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Validated HPLC Method for the determination of tinidazole in human serum and its application in a clinical pharmacokinetic study

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A high performance liquid chromatographic (HPLC) method for the determination of tinidazole in human serum using metronidazole as internal standard (IS) is described. Protein precipitation is used for the preparation of sample. Mobile phase consisting of 0.002 M phosphate buffer, methanol and acetonitrile mixture (85 : 7.5 : 7.5/v/v/v) was used at a flow rate of 1 ml/min on a C18 column. The eluate was monitored using an UV/Vis detector set at 320 nm. Ratio of peak area of analyte to IS was used for quantification of serum samples. The absolute recovery was greater than 95% over a concentration range of 0.5 to 30 μg/ml and the limit of quantitation was 0.05 μg/ml. The intra-day relative standard deviation (RSD) measured at 0.5, 5, 15 and 30 μg/ml ranged from 0.36 to 6.14%. The inter-day RSD ranged from 1.14 to 4.21%. The method is simple, sensitive and has been successfully used in a pharmacokinetic study conducted in healthy human volunteers.

1. Introduction

Tinidazole is a drug used for the treatment of *Trichomonas vaginalis* and other protozoal infections [1]. It is also active against anaerobic bacteria and its clinical use along with a drug against aerobic bacteria was well reviewed by Packard [2]. The drug has been useful preventing infections after colorectal surgery [2, 3], obstetric and gynecological surgery [2, 4] and oral surgery [5]. Tinidazole dosage by the oral route varies according to the infection treated. It is administered up to 2 g daily as a single dose or in divided doses.

A few analytical methods have been described to analyze tinidazole in the body fluids using HPLC [6–9], micellar liquid chromatography [10] and HPTLC [11] nevertheless the limit of quantitation was not given or given without validation or the recoveries were relatively low. To conduct the pharmacokinetic and bioavailability evaluation in a wide dosage range, a simple and sensitive HPLC assay method with UV detection has been developed for the quantitative determination of tinidazole in human serum using metronidazole as an internal standard.

2. Investigations, results and discussion

Chromatogram of serum sample obtained 24 h after oral administration of tinidazole to one of the volunteers is shown in Fig. 1. No endogenous interfering peaks were visible in blank serum at the retention times of tinidazole and metronidazole, thereby confirming the specificity of the analytical method. Both the analyte and the I.S. were well separated with retention times of 9.6 and 6.0 min, respectively. System suitability parameters for the method were as follows: Theoretical plates for tinidazole and I.S. were 636 and 1042, respectively, tailing factor was less than 1.5 for both tinidazole and I.S. and resolution between tinidazole and I.S. was 3.4.

The ratio of peak area of tinidazole to that of I.S. was used for the quantification of tinidazole in serum samples. The calibration curves were linear in the concentration range 0.05–100 μg/ml. The calibration/regression equation is $y = mx + c$, where y represents the peak area ratio of tinidazole to I.S., x represents the concentration of tinidazole, m is slope of the curve and c is the intercept. The equation of the calibration curve obtained from 10 points was $y = 0.355012 \times -0.01292$; ($r^2 = 0.9952$) and its calibration curve is shown in Fig. 2.

The LOQ, established by determining concentration of four spiked calibration standards having reproducibility with RSD $<20\%$ and accuracy of 80 to 120% was found to be 0.05 μg/ml. Using this method, it is possible to increase the sensitivity further by increasing serum/injection volume.

The intra-day precision of the assay was determined by analyzing four spiked serum samples at each concentration on the same day. For the determination of inter-day preci-

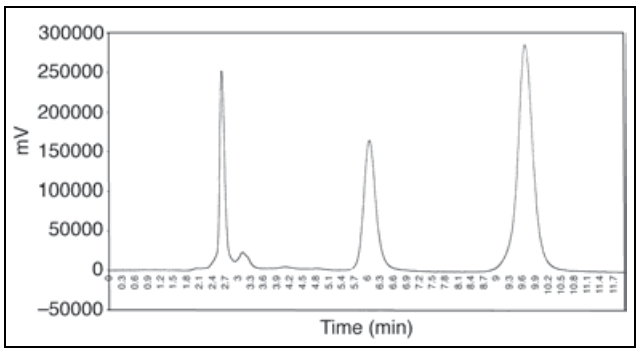

Fig. 1: Typical HPLC chromatogram for analysis of tinidazole: Serum sample from a subject collected 24 h after dosing. The respective concentration was 3.02 μg/ml

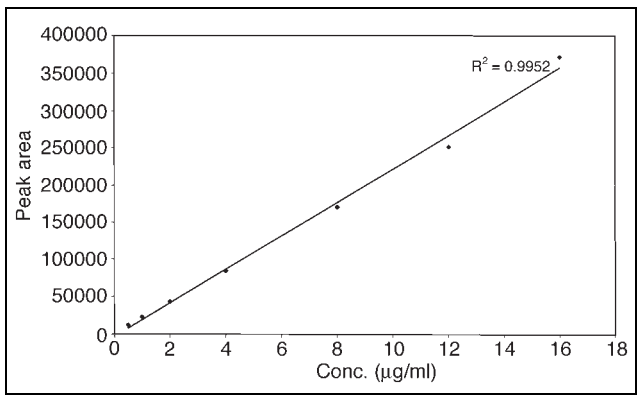

Fig. 2: Standard graph of tinidazole in serum

Table 1: Intra and inter-day precision of determination of tinidazole in human serum

Spiked concentration (μg/ml)	Day	Mean concentration (μg/ml)		
		Mean	Std Dev	R.S.D.
Intra-day variation (n = 4)				
0.5	0	0.512	0.031	6.05
	1	0.529	0.018	3.40
	5	0.487	0.021	4.31
	10	0.548	0.009	1.64
5	0	5.127	0.187	3.65
	1	4.785	0.293	6.12
	5	4.927	0.154	3.13
	10	5.216	0.019	0.36
15	0	14.195	0.814	5.73
	1	14.573	0.702	4.82
	5	15.592	0.957	6.14
	10	14.327	0.556	3.88
30	0	30.918	0.982	3.18
	1	29.227	1.312	4.49
	5	31.334	1.065	3.40
	10	28.061	1.251	4.46
Inter-day variation (n = 16)				
0.5		0.519	0.011	2.12
5		5.014	0.057	1.14
15		14.672	0.618	4.21
30		29.885	1.063	3.56

sion, spiked samples were analyzed on four different days. The intra-day RSD ranged from 1.64–6.05, 0.36–6.12, 3.88–6.14 and 3.18–4.49 for 0.5, 5, 15 and 30 μg/ml, respectively. The inter-day RSDs were 2.12, 1.14, 4.21 and 3.56% for 0.5, 5, 15 and 30 μg/ml, respectively (Table 1). These values were within limits (<15%) specified for inter and intra-day precision [12, 13].

The recovery of tinidazole from serum was estimated at concentrations of 0.5, 5, 15 and 30 μg/ml. Serum samples (in quadruplicates) containing tinidazole and IS were precipitated and analyzed. Four samples containing similar concentrations of tinidazole acetonitrile were directly injected and peak areas were measured. Absolute recovery was calculated by comparing the peak areas for direct injection of pure tinidazole with those obtained from serum samples spiked with the same amount of tinidazole and processed similarly. The absolute recoveries ranged from 93.7–103.5% (Table 2). The accuracy of the method was verified by comparing the concentrations of tinidazole measured in spiked serum with the actual concentrations added.

Serum concentrations of tinidazole in 12 human volunteers following oral administration of 500 mg tinidazole are shown in Fig. 3. Peak concentration of 11.71 ± 1.43 μg/ml (C_{max}, mean ± SD) for tinidazole, was reached at 2.75 ± 0.87 h (t_{max}, mean ± SD). The half-life was 16.98 ± 2.73 h. Systemic exposure $AUC_{(0-\infty)}$ was found to be 299.86 ± 47.70 μg.h/ml. These parameters are comparable with those reported earlier [14, 15].

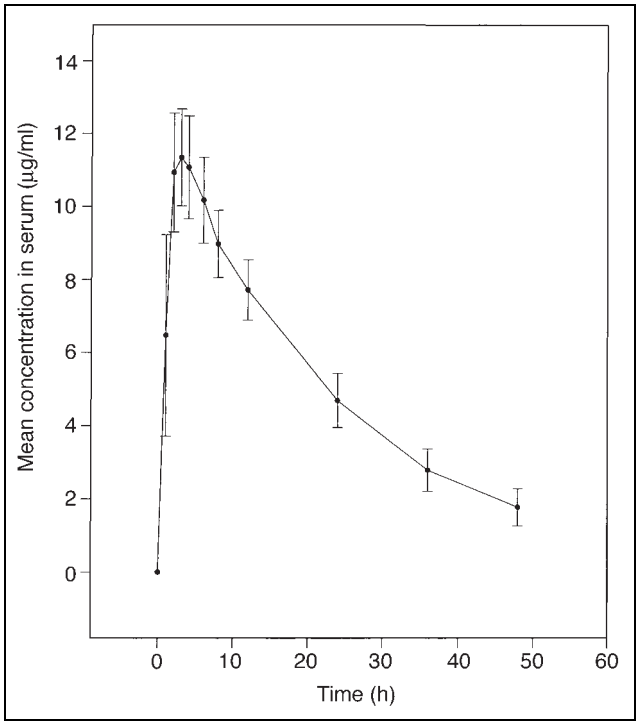

Fig. 3: Serum concentration versus time profile of tinidazole after 500 mg oral administration. The data points are mean ± SD of 12 observations

In conclusion, these experiments confirm that the presented method for the determination of tinidazole in human serum is simple, sensitive, specific, precise and accurate and requires a relatively small volume of serum (300 μl). The calibration curve was linear in the concentration range 0.05–100 μg/ml; hence the method is suitable for conducting pharmacokinetic studies with a wide range of dosage.

3. Experimental

3.1. Materials

Tinidazole and metronidazole pure samples were gifted by Novostat pharma, and Aristo pharmaceuticals, Mumbai, India, respectively. Methanol (SD-Fine Chemicals, Mumbai, India) and acetonitrile (Qualigens Chemicals, Mumbai, India) were of HPLC grade. Potassium dihydrogen orthophosphate was analytical reagent (AR) grade obtained from SD-Fine Chemicals, Mumbai, India. Tinidazole 500 mg tablets (Tiniba 500®) were obtained from Cadila Healthcare Limited, Ahmedabad, India. Double distilled water was used during entire HPLC procedure.

3.2. Standard solutions

Primary stock solutions of 1 mg/ml of tinidazole and metronidazole were prepared in methanol and stored at 4 °C. Appropriate dilutions of tinidazole were made in methanol to produce working stock solutions of 100, 10 and 1 μg/ml. These dilutions were used to spike serum in the preparation of calibration curves. The I.S. working stock solution (100 μg/ml) was made from primary stock solution using methanol for dilution. Calibration samples were prepared by spiking 300 μl of blank serum with an appropri-

Table 2: Absolute recovery and accuracy of determination of tinidazole in human serum

Concentration (μg/ml)	Absolute Recovery (%)		Accuracy (%)	
	Mean ± S.D. (n = 4)	Range (min – max)	Mean ± S.D. (n = 4)	Range (min – max)
0.5	96.5 ± 2.56	93.7–98.1	96.1 ± 1.83	93.7–98.3
5	98.1 ± 3.10	94.3–101.7	97.8 ± 2.19	94.8–99.9
15	99.7 ± 4.01	94.1–103.5	98.4 ± 2.27	95.2–100.4
30	99.1 ± 1.51	97.53–101.1	100.2 ± 1.68	98.6–102.3

ate amount of drug on the day of analysis. Samples for the determination of recovery, precision and accuracy were prepared by spiking control human serum in bulk of appropriate concentrations (0.5, 5, 15 and 30 μg/ml) and stored at −20 °C.

3.3. Extraction procedure

To 300 μl of serum samples, a methanolic solution of metronidazole equivalent to 0.6 μg was added as I.S. and shaken well. Then equivalent amount of acetonitrile solution (0.3 ml) was added for protein precipitation and mixed on a cyclo-mixer for 1 min and centrifuged at 4000 rpm using a table top centrifuge (Remi instruments, Mumbai, India) for 10 min. 20 μl of the supernatant was injected onto HPLC column.

3.4. Chromatographic conditions

The HPLC system (Shimadzu, Japan) consisted of a LC-10AT solvent delivery module, SPD-10A UV-Visible Spectrophotometric detector with LC10 software. The column used was Altech C18 (stainless steel column of length 25 cm and internal diameter of 4.6 mm packed with porous silica spheres of 5 μ diameter). A mobile phase consisting of potassium dihydrogen orthophosphate (0.002 M, pH 4.8), methanol and acetonitrile mixture (85 : 7.5 : 7.5 v/v/v) was used at a flow rate of 1.0 ml/min. The eluate was monitored at 320 nm. The sensitivity was set at 0.001 AUFS.

3.5. Linearity and limit of quantitation

The calibration samples were prepared by spiking 300 μl of control human serum with an appropriate amount of tinidazole and I.S. on the day of analysis. The lower limit of quantitation (LOQ) was defined as the lowest concentration at which the relative standard deviation and deviation from the nominal concentration were less than 20%.

3.6. Precision

Samples for the determination of precision were prepared by appropriately spiking control human serum in bulk, to get concentrations of 0.5, 5, 15 and 30 μg/ml. At each concentration 300 μl aliquots were distributed into screw-capped tubes and stored at −20 °C. Four replicates at each concentration were processed as described in the sample preparation on day 0, 1, 5 and 10 to determine the intra day and inter day reproducibility. The precision of the method at each concentration was calculated as the RSD.

3.7. Recovery and accuracy

The recovery from serum samples was determined by comparing the amount of tinidazole from serum samples with that of recovery standards, which were processed similarly without serum matrix (using acetonitrile, instead). The accuracy of the procedure was determined by expressing the mean calculated concentration as a percentage of the spiked/nominal concentration.

3.8. Application to clinical pharmacokinetic study

The assay method was used to determine tinidazole concentrations in serum following oral administration of a tinidazole 500 mg tablet to 12 healthy male human volunteers after an overnight fast. Blood samples (5 ml) were withdrawn from the ante cubital vein at the intervals of 0, 1, 2, 3, 4, 6, 8, 12, 24, 36, and 48 h after drug administration. The samples were allowed to clot and were centrifuged at 4000 rpm for 10 min. The serum was separated and stored at −20 °C until the commencement of analysis.

Pharmacokinetic parameters like peak serum concentration (C_{max}), time to reach peak concentration (t_{max}), area under the curve (AUC), elimination half-life ($t_{1/2}$), volume of distribution (Vd/f) and total clearance (CL/f) for tinidazole were obtained for each subject using a computer program RAMKIN (Krishna, unpublished work) meant for calculation of model independent parameters.

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