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Short communication

Determination of centpropazine and its metabolite in rat serum by high-performance liquid chromatography and fluorescence detection

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Abstract

A new HPLC assay method was developed for the simultaneous assay for centpropazine (antidepressant) and its hydroxylated metabolite (II) to assess their pharmacokinetics and metabolism characteristics. Rat serum samples were extracted with ether, backwashed with *n*-hexane and injected onto the HPLC system, which used a C_{18} column, gradient elution and fluorescence detection at 250 Ex/350 nm Em. Variations in intra- and inter-batch accuracy and precision were within acceptable limits of $<\pm 20\%$ at low and $<\pm 15\%$ at higher concentrations. Samples were stable in autosampler prior to injection and after multiple freeze–thaw cycles. Linearity was observed between 0.625 and 20 ng/ml for both I and II in serum. Overall the method developed was highly sensitive and could be employed for a wide range of studies.

Keywords: Centpropazine

1. Introduction

Centpropazine, *R*/*S* (±), 1-(*p*-propionylphenoxy)-3-(N^4-phenyl-piperazinyl) propane-2-ol (I) (Fig. 1) is an anti-depressant agent synthesized in this institute [1,2]. Renewed interest of the institute in this molecule which has shown wider safety margin in acute toxicity studies as compared to imipramine and was safer than tricyclics in overdosage by suicidal patients, a persistent risk in severely depressed patients, demanded the establishment of its pharmacokinetics and metabolism parameters. Given chronically to rats, centpropazine like imipramine depressed the density of 5-HT_1 and 5-HT_2 receptors in the cortex but differed from imipramine in producing no β-down regulation [3]. The racemate of this

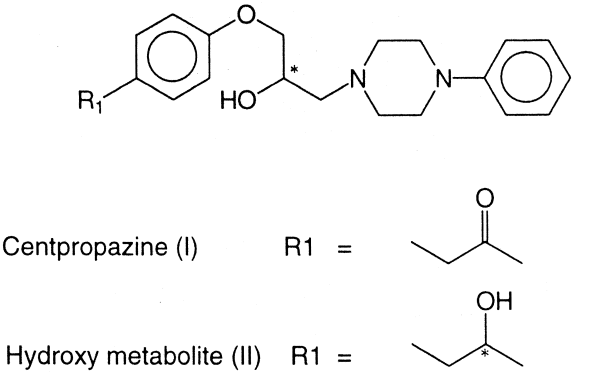

Fig. 1. Chemical structure of the analytes (I and II).

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compound has been granted marketing permission in India as an oral antidepressant drug. Preliminary pharmacokinetic studies indicated that I has very poor oral bioavailability owing to the low serum levels observed after administering 120 mg to patients. Hence it was necessary to investigate the possible reasons further. A method for estimation of I alone in serum using isocratic conditions (LOQ 2.5 ng/ml) and UV detection has been reported [4] which was not sensitive enough to detect levels as low as 2 ng/ml after oral dose of I in rats. Since determination of the bioavailability of I in rats and humans was important for its further development, priority was accorded to the development and validation of more sensitive assay method (LOQ 0.625 ng/ml) for the parent drug and one of its major metabolite (II) which was characterised and synthesised in our lab. Inclusion of the metabolite in the validation program will enable us to characterise the pharmacokinetics and metabolic features of the drug in vivo in a much better way.

Here we report a new gradient HPLC assay method with fluorescence detection for the estimation of the drug and its metabolite 1-*p*-(α-hydroxypropyl)-phenoxy-3-N^4-phenyl-piperazinyl) propane-2-ol (*R*/*S*, ±) (II) (Fig. 1). The method was found to be applicable to detect very low levels of I and II (both as sum of *R* and *S* forms) after a single oral dose of I at 40 mg/kg in rats. Resolution of other unknown metabolites from I and II was also accorded priority.

2. Experimental

2.1. Chemicals

Pure reference standards (I and II) (assay >99%) was synthesised and purified in this institute. HPLC grade *n*-hexane, acetonitrile, methanol were obtained from Ranbaxy Labs (Delhi, India). Diethyl ether (Solvent ether) was purified before use by washing with potassium hydroxide followed by distillation. Triple distilled water obtained from all quartz distillation unit was used to prepare the mobile phase, buffers and reagents. Drug free rat 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 and was used within 7 days. Mixed stock solution of I and II (200 μg/ml) were prepared by dissolving 10 mg of the compounds in 50 ml acetonitrile.

2.2. Instrumentation

A low pressure gradient HPLC, Shimadzu, Japan (LC-10ATvp) with CBM-10A (Communication Bus Module), FCV10Alvp (quaternary valve) and DGU-14 (degasser) was used to pump the mobile phase [Valve A: 100% phosphate buffer, (25 m*M*, adjusted to 3.5 with orthophosphoric acid); Valve B: 70:30% v/v acetonitrile: phosphate buffer] at 1.2 ml/min. Chromatographic separations were performed on a C_{18} reversed-phase column (Spheri-5, 5 μm, 220×4.6 mm I.D.) preceded by a guard column (30×4.6 mm I.D.) (Perkin-Elmer, Norwalk, CT, USA) of the same material. A gradient system was used for optimum separation of the analytes. In this system the acetonitrile content was increased from 35 to 45% in 20 min and reached 65% in 45 min. The increase in both the steps was in linear fashion. The acetonitrile content was then decreased to 35% by 55-min post injection. Flow rate was 1.2 ml/min. Re-equilibration time was 10 min between runs. Mobile phase solvents were filtered and degassed before use. Samples were injected by SIL-10ADvp auto-injector fitted with 50 μl loop. After elution the compounds were monitored using a model RF-10Axl, spectrofluorimetric detector set at 250 nm (Ex)/350(Em). Chromatographic peaks were integrated using Class LC10 work station (Shimadzu, Japan).

2.3. Sample preparation

To drug-free or spiked serum (2 ml) was added 100 μl 2 *M* KOH and 6 ml of extraction solvent (diethyl ether) in 20 ml glass tube. The tubes were vortex mixed for 1 min and centrifuged at 1000 *g* for 5 min. The organic layer was transferred to another tube by freezing the aqueous layer in liquid nitrogen and evaporated to dryness under reduced pressure in speed vac concentrator (Savant Instrument, Farmingdale, NY, USA) below 40°C. The residue was acidified with 300 μl 0.5 N hydrochloric acid and washed with 2×2 ml of *n*-hexane. The acid layer

was basified with 200 μl of 2 *M* KOH and extracted with 3 ml ether. The organic layer 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 0.1 ml acetonitrile and injected onto HPLC.

2.4. Calibration, precision, accuracy and recovery

The stock solution of I and II were diluted in acetonitrile to prepare standards comprising six concentrations from 12.5 to 400 ng/ml (12.5, 25, 50, 100, 200 and 400 ng/ml). These were diluted in drug-free serum to achieve concentrations ranging from 0.625 to 20 ng/ml (0.625, 1.25, 2.5, 5, 10 and 20 ng/ml). Calibration curves were constructed as the peak area vs. I or II concentration and linear weighted (weight=$1/x$) least-squares regression was performed to determine the slope and intercept. The validation programme for the new HPLC method for I and II included within and between precision and accuracy studies on three different days, autosampler stability and freeze–thaw (f–t) effects. Accuracy and precision of the assay method were studied at low (1.25 ng/ml); medium (5 ng/ml); high (20 ng/ml) concentration levels. Triplicate serum samples at each of the three concentration levels were processed and analysed in each run and three such batches were assessed. Variations in accuracy and precision were expressed as % bias and relative standard deviation (RSD), respectively. Acceptance limits of ±20% at the lowest limit of quantitation (LLOQ) and ±15% at other concentrations in the calibration range were used for validation [5]. The recovery of both the compounds was assessed by comparing the peak areas at different concentration levels with the standard curve obtained by analyzing the corresponding standard dilutions in acetonitrile.

2.5. Stability studies

2.5.1. Freeze–thaw (f–t) stability

Stability studies of I and II in serum were evaluated up to three freeze–thaw cycles by protein precipitation method as reported earlier [4]. Three sets of 0.156, 0.625 and 2.5 μg/ml were stored at −30°C, analysed on day 0 (no f–t cycle) and after one, two and three f–t cycles. Thawing was achieved by keeping the tubes at room temperature for 30 min. The samples were analysed by diluting them in acetonitrile (1:2) and centrifuging at 5000 rpm for 10 min at 4°C. The supernatant was directly injected into HPLC. Concentrations obtained from the spiked serum samples not subjected to f–t cycles were

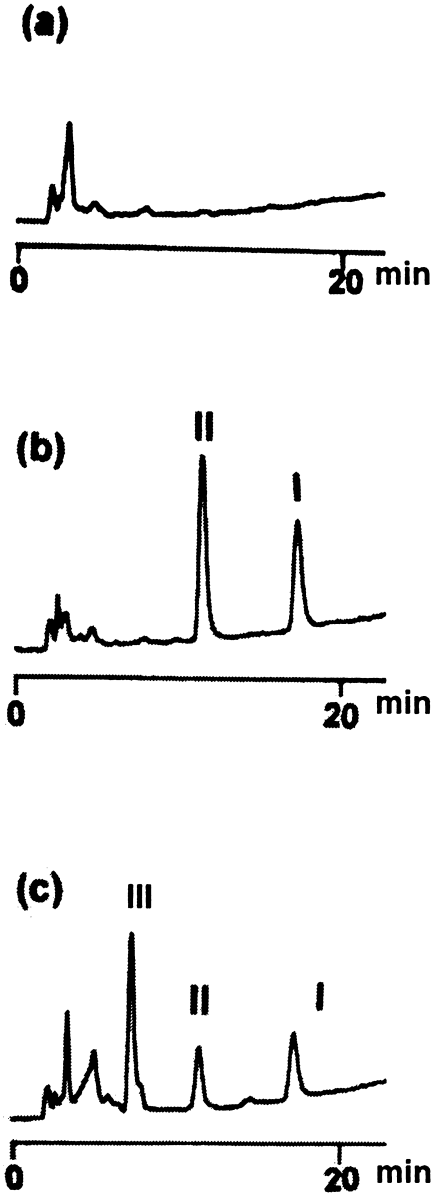

Fig. 2. Chromatograms of (a) blank rat serum (b) spiked rat serum (10 ng/ml of I and II) (c) 30 min post dose serum sample containing 5 ng/ml I and 3 ng/ml II.

considered 100% and those calculated on analysis after subsequent f–t cycle were compared with the initial concentrations.

2.5.2. Stability in autosampler

Stability of I and II in autosampler prior to injection was evaluated by comparing the chromatograms and peak areas of the QC (low, medium and high) samples injected immediately after reconstitution (0 h) and at the end of analysis of all samples. (Maximum loading at one time was 12 h, i.e., 12 samples.)

2.6. Application of the assay in rats

Application of the new HPLC assay method(s) was demonstrated by determining the concentrations of I and II in male Sprague–Dawley rats. Briefly the rats were administered single oral (40 mg/kg) dose (Table 2). Blood samples were collected form rats by vena cava between 0 and 480 min post dose. Serum required for analysis was separated and stored at −30°C until analysis.

3. Results and discussion

Fig. 2 shows the chromatograms obtained for blank serum sample, calibration standard of 20 ng/ml and a sample containing 5 and 3 ng/ml of I and II, respectively. I and II had retention time of ~11 and 16 min, respectively, using the chromatographic conditions discussed above. The total chromatographic run time was prolonged to 45 min to allow for late eluting peaks. The high drift in the baseline was observed due to the high mode of sensitivity in which the detector was operated in gradient conditions. The absence of similar drift in the UV detector supports our above said statement. However, this drift did not hinder in the reliable quantitation of I and its metabolites in serum. Calibration curves ($n=3$) were linear over 0.625–20 ng/ml for I ($y=68575x+9484$, $r=0.99$) and II ($y=106696x+9648$, $r=0.98$). The mean recovery, precision and accuracy data were consistent and in the acceptable limits (Table 1). The use of *n*-hexane in backwashing enabled the removal of several endogenous components thus permitting the reliable estimation of I and II. The unidentified metabolite (III) which is relatively more polar than I and II was also well resolved. Use of 25 m*M* and pH 3.5 phosphate buffer helped in the elution of the analytes as sharp peaks. The retention time of I and II shifted from 11 and 17 min to 18 and 26 min, respectively, when the molarity of the buffer was reduced from 25 to 10 m*M*. However, an increase in the molarity from 25 to 50 m*M* improved the peak shape by a marginal 5%. No trend was observed in the analytes upon storing them at −30°C for 72 h and the deviations were less than ±20%. The absence of extra peaks or loss of

Table 2
Serum levels of I and II in rats receiving single oral dose of 40 mg/kg

Time (min)	Concentration (ng/ml)	
	I	II
15	5.1	1.5
30	4.4	2.8
90	2.1	1.9
120	0.84	0.65
240	0.63	NQ[a]

[a] NQ not quantitated (below LLOQ).

Table 1
Precision, accuracy and recovery of I and II in serum

Analyte	Concentration (ng/ml) ($n=3$)	Recovery (%)	Bias		RSD(%)	
			Within run	Between run	Within run	Between run
I	1.25	98.39±1.65	−3.8	−3.3	3.7	6.5
II	1.25	96.04±1.53	−3.6	−3.2	7.2	4.2
I	5	96.12±4.12	−0.13	−8.5	3.8	6.3
II	5	93.34±5.06	−4.7	−4.6	3.2	9.1
I	20	97.42±2.09	−4.07	−4.1	3.4	8.2
II	20	94.03±5.31	2.9	3.1	4.8	4.5

recovery due to degradation of the analytes when injected via autosampler indicates their stability.

Although the hydroxy metabolite has two assymetric centres, implying possible four enantiomers in vivo, these were not resolved on the present HPLC system. For the estimation of the levels of I and II in oral pharmacokinetic studies the assay method needed 2 ml serum, because of its extensive presystemic first pass metabolism. In other pharmacokinetic studies (intraperitoneal, intravenous), 0.5–1 ml serum was sufficient for their quantitation in view of the higher bioavailability exhibited by I in these routes. Preliminary analysis of human samples (after a single oral dose of 40 and 120 mg) using the gradient system indicated the presence of same metabolites implying that these assay conditions can also be applied to a wide range of clinical studies of centpropazine.

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