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Development and validation of a new high-performance liquid chromatographic assay of centpropazine, a new antidepressant compound, in serum☆

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Abstract

Centpropazine is a new anti-depressant compound developed by Central Drug Research Institute, Lucknow (India). We report here the development and validation of a new HPLC assay of a parent drug in the serum of humans, monkeys and rats for pharmacokinetic studies. Centpropazine was eluted on a C_{18} column with a mobile phase consisting acetonitrile–phosphate buffer (60:40) pumped at 1.5 ml min^{-1} flow rate and quantitated by UV detector at 270 nm. Considering the sample volume available from different species and to enhance the sensitivity of assay, three sample clean up methods requiring 0.05, 0.5 and 4 ml serum for a linear quantitation range of 312.5 ng ml^{-1}–5 μg ml^{-1}, 0.04–2.5 μg ml^{-1} and 2.5–80 ng ml^{-1} respectively were developed. The lowest limit of quantitation of the method was 2.5 ng ml^{-1} requiring 4 ml serum, 40 ng ml^{-1} requiring 0.5 ml serum and 312 ng ml^{-1} requiring 50 μl serum sample. All these methods were fully validated in human serum and extended to monkey and rat serum. The recovery of centpropazine at 5, 80, 625, 1280 and 2500 ng ml^{-1} ranged between 92 and 105%. The within and between run variability in precision and accuracy were less than 10% and the drug in serum was stable up to three freeze-thaw cycles. Overall the method is simple, quick and robust for biopharmaceutical applications. The method was applied to analyse concentrations of centpropazine in rat serum after administering single 20 mg kg^{-1} peroral and 5 mg kg^{-1} iv dose. The chromatograms of treated rat serum exhibited three well resolved peaks of metabolites and one of them was identified as hydroxy-metabolite of centpropazine.

Keywords: Antidepressant; Centpropazine; HPLC; Bioanalysis; Validation

1. Introduction

Centpropazine, 1-(*p*-propionylphenoxy)-3-(N^4-phenyl-piperazinyl) propane-2-ol (Fig. 1), is a new anti-depressant agent synthesised in this institute [1,2]. The racemate of this compound has been granted marketing permission in India as an oral

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antidepressant drug. Structurally it is different from classical tricyclic anti-depressants and has very little autonomic effects in animals [3]. It was found to be safe and well tolerated in human up to 200 mg after single dose and up to 80 mg day^{-1} in multiple dose studies [4]. In multicentric trial, anti-depressant response of centpropazine was more than 80% at the end of 4 weeks medication in 42 patients suffering from depression [5].

Physicochemical properties such as IR and UV characteristics and analytical methods for centpropazine in bulk drug and formulations have been reported [6,7]. However to date there is no reported assay method of centpropazine in biosamples. To investigate serum levels and pharmacokinetic behaviour of centpropazine in laboratory animals and humans we have developed and validated the present HPLC assay in biological matrix (serum) of animals and humans. During development of the assay method, analysis of serum samples from human subjects receiving single 40 mg oral dose indicated very low serum levels (<2.5 ng ml^{-1}). Hence, prior to HPLC analysis it was necessary to enrich the drug in the matrix during the sample preparation step to achieve high sensitivity. At the same time in small experimental animals like rats; large volumes of frequent samples (serum) are not available for pharmacokinetic studies. Hence for clinical as well as preclinical pharmacokinetic studies, an HPLC assay method using 0.05, 0.5 and 4 ml serum was developed and validated. The physico-chemical properties such as lipophilicity (log P), pKa, and solubility of centpropazine were also determined to help in developing its sample clean-up and HPLC assay in biological samples.

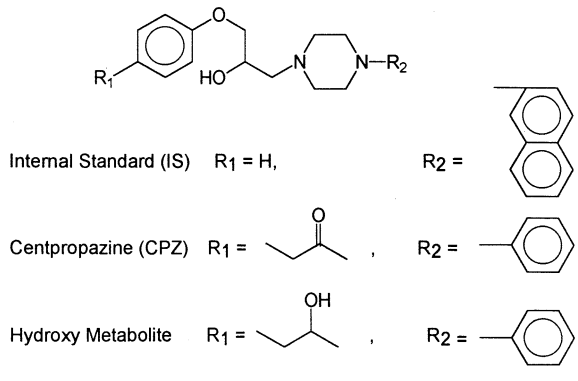

Fig. 1. Chemical structure of centpropazine, its hydroxy metabolite and internal standard.

2. Experimental

2.1. Chemicals and reagents

The reference standard of centpropazine and Internal Standard, 1-phenoxy-3-(N^4-β-napthyl-piperazinyl) propane-2-ol (IS; Fig. 1) were synthesised and purified ($>99\%$) in this institute. HPLC grade n-hexane, acetonitrile, methanol were obtained from E. Merck (Bombay, India). 1-Octanol was obtained from E. Merck (Germany). HPLC grade chloroform, isopropanol, ethyl acetate (ExcelaR grade), orthophosphoric acid (ExcelaR grade) were obtained from Sarabhai M. Chemicals (Baroda, India). Diethyl ether (Solvent Ether) was purified before use by washing with potassium hydroxide followed by distillation. Triple distilled water obtained from an all quartz distillation unit was used to prepare the mobile phase, buffers and reagents.

Blood from untreated healthy volunteers was procured through a local blood bank. Serum was separated and pooled. Drug free rat and monkey serum pool was prepared from the blood collected from young, healthy animals of the laboratory animal division of this institute. The serum was stored at $-30°C$.

2.2. HPLC system and chromatographic conditions

The system used for this work consisted of a HPLC pump (Kontron, Model 600, Zurich, Switzerland) coupled with a Uvikon 730S LC variable wavelength detector (Kontron, Zurich, Switzerland) set at 270 nm. Samples were injected through a 50 μl loop injector (Rheodyne, Model 7125, Cotati, CA, USA). Chromatograms were recorded and integrated by Nelson Analytical Chromatography Software (Nelson Analytical, Cupertino, CA, USA). Separations were achieved on a reverse phase C_{18} column (Spheri-5, 5 μm,

220 × 4.6 mm ID) preceded by a guard column (30 × 4.6 mm) (Pierce Chemical Co., Rockford, IL, USA) packed with the same material. A few chromatograms of treated rat serum were also recorded and integrated with Waters model 991 photo diode array detector. Savant Speed-vac concentrator; model SVC-200H (Savant Instruments, NY, USA) was used to evaporate the organic solvent. Mobile phase consisted of phosphate buffer (pH 3.5, 25 mM) and acetonitrile (40:60 v/v). It was filtered and degassed before use and pumped at 1.5 ml min^{-1} flow rate.

2.3. Partition coefficient (Log P)

The determination of partition coefficient was carried out according to the method of Said et al. [8]. Phosphate buffer (pH 7.4, 20 mM) and 1-octanol were mutually saturated for 6 h on magnetic stirrer and separated. A solution of centpropazine (1 mg ml^{-1}) was prepared in octanol saturated with buffer. Volumes of 2 ml of buffer and octanol containing centpropazine were placed in sealed glass tubes ($n = 7$). These tubes were tumble mixed at the rate of about 100 inversions per minute for 2 h at 25°C. The organic and aqueous phases were separated by centrifugation at 2500 rpm for 20 min. Centpropazine concentration in aqueous and octanol phase were determined by HPLC after a suitable dilution in mobile phase.

2.4. Solubility in organic solvents

Solubility of centpropazine was determined in organic solvents such as: chloroform; methanol; acetonitrile; ethyl acetate; octanol; and n-hexane. An excess amount of centpropazine was placed along with 2 ml each of the solvent in the air tight screw capped glass tubes fitted with teflon liner. The tubes were sonicated and tumbled for more than 24 h at 25°C. The tubes were then centrifuged at 1000 × *g* for 15 min. Aliquots of supernatant were withdrawn and analysed by HPLC after suitably diluting with mobile phase.

2.5. pKa determination

Ionization constant of centpropazine was determined by the aqueous solubility method [9]. Buffers of different pH range (5–10) were prepared. Excess amount of centpropazine (approximately 1 mg) was placed along with 10 ml of each buffer in the air tight screw capped glass tubes fitted with teflon liner. The tubes were sonicated and tumbled at 25°C for 24 h and then centrifuged at 1000 × *g* for 15 min at 25°C. Aliquots of supernatant were withdrawn and analysed by HPLC after suitably diluting with mobile phase.

2.6. Stock and standard solutions

Stock solutions of centpropazine (800 μg ml^{-1}) and IS (200 μg ml^{-1}) were prepared separately in acetonitrile by dissolving 40 and 10 mg of the respective compounds in 50 ml. Subsequent working standards of centpropazine (80 μg ml^{-1}) and IS (8 μg ml^{-1}) were made in acetonitrile from stock solutions by appropriate dilution. Further dilutions of centpropazine were prepared from working standard (80 μg ml^{-1}) in mobile phase in the range of 3.2–0.1 μg ml^{-1} for the determination of recovery.

2.7. Calibration graph

Calibration and quality control (QC) samples of centpropazine from 2.5 ng ml^{-1} to 5 μg ml^{-1} in serum were prepared by adding varying volumes of stock and working standard solutions in appropriate volume of pooled drug free human serum so that the volume ratio of organic phase added to serum was less than 2%. Calibration and QC standards were stored at −30°C until analysis. Prior to HPLC analysis these analytical standards and QC samples were processed by three different methods according to three calibration ranges as outlined in the following sections:

2.7.1. Standard curve representing 2.5–80 ng ml^{-1} (method A)

To drug free or spiked serum (4 ml) was added 1 ml TDW, 200 μl; 2 M potassium hydroxide, 50 μl IS (8 μg ml^{-1}) and 10 ml extraction solvent (2% isopropanol in diethyl ether) in a 30 ml glass tube. The tubes were vortex mixed for 1 min and centrifuged at 1000 × *g* for 5 min. The organic

phase was transferred to another tube by freezing the aqueous layer in liquid nitrogen and evaporated to dryness in speed vac concentrator. The residue was acidified with 300 μl of 0.5 M hydrochloric acid and washed with 2 ml n-hexane. The acid layer was basified with 200 μl of 2 M potassium hydroxide solution and extracted with 3 ml ether. The organic phase was transferred to another tube by freezing the aqueous layer in liquid nitrogen and evaporated to dryness in speed vac concentrator. The residue was reconstituted in 100 μl mobile phase and injected onto the HPLC system fitted with 50 μl loop. The internal standard method was used for quantitation. The calibration curve was obtained by weighted regression $(1/x)$ of the peak height ratio of centpropazine and IS versus concentration with Microsoft Excel version 5.0 on IBM PC computer.

2.7.2. Standard curve representing 40 ng ml^{-1}–2.5 μg ml^{-1} (method B)

To drug free or spiked serum (0.5 ml) was added 50 μl of 2 M potassium hydroxide and 3 ml diethyl ether. The tubes were vortex mixed for 1 min and centrifuged at $1000 \times g$ for 10 min. The organic phase was separated by snap freezing the aqueous layer in liquid nitrogen and evaporated in speed vac concentrator at 40°C. The residue obtained after single extraction was reconstituted in 100 μl mobile phase and injected onto the HPLC fitted with the fixed 50 μl loop. The external standard method was used in quantitation. The calibration curve was obtained by weighted regression $(1/x)$ of the peak height of centpropazine versus concentration on Microsoft Excel.

2.7.3. Standard curve representing 312.5 ng ml^{-1}–5.0 μg ml^{-1} (method C)

To 50 μl drug free or spiked serum in 0.5 ml eppendorf tubes, 100 μl acetonitrile was added. The tubes were vortex mixed and left in the dark for 30 min. These tubes were centrifuged at $5000 \times g$ for 20 min at 0°C and 100 μl of the clear supernatant was injected onto the HPLC. Calibration curve was plotted as outlined in method B.

2.8. Freeze thaw (f–t) stability

QC samples (0.5 ml) at 625 ng ml^{-1} and 2.5 μg ml^{-1} concentration were stored at −30°C in glass tubes. One set comprising triplicate samples of each concentration were analysed on day 1, immediately after spiking (without being frozen), and other samples after 1, 2 and 3 f–t cycles. Thawing was achieved by keeping the samples tubes at ambient temperature for 0.5 h. The percent loss of drug during f–t cycles was determined by comparing the concentrations with that of day 1.

2.9. Method validation

The validation programme for the new HPLC method for centpropazine included within and between precision and accuracy studies on four different days and f–t effects (three cycles). These studies were carried out in triplicates and at two concentrations low: 5 ng ml^{-1}, method A; 80 ng ml^{-1}, method B; 625 ng ml^{-1}, method C; and high: 80 ng ml^{-1}, method A; 1.28 μg ml^{-1}, method B; 2.5 μg ml^{-1}, method C.

2.10. Lowest limit of detection (LOD) and quantitation (LOQ)

The detection limit of the HPLC assay (LOD) of centpropazine after each sample clean up method was estimated as the drug quantity in serum which correspond to three times the baseline noise. The lowest limit of quantitation (LOQ) was defined as the concentration quantity of the sample which was quantified with less than 20% deviation in precision.

2.11. Accuracy and precision

The accuracy of each sample preparation method was determined by injection of calibration samples and three QC samples on four different days ($n = 12$; three each of low and high concentration). The precision was determined by one way ANOVA as within and between % RSD. The accuracy was expressed as % bias:

Table 1
Solubility of centpropazine in organic solvents at 25°C

Solvent	Solubility (mg ml^{-1})
Chloroform	88.75
Ethyl acetate	2.87
Acetonitrile	2.28
Methanol	1.82
n-Octanol	0.72
n-Hexane	0.010

$$\text{Bias} = \frac{(\text{Observed concentration} - \text{Nominal concentration}) \times 100}{\text{Nominal concentration}}$$

2.12. Analysis of centpropazine in monkey and rat serum

HPLC assay method developed and validated in human serum was extended in rat and monkey serum by examining the chromatograms for the possible interference and comparing the recovery of centpropazine from these serum matrices. Blanks and spiked serum standards of each species fortified with centpropazine at high and low concentrations of each method in triplicates were processed as described under the sample preparation and analysed on HPLC. Patterns of the chromatograms of the blank serum of all the species were compared and checked for interference. Recovery from the spiked serum of rat and monkey by each method was compared with the corresponding human serum standard.

2.13. Application of assay in rats

Application of the new HPLC method(s) was demonstrated by determining the concentration of centpropazine in male Sprague–Dawley rats. The rats (250 ± 20 g) were procured from the Laboratory Animal Division of the Institute and acclimatised for 3 days prior to study with 12 h alternate dark and light cycle. Each rat received either single oral (20 mg kg^{-1}) or bolus iv (5 mg kg^{-1}) dose via caudal vein. Two blood samples were collected from each rat by cardiac puncture and finally from vena cava (terminal) so as to get a minimum of five samples at each time point (0, 0.08, 0.5, 0.75, 4 and 24 h post dose). Serum required for analysis was separated and stored at -30°C until analysis.

3. Results and discussion

3.1. Log P, pKa and solubility

The Octanol buffer (pH 7.4) partition coefficient (P) and log P were found to be 1426 ± 43 and 3.15 ± 0.013 respectively. The pKa of centpropazine determined by solubility method was found to be 6.98 ± 0.086. Aqueous solubility of centpropazine was pH dependent and increased with decrease of pH from 9 to 5. In organic solvents solubility of centpropazine was found to decrease among the series from chloroform > ethylacetate > acetonitrile > methanol > 1-octanol > n-hexane. Solubilities of centpropazine in these solvents are given in Table 1.

3.2. Assay development

The successful analysis of drugs in the biological fluids in HPLC relies upon the optimisation of the sample preparation, chromatographic separation and post column detection. Each of the three steps was carefully optimised for developing a sensitive, selective, reproducible and robust assay of centpropazine in 0.05, 0.5 and 4 ml serum matrix. The compound has native fluorescence in mobile phase (excitation = 250 nm, emission = 340 nm). UV scan of centpropazine in mobile phase exhibited optimum absorption at 270 nm. Various mobile phases with different compositions were tried to elute the drug on C_{18} column and the chromatograms were studied for peak shape, sensitivity and selectivity. With mobile phase containing 60% acetonitrile and 40% buffer (pH 3.5) at a flow rate of 1.5 ml min^{-1} the compound eluted as a sharp peak. Sample clean-up of 4 ml of serum was done by single direct

extraction with ether fortified with 2% IPA. Addition of 2% IPA in extraction solvent minimised the emulsification of serum during extraction step and 1 ml water was added to reduce the viscosity of serum to improve the extraction efficiency. The chromatograms from 4 ml of the drug free serum exhibited an interference peak at the retention time of centpropazine. This endogenous interfering peak of serum was removed by back extraction with hexane and ultimately the centpropazine was enriched 40 times in the final step. The low solubility of centpropazine in hexane was an advantage in using it for back washing. No interference was observed with methods involving 0.5 ml and 50 μl serum. The representative chromatograms obtained with each of these methods for human, monkey and rat serum are compiled in Figs. 2a,b, 3a,b, and 4a,brespectively. Calibration curve equations of the three extraction methods during validation are given in Table 2.

Internal standard was added only in sample clean-up steps of method A (using 4 ml serum) to compensate the losses during multiple steps. With almost 100% recovery, it was found to have no effect on precision and accuracy of method B and C. Hence IS was not added in method validation by method B and C.

3.3. Assay validation in human serum

3.3.1. Recovery

The recoveries of centpropazine from spiked serum samples were calculated by comparing peak heights at low and high concentration levels with those obtained from the analysis of corresponding standard dilutions in mobile phase injected directly. The recovery of centpropazine was complete (range 94–105%) by three sample clean up methods as given in the Table 3.

3.3.2. Accuracy and precision

Table 2 summarises the within and between run precision and accuracy for the determination of centpropazine in fortified human serum by three

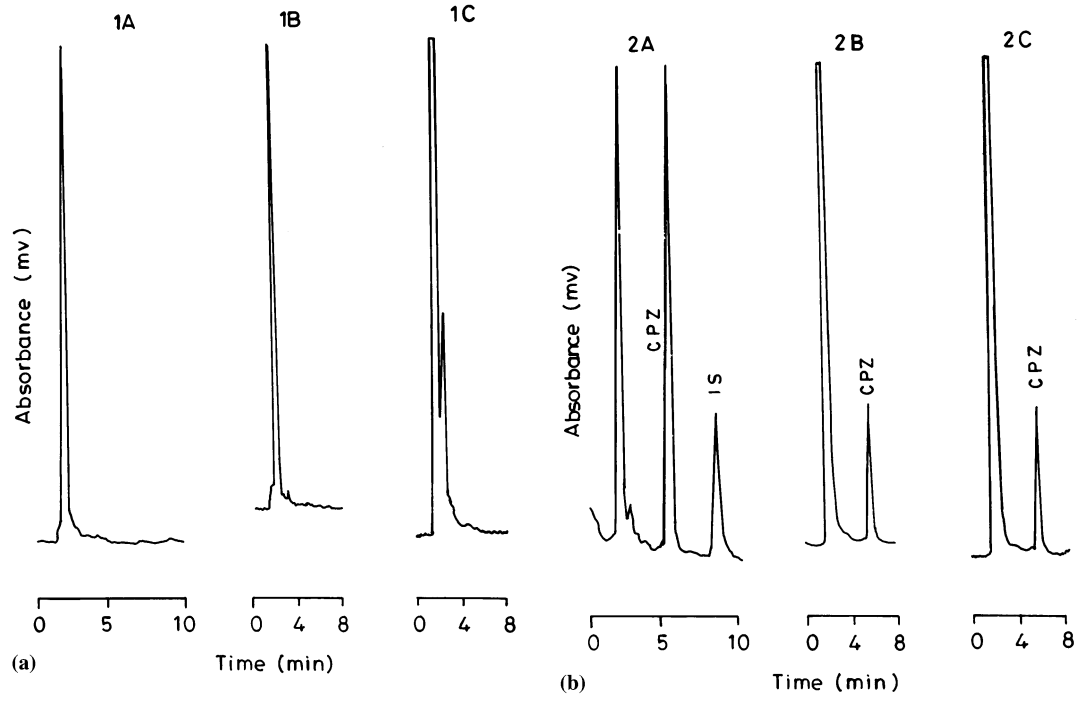

Fig. 2. (a) Representative chromatograms of (1) drug free human serum samples processed by method (A) 4 ml, (B) 0.5 ml and (C) 0.05 ml samples. (b) Representative chromatograms of (2) fortified human serum samples processed by method (A) 4 ml, (B) 0.5 ml and (C) 0.05 ml samples.

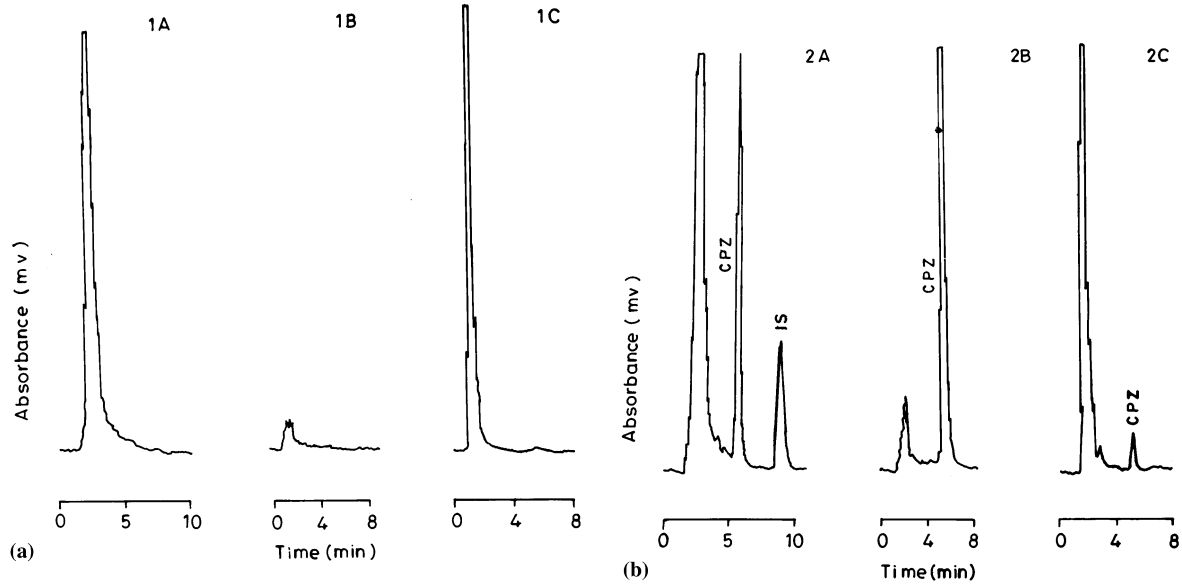

Fig. 3. (a) Representative chromatograms of (1) drug free monkey serum samples processed by method (A) 4 ml, (B) 0.5 ml and (C) 0.05 ml samples. (b) Representative chromatograms of (2) fortified monkey serum samples processed by method (A) 4 ml, (B) 0.5 ml and (C) 0.05 ml samples.

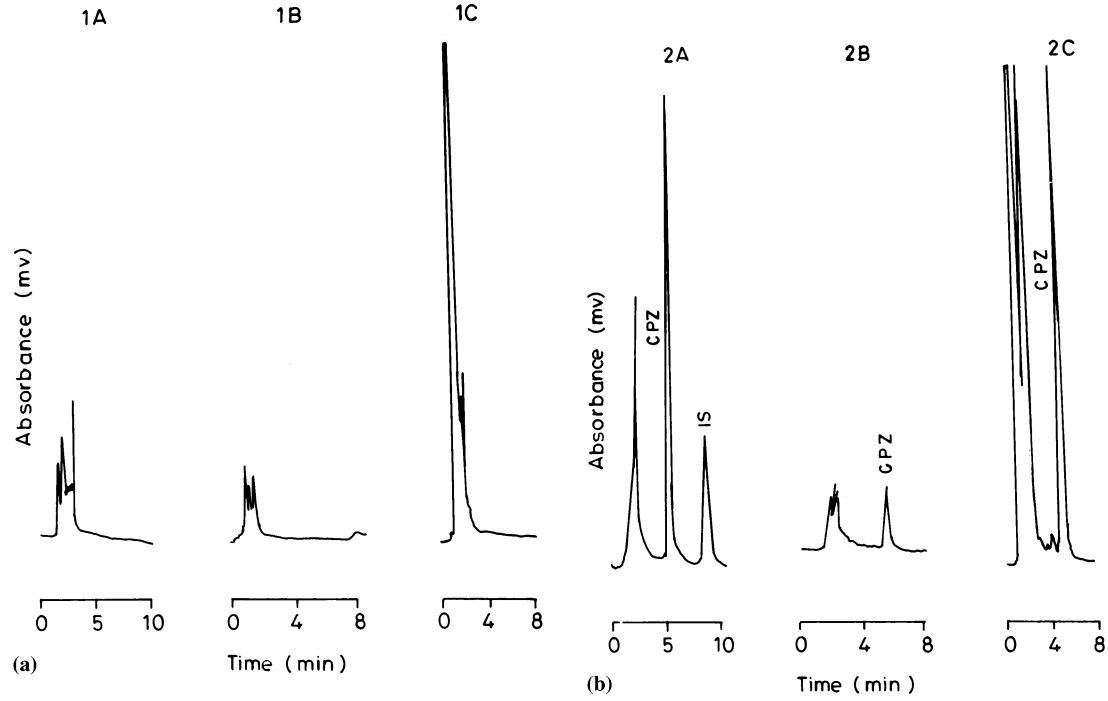

Fig. 4. (a) Representative chromatograms of (1) drug free rat serum samples processed by method (A) 4 ml, (B) 0.5 ml and (C) 0.05 ml samples. (b) Representative chromatograms of (2) fortified rat serum samples processed by method (A) 4 ml, (B) 0.5 ml and (C) 0.05 ml samples.

Table 2
Calibration curve equations of three extraction methods during assay validation of centpropazine

Extraction method	Equation	Weight	Slope (m)	Intercept (c)
Method A (4 ml)	$y=mx+c$	$1/x$	0.04	0.006
Method B (0.5 ml)	$y=mx+c$	$1/x$	60.40	−83.74
Method C (0.05 ml)	$y=mx+c$	$1/x$	4.035	27.28

sample clean up methods. Within and between run RSD% (precision) by all the methods at low concentration were less than 10 and 7% at high concentration. The accuracy of the method expressed as % bias for within and between run were less than 7% at low and high concentrations.

3.3.3. Freeze–thaw Stability

Stability of centpropazine in serum samples was monitored at −30°C up to 6 months and its concentrations did not change up to three f–t cycles. The f–t stability results plotted in Fig. 5 indicate less than 1% deviation from the nominal concentration at both high and low concentration levels.

3.4. Validation of HPLC assay in monkey and rat serum

The HPLC method as described above in human serum was extended in monkey and rat serum by a two step method: First; it was ensured that the chromatograms of drug free monkey and rat serum processed by the method of human serum did not elute any peaks of endogenous components of serum in the region of interest, secondly the recovery of centpropazine was 100% as compared to human serum by all the three methods of sample clean up at high and low concentrations. The recovery experiments in triplicate indicated insignificant difference in recovery of centpropazine at high and low concentrations in rat and monkey and human serum by all the three methods.

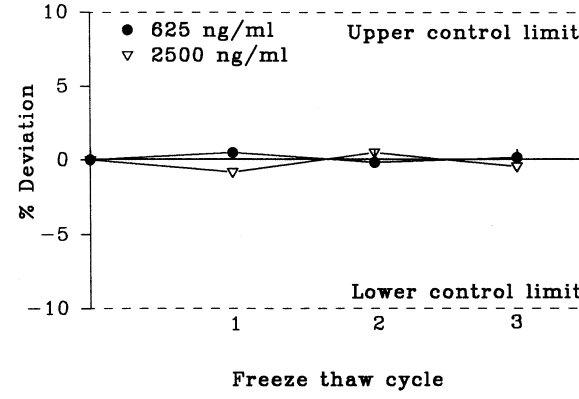

Fig. 5. Effect of freeze thaw cycles on the stability of centpropazine in human serum.

Table 3
Precision, accuracy and recovery of centpropazine in spiked human serum

Method	Concentration (ng ml^{-1}) ($n=3$)	Recovery (%)	Bias (%)		RSD (%)	
			Within-run	Between-run	Within-run	Between-run
A	5	100.4 ± 6.5	−1.1	−1.3	9.2	9.2
	80	98.8 ± 3.7	1.6	2.2	5.1	4.6
B	80	104.4 ± 6.3	6.1	6.4	2.6	1.0
	1280	102.5 ± 3.4	5.4	6.3	1.7	2.0
C	625	102.9 ± 6.9	5.2	4.6	1.0	3.6
	2500	94.8 ± 2.01	2.0	2.5	0.8	6.4

Table 4
Serum levels of centpropazine in rats receiving single bolus dose by oral (20 mg kg^{-1}) and iv (5 mg kg^{-1}) route[a]

Time (h)	Concentration (ng ml^{-1})	
	iv	oral
0.08	1880	ND
0.5	327	ND
0.75	184	ND
1	100	ND
4	2.2	4
24	ND	ND

[a] ND, below quantitation limit.

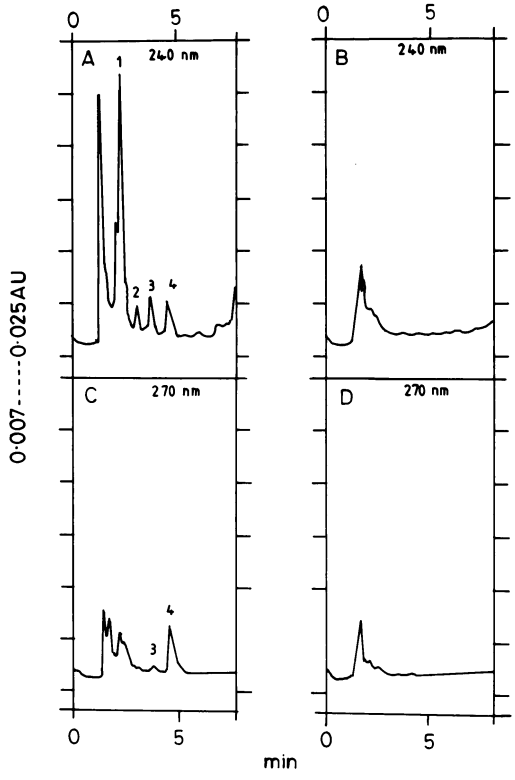

Fig. 6. Representative chromatograms (A and C = 45 min sample; B and D pre dose sample) on photo diode array detector from serum of rat receiving single 5 mg kg^{-1} iv dose of centpropazine and processed by method B.

3.5. Application of the HPLC assay

Serum samples of rats receiving single 5 mg kg^{-1} iv dose and 20 mg kg^{-1} peroral dose were analysed for centpropazine by the proposed method using sample cleanup method A, B and C. The concentration–time profile is given in the Table 4. The chromatograms did not exhibit any interference from endogenous components of serum or the metabolites. However the chromatograms obtained on Photo diode array detector showed three well resolved peaks (peak 1, 2 and 3; Fig. 5) prior to centpropazine (peak 4). The peak response of these metabolites was maximum at 240 nm whereas the parent compound showed maximum peak response at 270 nm. The chromatograms of blank serum were devoid of endogenous interfering components. The representative chromatograms of blank and treated rat serum on Photo diode array detector are shown in Fig. 6. The retention time and UV spectra of peak 3 matched with its hydroxy metabolite,1-*p*-(α-hydroxypropyl)-phenoxy-3-(N^4-phenyl-piperazinyl) propane-2-ol (Fig. 1). This Hydroxy metabolite has two asymmetric centres, implying a possible four enantiomers in vivo. However these enantiomers were not resolved on the present HPLC system. The other two metabolites could not be characterised.

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