compared using the total area under the plasma level versus time curve ($AUC_{0\text{-}48}$, $AUC_{0\text{-}\infty}$), peak plasma concentration (C_{max}) and time to C_{max} (T_{max}). No statistically significant difference was found between the $AUC_{0\text{-}\infty}$, C_{max} and T_{max} values of the test and reference, Flagyl® ($p > 0.05$). The 90% CI for the ratio of the $AUC_{0\text{-}\infty}$ (0.94-1.07) and C_{max} (0.88-1.03) and the logarithmically transformed $AUC_{0\text{-}\infty}$ (0.99-1.01) and C_{max} (0.94-1.01) values of the generic product over those of Flagyl® was calculated to be within the acceptable limit of 0.80-1.20 and 0.80-1.25, respectively. It was, therefore, concluded that the generic metronidazole was bioequivalent with the innovator formulation.

Keywords: Metronidazole, HPLC, Pharmacokinetics, Bioavailability.

INTRODUCTION

Metronidazole [2 –(2- methyl-5- nitroimidazole-1-yl)ethanol, Fig.1] is an antimicrobial drug that is used to treat protzoal and anaerobic bacterial infections (1).

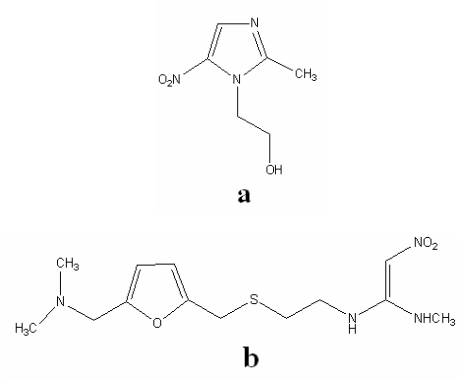

Figure 1. Chemical structure of metronidazole (a) and internal standard, ranitidine (b).

Metronidazole is readily and completely absorbed from the gastro-intestinal tract with the peak levels occurring at 1-2 hours after oral administration (2-4). It is widely distributed, appearing in most of the body tissues and fluids and is less than 20% bound to plasma proteins (1, 5-7). Metronidazole is metabolized in the liver by the side chain oxidation and glucuronide conjugation (3). The hydroxyl metabolite possesses 70% less activity than that of parent compound (8). Plasma half-life of metronidazole is 8 hours and longer in neonates and patients with renal failure (4, 7, 9-11). The majority of the dose of the drug is excreted in the urine mainly as metabolite and small amount appears in feces (1).

Bioavailability issues have been an increasing concern to drug regulatory authorities for assessment of the safety and efficacy of drug products (12). As the number of synonym drug products increase special attention in bioavailability issues becomes a major concern. Local drug regulatory authorities have therefore, issued guidelines to ensure adequate bioavailability studies in new drug applications for synonym drugs (13). The main purpose of the

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present study was to compare the relative bioavailability of generic metronidazole tablet preparation, manufactured by Amin, an Iranian pharmaceutical company, with that of the innovator Flagyl® (Milano-Pharmacia Upjohn). To achieve this goal a suitable high-performance liquid chromatography (HPLC) method for determination of metronidazole levels in plasma was required. Thus far, a large number of HPLC methods have been described to analyze metronidazole in body fluids (14-23). Nevertheless, these methods utilize large volume of matrices (14-17), involving organic extraction (17, 18) or solid phase extraction (19-21), which renders the methods highly sensitive for detection of drug in human saliva, gastric juice (16, 22) and gastric tissue samples (18) but unnecessary for bioequivalence and pharmacokinetic studies. Furthermore, these methods require a number of procedures for sample preparation such as liquid-liquid and solid phase extraction which complicate them. Some other HPLC methods which involve no organic extraction or do not incorporate internal standard might be simple and rapid but suffer from insufficient sensitivity to measure metronidazole concentration in plasma longer than 2 or 3 half-lives (16, 22, 23). So, a further aim of this study was to establish a simple, sensitive, rapid, reliable and specific HPLC assay for quantitation of metronidazole concentrations in plasma.

MATERIALS AND METHODS

Reagents and Solutions

Metronidazole was obtained from Sigma (UK), the internal standard, ranitidine was from Glaxo, Inc. (UK), hydroxychloroquine was from Sanofi Winthrop Pharmaceuticals (New York, NY), acetonitrile was from Fluka (Germany), methanol was from Aldrich (USA), hydrochloric acid and zinc sulfate were from Merck (Germany). All reagents and solutions were either HPLC or analytical grades. Metronidazole 250-mg tablets (Batch No: 555) were from Amin pharmaceutical company, Iran and Flagyl® 250-mg tablets (Batch No: 8006) was from Milano-Pharmacia Upjohn.

In-vitro Studies

Weight variation, content uniformity, assay and dissolution studies were all carried out according to USP XXV procedures (24). Samples were assayed by UV spectrophotometer at 278 nm.

Chromatographic Conditions

A reversed phase HPLC method was developed to quantitate plasma levels of metronidazole. The apparatus was a Shimadzu HPLC system model C-R6A (Japan), consisting of a model LC-6A intelligent solvent delivering pump, a computerized system controller, and a SPD-6AV UV detector. Chromatographic separation was performed using a µBondapak phenyl (300 × 3.9mm, Waters, Ireland) column. The mobile phase consisted of 5% acetonitrile in 0.1 M of KH_2PO_4 buffer (pH = 4.5). The aqueous phase was eluted at a flow rate of 1.5 ml/min and effluent was monitored at 324 nm. Quantitation was achieved by measurement of the peak area ratios of the drug to the internal standard.

Sample Preparation

To 200µl of plasma in a 10 ml test tube, was added 20µl of internal standard solution (250 µg/ml) and then the tube was vortexed. For protein precipitation 50µl of 10% zinc sulfate was added, samples were vortexed, placed in refrigerator for 15 min and centrifuged at 3000 rpm for 10 min. Supernatant layer was separated of which 50µl was injected onto the column and peak areas were recorded.

Calibration Procedure

A stock solution of metronidazole was prepared by dissolving 100 mg in 100 ml of methanol. This solution was used to prepare working standard solutions daily for different concentration between 1.5 and 300 µg/ml by dilution in deionized water. Stock solution of ranitidine (250 µg/ml) was also prepared in deionized distilled water. Calibration samples of metronidazole were prepared in blank plasma. To separate 180µl of blank plasma, were added first 20µl of metronidazole standard solutions at concentrations of 1.5, 15, 75, 150, 225, and 300 µg/ml and then 20µl of internal standard at fixed concentration of 250 µg/ml to obtain metronidazole standard concentrations ranging from 0.15 to 30 µg/ml. After precipitation of proteins of all calibration samples 50 µl of supernatant layer were injected onto the column. The calibration curve was obtained by plotting peak ratios of metronidazole to internal standard versus metronidazole concentrations. Plasma drug concentrations in samples were calculated by determination of the peak area ratio of metronidazole to internal standard and comparing the ratio with those of the standard curve which was obtained after analysis of calibration samples.

Within-day Variability

The within-day variability of the assay was determined by repeated analysis of samples for quality control at concentration of 0.15 to 30 µg /ml on the same day.

Between-day Variability

The between-day variability of the assay was determined by repeated analysis of samples for

quality control at concentrations of 0.15 to 30 µg /ml on 3 consecutive days.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

The parameters LOD and LOQ were determined using the signal-to-noise ratio by comparing test results from samples with known concentrations of analyte to blank samples. The analyte concentration that produced a signal-to-noise ratio of 3:1 was accepted as the LOD. LOQ was identified as the concentration that produced a signal-to-noise ratio of 10:1.

In-vivo Study Design

The ethics committee on human studies of the Isfahan University of Medical Sciences approved the study. Twelve healthy adult male volunteers aged between 21 to 24 years and weighing from 63 to 83 kg participated in the study. On the basis of medical history, clinical examinations and laboratory tests including hematology, blood biochemistry and urine analysis, no subject had a history of evidence of hepatic, renal, gastro intestinal or hematological deviations, or any acute or chronic disease or drug allergy. The subjects were instructed to abstain taking any medication at least 2 weeks prior to and during the study period. Informed consent was obtained from the subjects after explaining the nature and purpose of the study. The protocol which was used was the conventional, two way, crossover study with six subjects in each of the treatment group. In the first trial period, after an overnight fasting, subjects were given a single dose of two 250-mg tablets of either formulation (Reference or Test product) in a randomized fashion with 200 ml of water. Food and drinks (other than water, which was allowed after 2h) were not allowed for 4 hours after dosing to all volunteers. Approximately 2 ml blood samples were drawn into heparinized tubes through an indwelling canola before (0 hr) and at 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12, 24, 36 and 48 h after dosing. The blood samples were centrifuged at 3000 rpm for 15 min, plasma samples were separated and kept frozen at -20^0C in coded glass tubes.

Pharmacokinetic Analysis

The elimination rate constant (k_E) was obtained from the least square fitted terminal log-linear portion of the plasma concentration-time profile. The area under the curve to the last measurable concentration (AUC_{0-t}) was estimated by the linear trapezoidal rule. The area under the curve extrapolated to infinity ($AUC_{0-\infty}$) was calculated by equation of $AUC_{0-t} + C_t / k_E$ where C_t is the last measurable concentration. The peak plasma concentration (C_{max}) and time to peak (t_{max}) were determined by inspection of the individual drug plasma concentration-time profiles.

Statistical Analysis

For the purpose of bioequivalence analysis, AUC_{0-t} , $AUC_{0-\infty}$, and C_{max} were considered as primary variables. Two-way ANOVA for crossover design was used to assess the effect of formulations, periods, sequences, and subjects on these parameters. A difference between two related parameters was considered statistically significant for a P-value equal to or less than 0.05. The 90% confidence intervals of the ratio of pharmacokinetic parameters of test to reference products as well as those which were transformed logarithmically were also estimated (25-27). All statistical analyses were performed by using SPSS 10.

RESULTS AND DISCUSSION

In vitro studies

All products met the pharmacopoeia specifications for weight variation, assay, and content uniformity. Dissolution profiles of two formulations which were studied are shown in figure 2. The dissolution test revealed that after 60 minutes 95.26 ± 1.73 of stated metronidazole was released from Flagyl® tablets and 96.02 ± 2.15 from Amin tablets, respectively (n = 6). Therefore, either formulations met the USP dissolution specifications (17) stating that not less than 85% of drug content should be released within 60 minutes.

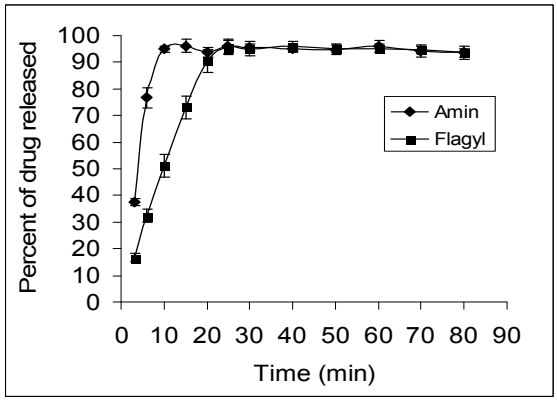

Figure 2. Dissolution rate profiles of Flagyl® (reference) and Amin (test) products.

HPLC assay

Fig.3 shows some chromatograms of blank human plasma (a), blank plasma spiked with ranitidine as internal standard (b), the lowest (0.15 µg/ml) (c) and the highest (30 µg/ml) (d) metronidazole concentration in plasma, and concentration of metronidazole in plasma of a healthy subject 8 hrs after ingestion of metronidazole tablets (e).

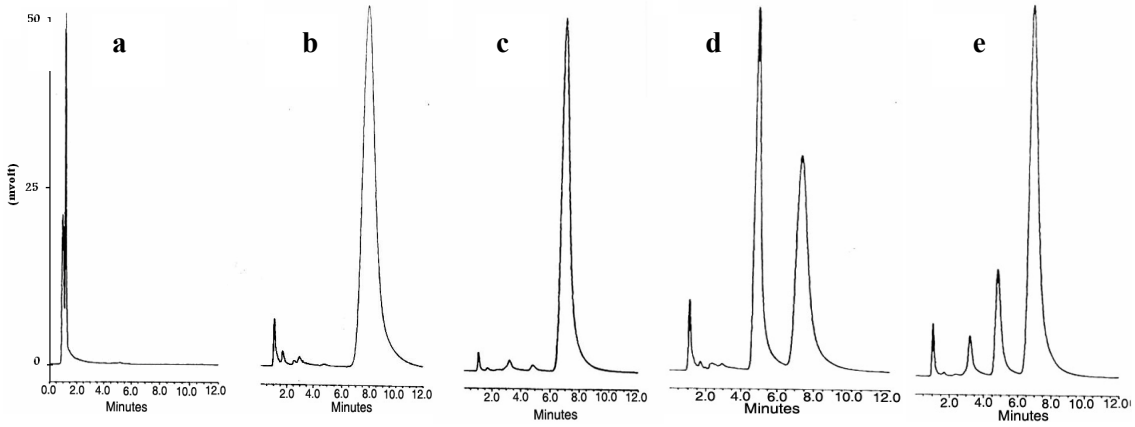

Figure 3. Chromatograms of blank human plasma (a), blank plasma spiked with internal standard, ranitidine (b), the lowest (0.15 µg/ml) concentration of metronidazole in plasma (c), the highest concentration of metronidazole in plasma (30 µg/ml) (d), and metronidazol concentration in plasma from a healthy subject 8 hrs after ingestion of metronidazole tablet (e) . The retention times for metronidazole and ranitidine (internal standard) are 4.9 and 7.2 minutes, respectively.

Table 1. Within-day and between-days variability of the HPLC assay for determination of metronidazole concentrations in plasma**.**

C (ug/ml)	Within-day variability				Between-day variability			
	Mean	SD[a]	CV%[b]	Error%	Mean	SD	CV%	Error%
0.15	0.169	0.006	3.79	12.5	0.164	0.010	6.16	9.04
0.75	0.755	0.075	9.83	0.66	0.744	0.057	7.65	4.73
1.5	1.498	0.066	4.39	0.17	1.461	0.089	6.09	12.0
7.5	7.395	0.041	5.54	1.40	7.486	0.432	5.77	4.85
15	14.95	0.959	6.41	0.33	14.78	0.755	5.10	1.22
22.5	22.35	1.730	7.74	0.66	22.47	1.541	6.85	3.08
30	29.81	1.548	5.19	0.63	29.68	1.371	4.62	1.40

a, Standard deviation, b, Coefficient of variation

Table 2. Pharmacokinetic parameters of the two products of metronidazole (Amin company) and Flagyl® tablets administred orally to twelve healthy volunteers.

Prameters	Test (Mean ± SD)	Ref (Mean ± SD)	Test / Ref (Mean ± SD)	$CI_{90\%}$ Test / Ref	$CI_{95\%}$ Test / Ref	P-value
C_{max} (µg/ml)	9.99 ± 1.34	10.61 ± 1.43	0.95 ± 0.14	0.880 – 1.026	0.864 – 1.043	0.218
Log C_{max}	1.00 ± 0.058	1.02 ± 0.059	0.98 ± 0.06	0.944 – 1.011	0.936 – 1.019	0.196
AUC_{0-48} (µg.h/ml)	131.74 ± 15.97	132.22 ± 22.24	1.01 ± 0.12	0.947 – 1.073	0.933 – 1.087	0.918
Log AUC_{0-48}	2.12 ± 0.054	2.12 ± 0.072	1.00 ± 0.02	0.988 – 1.014	0.985 – 1.017	0.829
$AUC_{0-\infty}$ (µg.h/ml)	136.73 ± 17.52	137.45 ± 23.05	1.01 ± 0.12	0.944 – 1.067	0.930 – 1.081	0.881
Log $AUC_{0-\infty}$	2.13 ± 0.057	2.13 ± 0.071	1.00 ± 0.02	0.987 – 1.013	0.984 – 1.016	0.910
T_{max} (hrs)	2.17 ± 0.91	2.17 ± 0.86	1.13 ± 0.55	0.843 – 1.410	0.784 – 1.482	1.000
$T_{1/2}$ (hrs)	9.15 ± 1.84	9.10 ± 0.64	1.015 ± 0.25	0.884 – 1.147	0.854 – 1.177	0.934

$T_{1/2}$, elimination half-life, AUC_{0-48}, area under the curve to the last measurable concentration,
$AUC_{0-\infty}$, area under the curve extrapolated to infinity, C_{max}, peak plasma concentration, T_{max} , time to peak plasma concentration

All chromatograms were free from any interferences at the retention times of metronidazole or internal standard, and both compounds were eluted as completely and appeared as two separate resolved peaks without peak tailing in such a way that it was possible to calculate peak height or peak area of standard curves. The retention times for metronidazole and ranitidine (internal standard) were 4.9 and 7.2 min, respectively. Linear relationships were found when the peak area ratios of metronidazole to the internal standard were plotted versus the metronidazole plasma concentration ranging from 0.15 to 30 µg/ml (r^2 = 0.9999). The results of within- and between-day variability are presented in Table 1. Relative errors were within 1.22 to 12.0 % and the CV% ranged from 4.62 to 7.65. Results of coefficients of variation and percent errors indicate that method is reproducible within day and between days. The limits of quantitation and detection of the method were 0.04µg/mL (almost 10 times greater than intercept obtained from standard curve) and 0.005µg/mL, respectively. Metronidazole was measurable at the first sampling time (0.25 h) and after 6 half-lives in all volunteers. The described method utilizes no organic extraction which makes the method rapid and simple. The use of $ZnSO_4$ followed by cooling steps in sample preparation eliminated the problem of additional precipitation which are being observed upon standing of the supernatant when automatic injectors are used (28). By using zinc sulfate rather than perchloric acid or trichloroacetic acid for protein precipitation, column durability is greatly extended (29). Large volume of acetonitrile (500 µl), relative to zinc sulfate volume (50 µl) was needed to precipitate the plasma proteins which results in dilution of samples (initially 200 µl) and consequently increases the limit of quantification of the assay. The use of internal standard increases the accuracy of the assay whose availability is an important issue in HPLC assays. In the present study, ranitidine was employed as internal standard and in a separate study very satisfactory results were obtained when hydroxylchloroquine sulfate was used as internal standard (results are not shown). In the absence of ranitidine, therefore, the use of hydroxychloroquine is recommended.

In vivo studies:

Metronidazole was well tolerated by the subjects and unexpected incidents that could have influenced the outcome of the study did not occur. All volunteers who started the study continued to the end and were discharged in good health. The mean concentration-time profile following oral administration of two different metronidazole preparations are plotted in Fig. 4. The mean pharmacokinetic parameters for the brands of metronidazole tablets are summarized in Table 2. The parameters t_{max} and $AUC_{0-\infty}$ correspond to the respective rate and extent of drug absorption, and C_{max} is related to both of these two processes (30), and all three measures being essential for comparison of the bioavailability of the two preparations.

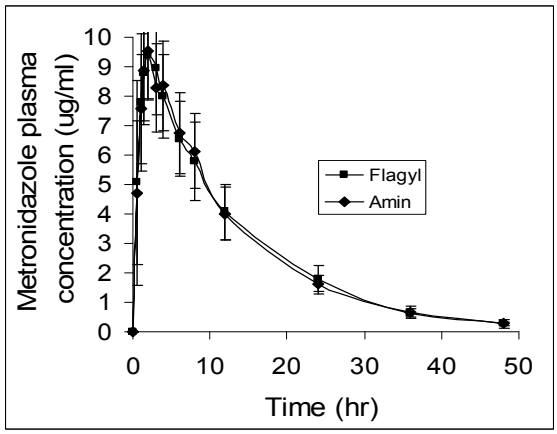

Figure 4. The mean plasma metronidazole levels vs time profiles following ingestion of a single dose of two 250-mg tablet for test (Amin) and reference (Flagyl®) tablet products to 12 healthy volunteers. Data is shown as mean ± SD.

The AUC_{0-48} and $AUC_{0-\infty}$ for two products were not statistically different (P > 0.05), suggesting that the plasma profiles generated by Flagyl® were comparable to those produced by metronidazole manufactured by Amin company. ANOVA for these parameters, after log-transformation of data, showed no statistically significant difference between the two formulations. ANOVA did not reveal any considerable differences in periods, formulations, or sequences (P > 0.05). Ninety percent confidence intervals of the ratio of the $AUC_{0-\infty}$ of two formulations (94.4 – 106.7 %) were found to be within the FDA acceptable range of 80-120% for bioequivalance evaluation. The C_{max} values of two products were also analyzed by the ANOVA procedure, indicating no statistically difference between generic formulation and reference. Furthermore there was no significant difference with regards to periods and sequences (P > 0.05). The ninety percent confidence intervals of the ratio of C_{max} of two formulations were 88 – 102.6 %, which lies within the FDA acceptable range of 80-120% (Table 2).

In summary, a rapid, practical and sensitive HPLC method is described for determination of metronidazole in human plasma. Addition of zinc sulfate followed by cooling allowed efficient

precipitation of plasma proteins, increased volume of injection, lowered the possibility of additional protein precipitation upon standing which improved the limit of quantitation. The short time of analysis, simplicity, and sufficient sensitivity makes the method particularly useful for pharmacokinetic and bioequivalent studies of metronidazole even following oral single dose (one tablet) of the drug rather than two tablets. In conclusion based on estimated pharmacokinetic parameters and statistical analyses, it was found that the metronidazole tablets manufactured by Amin Company were bioequivalent to Flagyl®, manufactured by Milano – Pharmacia Upjohn, and that generic and reference products may be considered equally effective in medical practice.

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REFERENCES

1. Reynolds JEF. Martindale, The Extra Pharmacopoeia, Vol. 33, The Royal Pharmaceutical Society, Great Britain, London; 2002. p. 594 - 596.
2. Ralf ED. Clinical pharmacokinetics of metronidazole. Clin Pharm 1983; 8:.43-62.
3. Tracy JW, Webster LT. Drugs used in the chemotherapy of protozoal infections. In : Hardman JG, Limbird LE, Gilman AG.(ed) Goodman & Gilman's The pharmacological basis of therapeutics , 10th ed., NewYork: Mc Graw – Hill; 2001. p. 1106-1107.
4. Fredricsson B, Hagstrom B, Nord CE, Rane A. Systemic concentrations of metronidazole and its main metabolites after intravenous, oral and vaginal administration. Gyneeol Obstet Invest 1987; 24:200-207.
5. Sattar MA, Sankey MG, Cawley ML, Kaye CM, Holt JE. The penetration of metronidazole into synovial fluid. Postgard Med J 1982; 58:20-24.
6. Nagar H, Berger SA, Hammar B. Penetration of clindamycin and metronidazole into the appendix and peritoneal fluid in children. Eur J Clin Pharmacol 1989; 37:209-210.
7. Bennett WM, Aronoff GR, Golper TA, Morrison G, Singer I, Brater DC. Drug prescribing in Renal Failure: Dosing Guidelines for Adults. 3rd ed. American College of physicians, philadelphia, 1994.
8. Gjerloff C, Arnold E. Pharmacokinetics of intravenous metronidazole in man. Acta Pharmacol Toxicol 1982; 51:132-135.
9. Jensen JC, Gugler R. Single and multiple dose metronidazole kinetics. Clin Pharmacol Ther 1983; 34:481-487.
10. Rubenson A, Rosetzsky A. Single dose prophylaxis with metronidazole in infants during abdominal surgery: a pharmacokinetic study. Eur J Clin Pharmacol 1986; 29: 625-628.
11. Houghton GW, Dennis MJ, Gabriel R. Pharmacokinetics of metronidazole in patients with varying degrees of renal failure. Br J Clin Pharmacol 1985; 19:203-209.
12. Eranko PO, Palva ES, Konno K, Venho VMK. Regulatory control of synonym drug bioavailability: Bioinequivalency of Nifedipine preparations, Pharmaceutic Med 1990; 4:197-205.
13. Emami J, Varshosaz, J, Flamarzian M, Tahvilian R, High performance liquid chromatographic determination, pharmacokinetic and comparative bioavailability studies of cisapride. J Pharm Biomed Anal 2003; 33:513 – 520.
14. Adamovics J. Rapid determination of metronidazole in human serum and urine using a normal-phase high-performance liquid chromatographic column with aqueous solvents. J Chromatogr 1984; 309(2):436-440.
15. Rona K, Simple BG. Liquid chromatographic method for the determination of ornidazole and metronidazole in human serum. J Chromatogr 1987; 420(1):228-230
16. Jessie MJ, Barrett DA, Shaw PN, Spiller RC. 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- Biomed Appl 1996; 677(2):374-379.
17. Galmier MJ, Frasey AM, Bastide M, Beyssac E, Petit J, Aiache JM, Lartigue-Mattei C. Simple and sensitive method for determination of metronidazole in human serum by high-performance liquid chromatography. J Chromatogr B- Biomed Sci Appl 1998; 720(1-2):239-243.
18. Wibawa JD, Shaw PN, Barrett DA. Quantification of metronidazole in small-volume biological samples using narrow-bore high-performance liquid chromatography. J Chromatogr -B Biomed Sci Appl 2001; 761(2):213-219
19. Venkateshwaran TG, Stewart JT. Determination of metronidazole in vaginal tissue by high-performance liquid chromatography using solid-phase extraction. J Chromatogr-B Biomed Appl 1995;672(2): 300-304

20. Yeung PK, Little R, JiangY, Buckley SJ, Pollak PT, Kapoor H, Veldhuyzen-van-Zanten SJ. A simple high performance liquid chromatography assay for simultaneous determination of omeprazole and metronidazole in human plasma and gastric fluid. J Pharm Biomed Anal. 1998; 17(8):1393-1398.
21. Sorensen LK, Hansen H. Determination of metronidazole and hydroxymetronidazole in trout by a high-performance liquid chromatographic method. Food Addit Contam 2000; 17(3):197-203.
22. Pollak, PT. A liquid chromatography assay for the study of serum and gastric juice metronidazole concentrations in the treatment of Helicobacter pylori. Ther.Drug Monit. 1996; 18(6):678-87.
23. Menelaou A, Somogyi AA, Barclay ML, Bochner F. Simultaneous quantification of amoxycillin and metronidazole in plasma using high-performance liquid chromatography with photodiode array detection. J Chromatogr-B Biomed Sci Appl 1999; 731(2):261-266.
24. The United States Pharmacopoeia, 25th edition; 2002.
25. Westlake WJ. Use of confidence intervals in analysis of comparative bioavailability trials. J Pharm Sci 1972; 61 (8):1340-1341.
26. Rockville MD. FDA guidance on statistical procedures for bioequivalence studies using a standard two-treatment crossover designs, Division of Bioequivalence, office of Generic Drugs, Center for Drug Evaluation and Research, Food and Drug Administration; 1992.
27. Chow S. Individual bioequivalence: a review of the FDA draft guidance. Drug lnf J 1999; 33:435-444.
28. Wheeler LA, De-Meo M, Halula M, George L, Heseltine P. Use of high-pressure liquid chromatography to determine plasma levels of metronidazole and metabolites after intravenous administration. Antimicrob Agents Chemother 1978; 13(2):205-209.
29. Nilsson-Ehle I, Ursing B, Nilsson-Ehle P. Liquid chromatographic assay for metronidazole and tinidazole: pharmacokinetic and metabolic studies in human subjects. Antimicrob. Agents Chemother 1981; 19(5): 754-60.
30. Yuen KH, Phen KK, Chan KL, Toh WT. Pharmacokinetics and bioequivalent study of a generic metoprolol tablet preparation. Drug Dev Ind Pharm 1998; 24 (10):955-961.