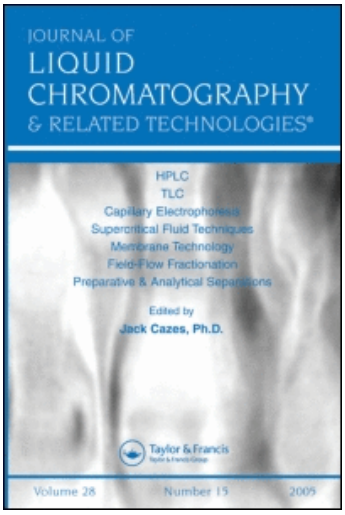

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High Performance Liquid Chromatographic Determination of Fenoverine in Human Serum: Application to Pharmacokinetic Study

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Abstract: A simple, specific, precise, sensitive, and rapid high performance liquid chromatographic method with UV detection was developed and validated for quantification of fenoverine in human serum. The developed method employed liquid-liquid extraction of fenoverine from serum with dichloromethane using chlorpromazine hydrochloride as internal standard (I.S). Chromatographic separation was accomplished within 10 min on an inertsil ODS column employing acetonitrile, water, and 0.375% v/v triethylamine (pH adjusted to 2.5 with orthophosphoric acid) in the ratio of 41:59 v/v at a flow rate of 1 mL min^{-1}. Retention times of fenoverine and internal standard are 6.76 and 8.19 min, respectively. The proposed method was linear in the range of 5 to 2000 ng mL^{-1}, specific and sensitive with limit of quantification of 5 ng mL^{-1}, better than the reported method. The intra-day and inter-day coefficient of variation of the assay method was found to be 2.30 to 2.84 and 1.01 to 2.34, respectively, with high accuracy and precision results. This validated method was applied successfully for pharmacokinetic study of fenoverine in human serum. Such a method would be ideally suitable for the estimation of the drug for pharmacokinetic studies in human volunteers after oral administration of a single or multiple dose(s) of fenoverine.

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Keywords: Fenoverine, Human serum, Pharmacokinetics, Reversed phase HPLC

INTRODUCTION

Fenoverine {10-[(4-piperonyl-1-piperazinyl) acetyl] phenothiazine derivative} (Figure 1) is a powerful musculotropic spasmolytic agent that is more effective than papaverine. Fenoverine synchronizes the smooth muscle motility by modulating the cellular calcium flow and is effective in the management of spasmodic conditions of the gastrointestinal,[1] colon,[2] bile duct, and urogenital tract.[3] The drug has antiemetic, antidiarrheal, and analgesic activity, and is exempt of anticholinergic activity.[4]

Few reports of high performance liquid chromatographic methods are available for quantification of fenoverine in biological fluids[5] and tissue samples.[6] The reported method in plasma[5] utilized a cyano (CN) column with a high retention time of 15.2 min for fenoverine at flow rate of 1.5 mL min^{-1}. Thus, the reported method is not economical both in terms of time and mobile phase consumption. The main objective of the present study is to develop a simple, specific, precise, economic, reliable, rapid, high performance liquid chromatographic method for fenoverine in human serum by employing the widely used RP ODS column.

The optimization of a simple liquid-liquid extraction procedure for fenoverine in serum is studied to obtain a good extraction recovery. The various parameters, such as concentration of sodium hydroxide used as a reagent to control pH of serum samples and precipitate the drug and organic solvent used as extractant along with mixing time of the solutions were optimized. The proposed method is applicable for determination of fenoverine in human serum to assess pharmacokinetic parameters in healthy human volunteers.

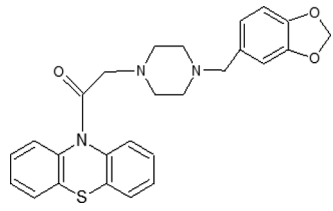

Figure 1. Structure of Fenoverine.

EXPERIMENTAL

Chemicals and Reagents

Fenoverine was a kind gift from M/s Euro drugs, Hyderabad, India. Chlorpromazine hydrochloride, internal standard was obtained from M/s Sun pharmaceutical Ind. Ltd. Baroda, India. Triethylamine was of analytical grade and obtained from M/s SD fine chemicals. Acetonitrile and methanol used were of HPLC grade (Rankem, India), orthophosphoric acid (GR grade) was obtained from E- Merck, India. Double distilled water was used during the entire HPLC procedure.

Standard Solutions

Primary stock solutions of $1\,mg\,mL^{-1}$ fenoverine and chlorpromazine hydrochloride (I.S.) were prepared in methanol (HPLC grade), dilutions of stock solution were also made with methanol and stored at 4°C and were found to be stable for several weeks. Standard solutions of fenoverine in human serum were made by spiking an appropriate volume of the diluted stock solution in 1 mL of serum, to produce working standard solutions of 5, 10, 100, 500, 1000, and $2000\,ng\,mL^{-1}$. In each sample, $100\,\mu L$ of $100\,ng\,mL^{-1}$ internal standard solution was added. The peak area ratio of fenoverine to that of I.S was plotted against the corresponding concentration to obtain the calibration graph. For the determination of recovery, stability, precision, and accuracy, the samples were prepared by spiking control human serum in blanks of appropriate concentrations (5, 500, and $1500\,ng\,mL^{-1}$) and stored at $-20°C$.

Sample Preparation

To 1 mL of serum with fenoverine, $100\,\mu L$ of $100\,ng\,mL^{-1}$ internal standard was added in a 10 mL clean glass tube and vortexed for one minute. Later, a volume of $300\,\mu L$ of 1N sodium hydroxide was added and mixed thoroughly, then 5 mL dichloromethane was added and vortexed for 5 min on a cyclomixer, followed by centrifugation at 3500 rpm for 10 min. The aqueous supernatant was removed and the residue was re-extracted with 1 mL of dichloromethane followed by centrifugation. The organic phase was separated in to a clean glass tube and evaporated to dryness in vacuum oven. The dried residue was reconstituted in $200\,\mu L$ of methanol and vortexed for 1 min and injected on to the HPLC column for analysis.

Application to Pharmacokinetic Studies in Human Volunteers

Pharmacokinetic study was conducted in healthy human volunteers. The assay method was used to determine fenoverine concentrations in serum following oral administration of 200 mg of a fenoverine floating mini tablet to healthy male human volunteers after an over night fast. Blood samples (5 mL) were withdrawn from the ante cubital vein at the intervals of 0, 0.5, 1, 2, 4, 6, 8, 10, 12, and 24 hr following drug administration. The samples were allowed to clot and centrifuged at 3500 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), and elimination half-life ($t_{1/2}$) for fenoverine were obtained using a computer program KINETICA (Innaphase corporation, 1999) meant for calculation of model independent parameters.

Chromatography

Quantitative HPLC was performed on an isocratic high performance liquid chromatograph (Shimadzu, Japan) consisting of a LC–10AT solvent module, SPD–10A, and UV-Visible spectrophotometric detector with LC 10 software. The analytical column C_{18} Inertsil ODS–3V (Column of length 25 cm and internal diameter of 4.6 mm, packed with particles of 5 μ diameter) was used for chromatographic separation. The composition of the mobile phase was acetonitrile, water, and 0.375% v/v triethylamine (pH adjusted to 2.5 with orthophosphoric acid) in the ratio of 41:59 v/v. The mobile phase was pumped from the solvent reservoir to the column at a flow rate of 1 mL min^{-1}, with run time of 10.0 min and column temperature was ambient. The volume of the injection loop was 20 μL. Prior to injection of drug solutions, the column was equilibrated for at least 30 min with the mobile phase flowing through the system. The eluents were monitored at 254 nm and data was acquired, stored, and analyzed with the LC 10 software. The sensitivity was set to 0.0008 AUFS.

Validation and Stability Studies

Selectivity and Specificity

The selectivity and specificity of the method was determined from six different serum samples for any interfering peaks at the retention

time of fenoverine and internal standard. The calibration curve for linearity was obtained using six standards of drug (5, 10, 100, 500, 1000, and 2000 ng mL^{-1}). The concentration of samples was calculated from the equation using regression analysis, $Y = mX + C$, where X = concentration of fenoverine, Y = peak ratio of fenoverine/I.S., m = slope of the calibration curve, and C = intercept of calibration curve.

Precision and Accuracy

The intra and inter-run precision and accuracy of the assay ($n = 6$) were determined by percent C.V and percent error values, respectively, based on reported guidelines.[7] Samples containing 5, 500, and 1500 ng mL^{-1} concentrations were spiked for the determination of precision and accuracy. Six replicates at each concentration were prepared from primary stock solutions on day 1, 3, 5, and 10 to determine intra-day and inter-day precision and accuracy. The intra-day precision of the assay was determined by analyzing six spiked samples at each concentration on the same day, and precision was determined by analyzing the samples on four different days. The percent error values were calculated by the following equation.

$$\text{Percent error} = \frac{(\text{Calculated concentration} - \text{Added concentration})}{\text{Added Concentration}} \times 100$$

Extraction Efficiency

The efficiency of the extraction method to recover fenoverine and I.S from serum was tested using samples containing 5, 500, and 1500 ng mL^{-1} fenoverine and appropriate concentrations of I.S. These samples were then subjected to the sample preparation procedure explained above. The areas of fenoverine and I.S in the extracted samples ($n = 3$) were then compared with those of unextracted samples ($n = 3$) containing equivalent concentrations of fenoverine and I.S in the injected solutions.

Stability Studies

Within-run stability was tested by reanalyzing quality control (QC) samples at three different concentration levels kept at 4°C. Long-term and freeze–thaw stability studies were evaluated together by analyzing QC samples at low, medium, and high concentrations, which were stored at 4°C for a week and then followed by three freeze–thaw cycles, each of which stored at −20°C for 24 h and thaw at room temperature.

RESULTS AND DISCUSSION

Chromatographic Separation

Under the experimental conditions used in this study, reproducible chromatographic separations between fenoverine and chlorpromazine were obtained in a mobile phase composed of acetonitrile, water, and 0.375% v/v triethylamine, pH adjusted to 2.5 with orthophosphoric acid in the ratio of 41:59 v/v. Representative chromatograms of a blank serum sample, a serum sample spiked with fenoverine and a volunteer sample are shown in Figure 2a, 2b, and 3, respectively. The retention times of fenoverine and chlorpromazine (I.S) were 6.76 and 8.19 min, respectively. No interference peaks were observed with either fenoverine or the internal standard in the chromatogram. System suitability parameters for the method were as follows: theoretical plates for fenoverine and I.S were 1587 and 1707, respectively. Tailing factor was less than 0.45 for both fenoverine and I.S.

There is not much information available on the quantitative analysis of fenoverine in dosage forms and in biological fluids. The proposed method is advantageous to that of reported method,[5] in which the retention times of fenoverine and chlorpromazine were 15.2 and 20.6 min with a flow rate of 1.5 mL min^{-1}. In our method, the retention times of fenoverine and chlorpromazine (I.S.) were 6.76 and 8.19 min, respectively, thus avoiding the loss of time and mobile phase.

Sample Preparation

Extraction of fenoverine from serum samples was carried by the liquid–liquid extraction procedure using dichloromethane as solvent. Sodium hydroxide is added to the sample to precipitate the drug from the serum, as it has a low solubility in alkaline conditions, and then addition of dichloromethane solubilized fenoverine with higher extraction recovery. Thus, dichloromethane was used as the extracting solvent.

Linearity and Sensitivity

The linearity of the detector response was assessed for various extracted serum standards over the range of 5 to 2000 ng mL^{-1}. The calibration curve of fenoverine exhibits excellent linearity and a high correlation coefficient. The equation of the calibration curve obtained from 6 points was $y = 0.0016x + 0.227$ ($r^2 = 0.9916$). The limit of quantification (LOQ), established by determining the concentrations of four spiked

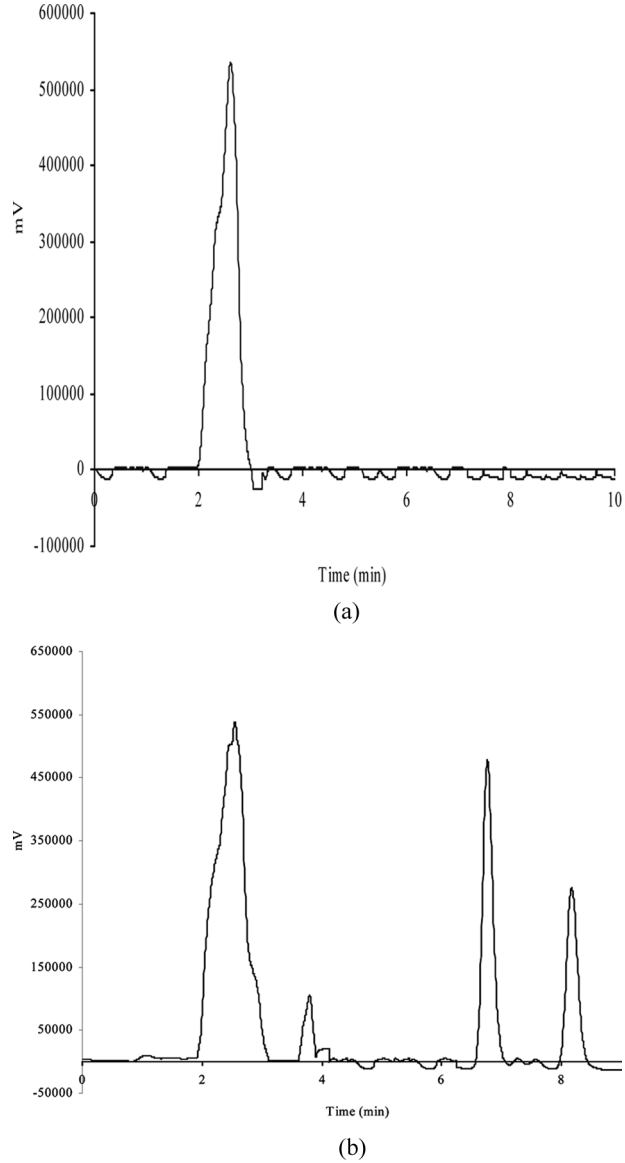

Figure 2. Representative chromatogram of (a) human blank serum and (b) serum with fenoverine and I.S.

calibration standards having a reproducibility with a coefficient of variation (%CV) less than 20% and an accuracy of 80 to 120%, was found to be $5\,ng\,mL^{-1}$. This LOQ was sufficient to determine fenoverine concentrations in human serum obtained in the pharmacokinetic study.

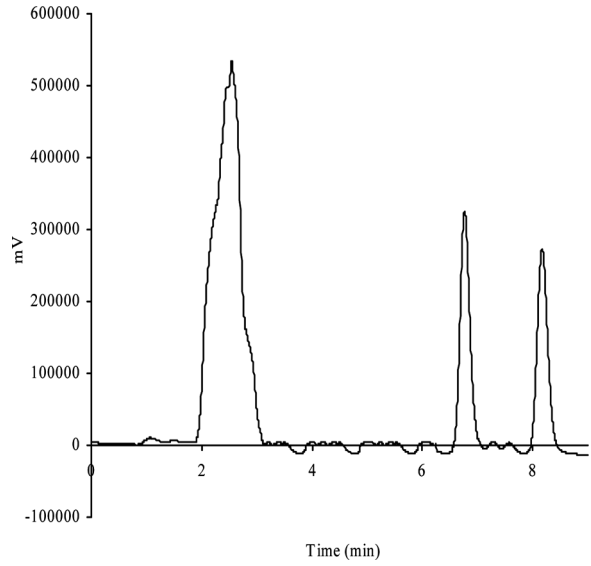

Figure 3. Chromatogram of volunteer sample containing fenoverine (RT, 6.76 min) and chlorpromazine (RT, 8.19 min.).

Precision and Accuracy

The precision and the accuracy were determined by analyzing serum samples spiked at concentrations of 5, 500, and 1500 ng mL^{-1}. The intra-day precision of the assay was determined by analyzing six spiked serum samples at each concentration on the same day. For the determination of precision, the samples were analyzed on four different days. The intra-day and coefficient of variation and error (%) values were shown in Table 1. These values were with in the limits (<15%) specified for inter and intra-day precision.[8,9]

Stability Studies

Stability results shown in Table 2, reveal that the analytes were stable for at least 7 days at 4°C after extraction. The mean concentrations for low, medium, and high QC samples after three freeze thaw cycles were 99.6, 100.0, and 99.7%, respectively. This was within the acceptable range of 90–110%. The drug was stable in serum during 7 days storage period at 4°C. The peak ratios of low, medium, and high standard samples were 102.6, 99.9, and 99.9, respectively. Oliver et al.[5] reported that the fenoverine did not show significant degradation in a commercial product

Table 1. Intra-day accuracy and precision of Fenoverine assay in proposed HPLC method

Concentration of Fenoverine ng ml^{-1}	Intra-day					
	Mean ± SD* ng ml^{-1}	% C.V	% Error	Mean ± SD* ng ml^{-1}	% C.V	% Error
5	4.97 ± 0.11	2.30	−0.006	5.02 ± 0.12	2.34	0.004
500	502.15 ± 3.85	2.69	0.004	501.13 ± 5.08	1.01	0.002
1500	1499.33 ± 5.13	2.84	0.000	1503.67 ± 16.76	1.11	0.002

*Readings are mean of six observations ($n = 6$).

at various temperatures with 75% relative humidity for 3 months. These results suggest that the drug is stable for long term storage.

Extraction Recovery

The recovery of fenoverine from serum was estimated at 5, 500, and 1500 ng mL^{-1} concentrations. Serum samples (in triplicates) containing fenoverine and I.S. were extracted and analyzed. Triplicate samples containing similar concentrations of fenoverine in mobile phase were directly injected and peak areas were measured. Absolute recoveries were calculated by comparing the peak areas of pure samples spiked and with the same amount of fenoveine in serum. The absolute recoveries ranged from 84.19–105.73 (Table 3). The liquid extraction procedure was simple, employing a single solvent with good reproducibility and high recovery. The recovery values indicate the suitability of dichloromethane as a solvent for good extraction recovery of the drug from serum.

Application of the Analytical Method to Bioavailability Study

The method described herein was utilized in a bioavailability study of fenoverine in human serum samples obtained after oral administration of

Table 2. Results of stability study ($n = 5$)

Fenoverine ng/ml	With in run stability		Freeze thaw stability	
	Mean ± SD (ng ml^{-1})	CV (%)	Mean ± SD (ng ml^{-1})	CV (%)
5	4.98 ± 0.06	1.205	5.13 ± 0.46	8.967
500	500.03 ± 1.65	0.330	499.52 ± 1.96	0.392
1500	1496.56 ± 10.27	0.706	1498.36 ± 15.34	1.024

Table 3. Recovery and accuracy of the proposed method

	Absolute recovery (%)		Accuracy (%)	
Conc ng/ml	Mean ± SD ($n = 3$)	Range (Min–Max)	Mean ± SD ($n = 3$)	Range (Min–Max)
5	85.75 ± 1.56	84.19 – 87.31	92.17 ± 1.63	90.54 – 93.80
500	94.63 ± 2.58	92.05 – 97.21	96.35 ± 2.74	93.61 – 99.09
1500	100.16 ± 5.56	94.61 – 105.73	100.28 ± 0.403	99.97 – 100.74

200 mg of floating mini tablets. The serum collected from the volunteer prior to the administration of the drug did not show the presence of any interfering endogenous peaks. A representative chromatogram of fenoverine extracted from serum is given in Figure 3. It is clearly evident from the figure that there were no interference peaks in analyte and I.S retention times in the real sample, indicating the specificity of the developed method. A typical serum concentration time plot of fenoverine from the healthy human volunteer is shown in Figure 4. The pharmacokinetic parameters determined are Tmax 2 h, Cmax 368.12 ng mL^{-1}, AUC 3861.11 ng mL^{-1} h^{-1}, and half-life of 8.33 h. Thus, the developed method was successfully applied for pharmacokinetic study in human volunteers.

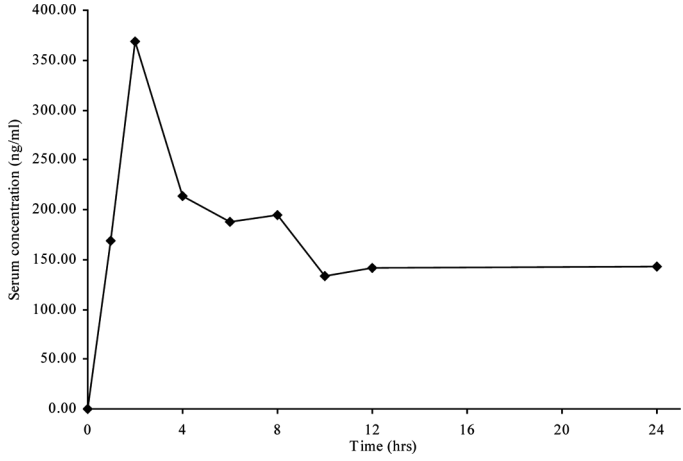

Figure 4. Serum Concentration-time profile of fenoverine after oral administration of 200 mg floating mini tablets.

CONCLUSION

A validated, specific HPLC method for the determination of fenoverine in human serum has been developed. Its specificity without interference peaks from endogenous components is very useful for quantitative analysis of fenoverine in human serum. The developed method was rapid with a run time of 10 min and with good reproducibility. This method required a sample volume of 1 mL for accurate and precise determination of fenoverine in human serum. Finally, the developed method has been successfully utilized for a bioavailability study of fenoverine sustained release tablets in healthy human volunteers. Also, the developed HPLC method is suitable for routine estimation of fenoverine in serum samples in the range of 5 ng to 2000 ng mL^{-1}.

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