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Validated HPLC method for determination of chlorzoxazone 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 chlorzoxazone in human serum using phenacetin as internal standard (IS) is described. Protein precipitation is used for preparation of the sample. A mobile phase consisting of acetonitrile and 0.5% acetic acid in water mixture (40 : 60 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 287 nm. Ratio of peak area of analyte to IS was used for quantification of serum samples. The absolute recovery was greater than 96% over a concentration range of 1 to 100 μg/ml and the limit of quantitation was 0.05 μg/ml. The intra-day relative standard deviation (RSD) measured at 1, 10, 50, and 100 μg/ml ranged from 0.9 to 5.1%. The inter-day RSD ranged from 0.6 to 3.0%. The method is simple, sensitive and has been successfully used in pharmacokinetic study conducted in healthy human volunteers.

1. Introduction

Chlorzoxazone is a benzazole derivative used as a muscle relaxant [1]. It is 6-hydroxylated by cytochrome P4502E1 (CYP2E1) [2]. Urinary excretion of 6-hydroxy chlorzoxazone is an index of CYP2E1 activity [3]. Interindividual variability of chlorzoxazone 6-hydroxylation in men and women and its relationship to the CYP2E1 genetic polymorphisms was well reviewed by Richard [4]. Chlorzoxazone is a substrate for CYP2E1. Interest in CYP2E1 arises because of its likely role in the activation of important human procarcinogens and protoxins [5, 6]. Usually, 2 g of chlorzoxazone are administered daily in divided doses.

A few analytical methods have been described to analyze chlorzoxazone in the body fluids using HPLC [7–9] and micellar liquid chromatography [10]. Nevertheless, the limit of quantitation was not given or given without validation or the recoveries were relatively low. The aim of this paper was to develop for the pharmacokinetic and bioavailability evaluation in a wide dosage range, a simple and sensitive HPLC method with UV detection for the quantitative determination of chlorzoxazone in human serum.

2. Investigations, results and discussion

Typical chromatograms corresponding to blank serum and chlorzoxazone spiked control serum are shown in Figs. 1 and 2. A chromatogram of serum sample obtained 2 h after oral administration of chlorzoxazone to one of the volunteers is shown in Fig. 3. No endogenous interfering peaks were visible in blank serum at the retention times of chlorzoxazone and phenacetin, thereby confirming the specificity of the analytical method. Both the analyte and the I.S. were well separated with retention times of 9.3 and 6.8 min, respectively. System suitability parameters for the method were as follows: Theoretical plates for chlorzoxazone and I.S. were 638 and 1045, respectively. Tailing factor was less than 1.5 for both chlorzoxazone and I.S. and resolution between chlorzoxazone and I.S. was 3.4.

The ratio of peak area of chlorzoxazone to that of I.S. was used for the quantification of chlorzoxazone in serum samples. The calibration curves were linear in the concentration range 0.05–100 μg/ml. The calibration/regression equation was $y = mx + c$, where y represents the peak area ratio of chlorzoxazone to I.S., x represents the concentrations of chlorzoxazone, m is slope of the curve and c is the intercept. The equation of the calibration curve obtained from 10 points was $y = 0.0999x + 0.025$; ($r^2 = 0.9998$).

The LOQ, established by determining concentration of four spiked calibration standards having reproducibility with RSD < 20% and accuracy of 90 to 105% was found to be 0.05 μg/ml. Using this method, it is possible to increase the sensitivity further increasing serum/injection volume.

The intra-day precision of the assay was determined by analyzing six spiked serum samples at each concentration

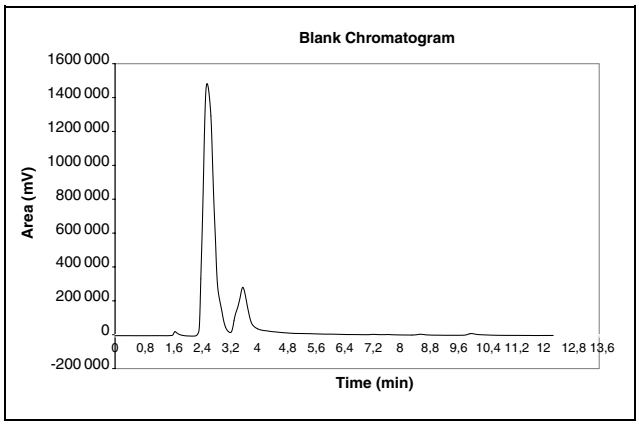

Fig. 1: Typical HPLC chromatogram for analysis of chlorzoxazone: blank serum

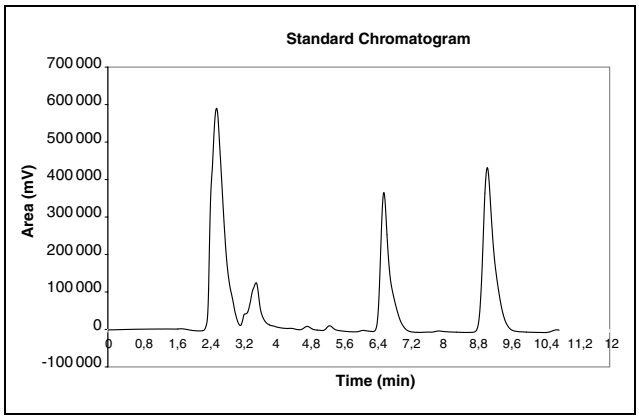

Fig. 2: Typical HPLC chromatogram for analysis of chlorzoxazone: blank serum sample spiked with 2 μg/ml

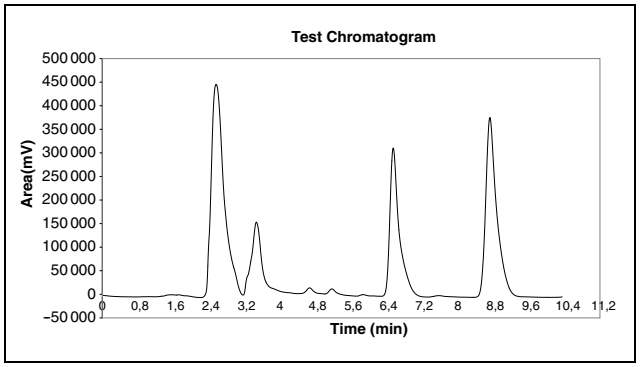

Fig. 3: Typical HPLC chromatogram for analysis of chlorzoxazone: Serum sample from a subject collected 2 h after dosing. The respective concentration was 3.12 μg/ml

on the same day. For the determination of inter-day precision, spiked samples were analyzed on four different days. The intra-day RSD and the inter-day RSDs are given in (Table 1). These values were within limits (<15%) specified for inter and intra-day precision [9, 10].

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

Spiked concentration (μg/ml)	Day	Mean concentration (μg/ml)		
		Mean	S.D	R.S.D.
Intra day variation (n = 6)				
1	0	1.09	0.03	2.752
	1	0.99	0.02	2.02
	2	0.97	0.05	5.154
	3	0.99	0.04	4.04
	4	0.98	0.03	3.061
10	0	10.91	0.10	0.916
	1	10.12	0.23	2.272
	2	10.85	0.30	2.765
	3	10.63	0.13	1.223
	4	9.98	0.27	2.705
50	0	49.78	1.62	3.25
	1	51.09	2.01	3.934
	2	50.18	1.98	3.945
	3	50.98	1.72	3.373
	4	50.72	2.08	4.101
100	0	102.38	1.98	1.934
	1	100.97	2.30	2.278
	2	103.09	2.69	2.609
	3	99.03	1.69	1.706
	4	100.67	2.98	2.960
Inter day variation (n = 16)				
1		0.99	0.03	3.03
10		10.63	0.19	1.787
50		51.01	0.32	0.627
100		101.92	0.78	0.765

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

Concentration (μg/ml)	Absolute recovery (%)		Accuracy (%)	
	Mean ± S.D. (n = 6)	Range (min – max)	Mean ± S.D. (n = 6)	Range (min – max)
1	93.26 ± 1.32	90.7–95.1	98.50 ± 1.81	95.7–100.3
10	98.93 ± 1.41	95.3–100.7	101.32 ± 0.99	100.8–98.9
50	97.07 ± 2.09	93.9–100.5	94.96 ± 1.20	93.2–96.4
100	95.98 ± 1.82	98.53–99.1	93.09 ± 0.82	92.6–94.3

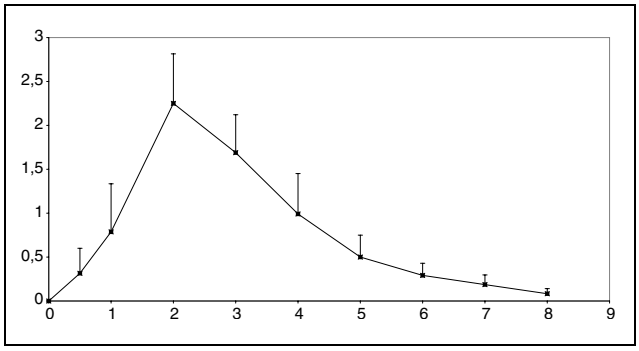

Fig. 4: Serum concentration versus time profile of chlorzoxazone after 250 mg oral administration. The data points are mean ± SD of 12 observations

The recovery of chlorzoxazone from serum was estimated at 1, 10, 50 and 100 μg/ml concentrations. Proteins in serum samples (in six replicates) containing chlorzoxazone and IS were precipitated and analyzed. Six samples containing similar concentrations of chlorzoxazone in methanol were directly injected and peak areas were measured. Absolute recovery was calculated by comparing the peak areas for direct injection of pure chlorzoxazone with that obtained from serum samples spiked with the same amount of chlorzoxazone and processed similarly. The absolute recoveries ranged from 90.7–101.5% (Table 2). The accuracy of the method was verified by comparing the concentrations of chlorzoxazone measured in spiked serum with the actual concentrations added.

Serum concentrations of chlorzoxazone in 12 human volunteers following oral administration of 250 mg chlorzoxazone are shown in Fig. 4. The peak concentration of 11.11 ± 4.41 μg/ml (C_{max}, mean ± SD) for chlorzoxazone reached at 1.45 ± 0.44 h (t_{max}, mean ± SD). The half-life was found to be 1.31 ± 0.38 h. Systemic exposure $AUC_{(0-\infty)}$ was found to be 34.2 ± 4.89 μg · h/ml. These parameters are comparable with those reported earlier [11, 12].

3. Experimental

3.1. Materials

Chlorzoxazone and phenacetin pure samples were gifted by Biological Evans, Hyderabad, India and Dr. Reddy's Laboratories, Hyderabad, India, respectively. Methanol (SD-Fine Chemicals, Mumbai, India) and acetonitrile (Qualigens Chemicals, Mumbai, India) are of HPLC grade. Acetic acid HPLC grade obtained from SD-Fine Chemicals, Mumbai, India. Chlorzoxazone 250 mg tablets (Parafon 250®) were obtained from Ethnor Pharmaceuticals Limited, Mumbai, India. Double distilled water was used during entire HPLC procedure.

3.2. Standard solutions

Primary stock solutions of 1 mg/10 ml of chlorzoxazone and 1 mg/ ml of phenacetin were prepared in methanol and stored at 4 °C. Appropriate dilutions of chlorzoxazone were made in methanol to produce working stock solutions of 100, 50, 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 the primary stock solution using methanol for dilution. Calibration samples were prepared by spiking 250 μl of blank serum with appropriate 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 (1, 10, 50, and 100 μg/ml) and stored at −20 °C.

3.3. Extraction procedure

To 250 μl of serum samples, a methanolic solution of phenacetin equivalent to 0.8 μg was added as I.S. and shaken well. Then equivalent amount (250 μl) of methanol solution was added for protein precipitation and mixed on a cyclo-mixer for 1 min and centrifuged at 13000 rpm using a Biofuge centrifuge (Heraeus, Germany) 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-10AVP solvent delivery module, SPD-10AVP UV-Visible Spectrophotometric detector with LC10A software. The column used was Wakosil II C18 (stainless steel column of length 25 cm and internal diameter of 4.6 mm packed with porous silica spheres of 5 μm diameter). A mobile phase consisting of acetonitrile and 0.5% acetic acid in water mixture (40 : 60 v/v) was used at a flow rate of 1.0 ml/min. The eluate was monitored at 287 nm. The sensitivity was set at 0.001 AUFS.

3.5. Linearity and limit of quantitation

The calibration samples were prepared by spiking 250 μl of control human serum with appropriate amount of chlorzoxazone 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 1, 10, 50, and 100 μg/ml. At each concentration 250 μl aliquots were distributed into screw-capped tubes and stored at −20 °C. Six replicates at each concentration were processed as described in the sample preparation on day 0, 1, 2, 3 and 4 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 chlorzoxazone 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.

3.8. Application to clinical pharmacokinetic study

The assay method was used to determine chlorzoxazone concentrations in serum following oral administration of chlorzoxazone 250 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, 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 h following the drug administration. The samples were allowed to clot and 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 chlorzoxazone were obtained for each subject using a computer program RAMKIN (Krishna, unpublished work) meant for calculation of model independent parameters.

Acknowledgements: The first and fourth author are grateful to the University Grants Commission, New Delhi, India, and the second author is grateful to the Council of Scientific and Industrial Research, New Delhi, India, and the third author is grateful to the All India Counsil for Techical Education, New Delhi, India for the financial assistance.

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Recceived June 10, 2002
Accepted August 12, 2002

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