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Validated HPLC Method for the Determination of Ornidazole in Human Serum and Urine

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A high performance liquid chromatographic method for the determination of ornidazole in human serum using tinidazole as internal standard is described. Protein precipitation is used for the preparation of sample. Mobile phase consisting of 0.002 M phosphate buffer (pH-4.8), methanol and acetonitrile mixture (70:10:20 v/v/v) was used at the flow rate of 1ml/min on a C18 column. The eluate was monitored using an UV/Vis detector set at 318 nm. Ratio of peak area of analyte to internal standard was used for quantification of serum samples. The absolute recovery was greater than 90% over a concentration range of 1 to 20 μg/ml and the limit of quantitation was 0.05 μg/ml. The inter- day relative standard deviation ranged from 1.02 to 3.70 at 1 μg/ml, 0.96 to 3.62 at 2 μg/ml, 3.33 to 5.01 at 10 μg/ml and 1.16 to 5.03 at 20 μg/ml and for intra-day, 1.03, 2.16, 1.95 and 1.23 at 1, 2, 10, 20 μg/ml, respectively. The method was found to be simple, sensitive and could be used in the pharmacokinetic study involving human volunteers.

Ornidazole (ORZ, 1-(3-chloro-2-hydroxypropyl)-2-methyl-5-nitroimidazole, fig. 1) is an antimicrobial agent for the treatment of infections due to trichomonas, amoebae, *Giardia lamblia*, and anaerobic bacteria[1-3]. Ornidazole is used like metronidazole (MTZ) in the management of susceptible protozoal and anaerobic bacterial infections. The longer half-life (12-14 h) in comparison to that of metronidazole (6-8 h) is of particular advantage[4]. The antimicrobial activity of ORZ is due to the reduction of the nitro group to a more reactive amine that attacks microbial DNA, brings about loss of helical structure of DNA and subsequent DNA breakage, thus inhibiting further synthesis and causing degradation of existing DNA. The reported HPLC methods of estimation of ORZ suffer from poor sensitivity, and higher detection limit[5]. Other techniques, including spectrophotometry[6-8] and electrochemistry[9] reported for determination of ORZ in pharmaceuticals and biological fluids also suffer from poor sensitivity. The present developed method using tinidazole (TNZ) as internal standard (IS) is simple, and could be used for the determination of ORZ in human serum and urine, with ease and higher sensitivity.

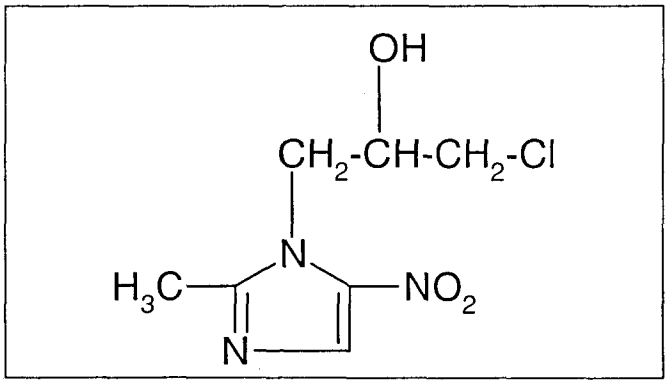

Fig. 1: Structure of ornidazole

MATERIALS AND METHODS

Ornidazole was extracted with methanol from 500 mg tablets (Orni 500®) supplied by Cadila Healthcare Limited, Ahmedabad. Tinidazole pure sample was a gift from Aristo Pharmaceuticals, Mumbai. Methanol and acetonitrile (E.

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Merck (India) Limited, Mumbai) were of HPLC grade. Potassium dihydrogen phosphate (analytical grade) was purchased from E. Merck (India) Limited, Mumbai. Double distilled water was used in the study. Serum was collected from healthy human volunteers.

Standard solutions:

Primary stock solutions of 1 mg/ml of ORZ and TNZ were prepared in methanol and stored at 4°. Appropriate dilutions of ORZ were made in methanol to produce working stock solutions of 100, 10, and 1 μg/ml. These dilutions were used to spike serum in preparation of calibration curves. Internal standard (TNZ, IS) was used directly from the stock solution (100 μg/ml). Calibration samples were prepared by spiking 250 μl of blank serum with an appropriate amount of drug on the day of analysis. Samples for the determination of recovery, precision and accuracy were prepared by spiking control serum in bulk with standard solutions of appropriate concentration (1, 10, 20 μg/ml) and stored at −80°.

Extraction procedure:

To 250 μl of serum sample, 10 μl of different concentrations of ORZ and methanolic solution of TNZ equivalent to 1 μg as IS were added and mixed. Methanol (250 μl) was added and the contents were vortexed for 1 min using a cyclomixer (Remi Instruments, Mumbai) and centrifuged at 13 000 rpm for 8 min at 37° in a Biofuge fresco centrifuge (Hereaus, Germany). An aliquot (20 μl) of the supernatant was injected into the HPLC column.

Chromatographic conditions:

An HPLC system (Shimadzu, Kyoto, Japan) equipped with LC-8A solvent delivery module and SPD-10A *VP* UV/Vis spectrophotometric detector was used. Wakosil II 5C-18RS-100A, 5 μm, 4.6x250 mm stainless steel column (SGE, Japan) was used for the analysis. Sensitivity was set at 0.001 aufs (absorbance units full scale). A mobile phase consisting of 0.002 M potassium dihydrogen phosphate buffer (pH 4.8), acetonitrile and methanol mixture (70:20:10 v/v/v) was used at a flow rate of 1.0 ml/min. Eluent was monitored using a UV/Vis detector set at 318 nm.

Recovery and accuracy:

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

Linearity, limit of quantification and precision:

The calibration samples were prepared by adding control serum with appropriate amounts of ORZ and TNZ on the day of analysis. The lowest concentration at which relative standard deviation (RSD) from the nominal concentration were less than 20% was taken as lower limit of quantitation (LLOQ). Precision of assay was determined by analyzing serum samples containing ORZ at four different concentrations of 1, 2, 10, and 20 μg/ml. Samples for precision study were obtained by spiking blank serum with the analyte solution at each concentration in bulk and the aliquots were stored in Eppendorf tubes at −4°. Four replicates at each concentration were processed as described, in the sample preparation on day 0, 1, 2 and 3 to determine the inter-day and intra-day reproducibility. The precision of the method at each concentration was calculated as the RSD.

RESULTS AND DISCUSSION

Typical chromatograms corresponding to blank serum and serum spiked with ORZ 10 μg/ml are shown in figs. 2 and 3, respectively. No endogenous interfering peaks were visible in blank serum at retention times of ORZ and the IS, there by confirming the specificity of the analytical method.

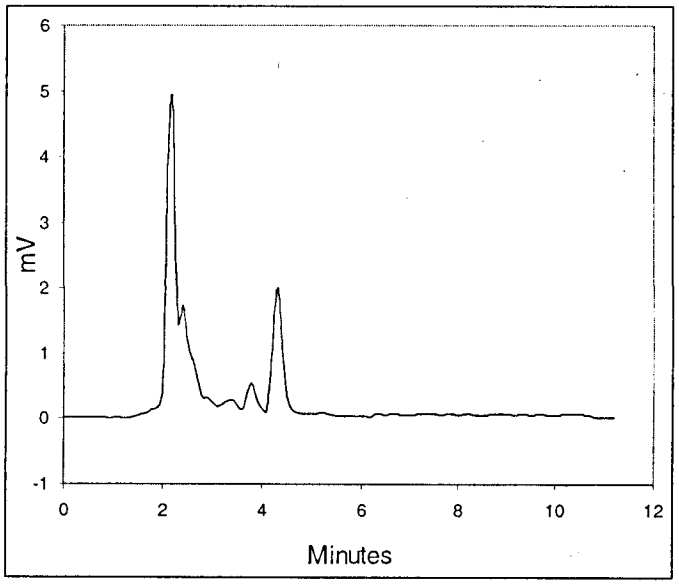

Fig. 2: Typical HPLC chromatogram for analysis of ornidazole: blank serum

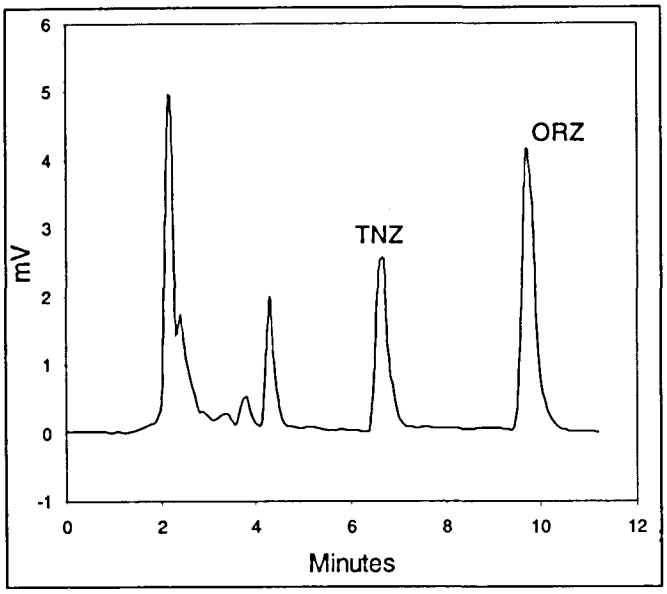

Fig. 3: Typical HPLC chromatogram for analysis of ornidazole: serum sample spiked with 10 μg/ml

TNZ stands for tinidazole and ORZ for ornidazole

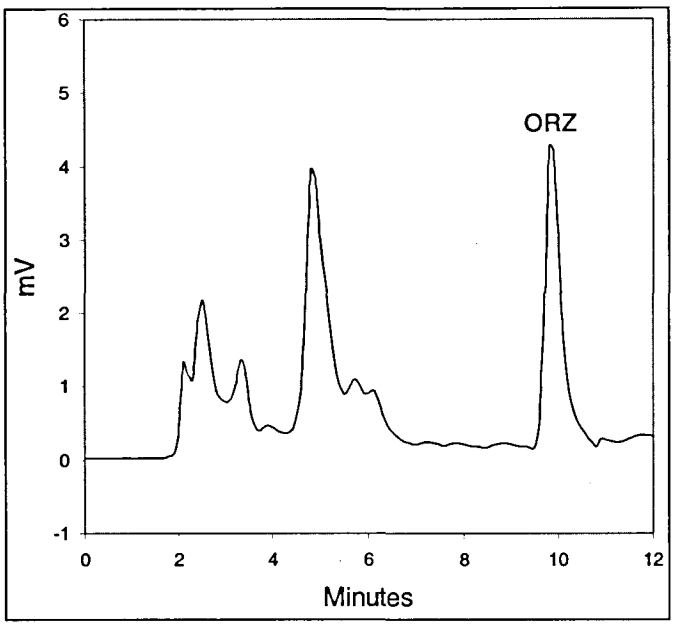

Fig. 4: Ornidazole in a urine sample

ORZ stands for ornidazole

Both analyte and the IS were well separated with retention times of 9.76 and 6.69 min, respectively. Chromatogram of ORZ in a urine sample is shown in fig. 4. System suitability parameters for the method were as : theoretical plates for ORZ and TNZ were 2024 and 2348. Tailing factors were less than 1.25 and 1.23 for ORZ and TNZ, respectively. Resolution between ORZ and TNZ was 3.07.

Peak area ratios of ORZ to that of IS were measured. A representative calibration graph of peak area ratio versus ORZ concentration in the range of 1 μg to 20 μg resulted in regression equation $y=0.165x+0.0436$ ($R^2=0.9961$). The lowest concentration with relative standard deviation (RSD) less than 20% was taken as LLOQ and was found to be 0.05 μg/ml.

The intra-day precision was determined by analyzing four spiked serum samples at each concentration in different time on the same day. For the determination of inter-day precision, spiked samples were analyzed on four different days (Table 1). These values are within the limits (<15%) specified for inter and intra day precision[10,11].

The recovery of ORZ was estimated at 1, 2, 10, and 20 μg/ml concentrations. Serum samples (in six replicates) containing ORZ and IS were precipitated and analyzed. Six samples containing similar concentrations of the compound in methanol were directly injected and peak areas were measured. Absolute recovery was calculated by comparing the peak areas for direct injection of pure ORZ in methanol with that obtained by serum samples spiked with the same amount of ORZ and processed similarly. The absolute recoveries and accuracies are given in Table 2. The accuracy of the method was verified by comparing the concentrations measured for ornidazole spiked in serum with the actual added concentrations.

These experiments confirm that the present method for determination of ORZ in serum samples is specific, accurate, precise and has high sensitivity. The calibration curve was linear and hence the method is suitable for analysis of serum samples in the concentration range of 1 to 20 μg /ml. This method was used for analysis of serum samples collected during a pharmacokinetic study. HPLC chromatogram of blank serum sample showed no peaks at retention time of ORZ and IS indicating that there was no interference from serum endogenous peaks. The developed HPLC method can be used for analysis of ornidazole serum samples during pharmacokinetic studies in human volunteers.

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TABLE 1: INTRA AND INTER-DAY PRECISION OF DETERMINATION OF ORNIDAZOLE IN HUMAN SERUM

Spiked Concentration (μg/ml)	Day	Measured concentration		
		Mean (μg/ml)	SD	RSD
Inter-day variation 1	0	0.98	0.01	1.02
	1	1.02	0.02	2.05
	2	1.35	0.05	3.70
	3	0.96	0.01	1.51
2	0	2.27	0.03	3.62
	1	2.21	0.18	3.13
	2	1.96	0.15	2.86
	3	2.20	0.01	0.96
10	0	10.13	1.67	3.33
	1	10.95	2.03	3.98
	2	10.01	2.51	5.01
	3	9.27	1.98	4.01
20	0	20.23	0.32	3.12
	1	19.87	0.49	4.96
	2	20.53	0.53	5.03
	3	20.27	0.12	1.16
Intra-day variation 1		0.97	0.01	1.03
2		2.12	0.23	2.16
10		9.63	0.97	1.95
20		20.62	0.12	1.23

SD: Standard deviation, RSD: Relative standard deviation and Mean value of 4 determinations

TABLE 2: RECOVERY AND ACCURACY OF DETERMINATION OF ORNIDAZOLE IN HUMAN SERUM

Concentration (μg/ml)	Absolute recovery (%) Mean ± S.D.	Accuracy (%) Mean ± S.D.	Range (min-max) (μg/ml)
1	92.49±1.34	99.67±1.97	0.92 - 1.23
2	98.97±1.07	98.32±1.32	1.95 - 2.13
10	99.12±2.31	97.01±0.99	9.78 - 10.32
20	98.97±1.07	98.32±1.32	19.34 - 20.8

SD: Standard deviation and Mean value of 6 determinations

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