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Short communication

A sensitive LC assay for the simultaneous determination of centbutindole and its metabolite in rat serum using fluorescence detection

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Abstract

Centbutindole (±) 2-γ-[(*p*-fluorobenzoyl)propyl]-1, 2, 3, 4, 6, 7, 12, 12a-octahydro-pyrazino (2′, 1′: 6, 1) pyrido [3, 4-b] indole (**I**), is a new neuroleptic agent developed by Central Drug Research Institute, India. In the present study, a high performance liquid chromatography (HPLC) assay method for the simultaneous assay for **I** and its metabolite (**II**) in rat serum was developed and validated. The present method requires only 1 ml of serum with detection levels similar to that reported earlier using 4 ml serum. This assay has been found to be more suited for pre-clinical as well as phase IV studies. Linearity was observed between 1.25 and 40 ng/ml for **I** and 0.625 and 20 ng/ml for **II** in rat serum. Recoveries were consistent for both the analytes over the concentration ranges studied. Variation in intra-and inter-batch accuracy and precision were within acceptable limits of ± 20% at lowest limit of quantitation, whereas at higher concentrations it was ± 15%. The assay method was employed for the study of the pharmacokinetics and metabolism of **I** in rats. The parent compound and its metabolites were quantitated in serum and could be monitored up to 24 h post dose.

Keywords: HPLC; Centbutindole; Hydroxy metabolite; Serum; Fluorescence

1. Introduction

Centbutindole, (±) 2-γ-[*p*-fluorobenzoyl)propyl]-1,2,3,4,6,7,12,12aoctahydropyrazino (2′,1′: 6,1) pyrido[3,4-b]indole (**I**) (Fig. 1), a racemic mixture, is a new neuroleptic agent synthesized and developed in this institute. The drug has been granted marketing permission by the drug controller of India. Pharmacologically it is a dopamine antagonist [1–3], like the other neuroleptic agents, in doses lower than other

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available drugs. Clinically it is safe, well tolerated and has fewer side effects [4].

A high performance liquid chromatography (HPLC) assay method for **I** has been reported for tablet formulation [5]. The only method previously developed to estimate the levels of **I** and **II** in serum for application to establish pharmacokinetics, involved handling of large quantities of serum (4 ml) which was necessary to detect low levels of **I** following administration of single oral dose of 3 mg of **I**. This assay method cannot be exploited for preclinical pharmacokinetics in rats as it would require withdrawing 8–10 ml of blood from the animal in-order to process 4 ml of serum resulting in excessive animal handling for the establishment of the pharmacokinetics. This necessitated the development of a method sensitive enough to quantitate drug and metabolite levels in lower serum volumes compared with previous method. Here we report a new isocratic HPLC assay method with fluorescence detection for the estimation of drug (**I**) and its metabolite (**II**) (Fig. 1) using 1 ml of serum. Recovery, accuracy, and precision of its metabolite were used as parameters of validation. The method was applied to study the pharmacokinetics and metabolism of **I** in rats after a single 4 mg/kg p.o. dose. The present assay has been found to be more economical and less time consuming as compared with earlier assay. Moreover, the present assay will also be helpful for phase IV studies due to low sample requirements.

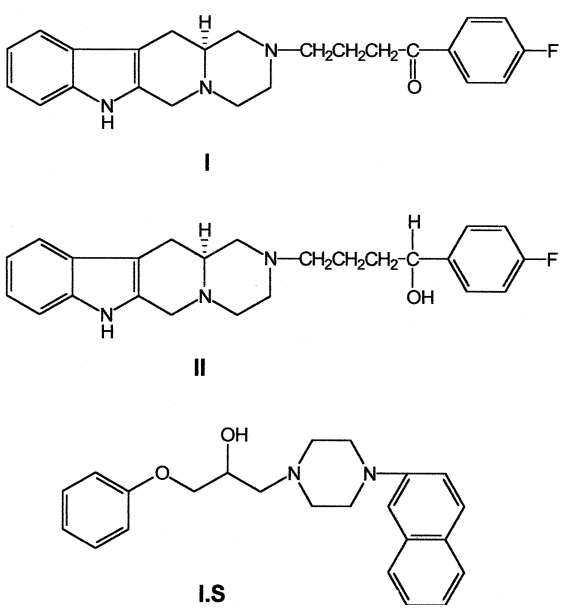

Fig. 1. Chemical structure of the analytes (**I** and **II**, and I.S).

2. Experimental

2.1. Materials

Pure reference standards (**I**, **II**) (assay $>99\%$) and 67/127 (used as I. S.) were synthesized in this institute. HPLC grade *n*-hexane, acetonitrile, methanol were obtained from Ranbaxy Laboratories (Delhi, India). Diethyl ether was purified before use 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 Sprague–Dawley rats from the laboratory animal division of this institute. The serum was stored at -60 °C and was used within 7 days.

2.2. Instrumentation

The HPLC system consisted of a Shimadzu LC-10ATvp solvent delivery system equipped with 7725i Rheodyne injector fitted with fixed 20 μI loop, SPD-10Avp dual wavelength UV-VIS detector coupled in series to RF-10A fluorescence detector. An SCL-10Avp System Controller (Shimadzu, Kyoto, Japan) was used as an interface to link the HPLC system with the software. The I. S. was monitored at an absorption wavelength of 270 nm on UV-VIS, whereas the analytes **II** and **I** were detected at $Ex_{\lambda}/Em_{\lambda}$ wavelength of 285/345 nm using RF-10A fluorescence detector. The chromatograms were integrated using CLASS VP-Series chromatographic software (Shimadzu, Kyoto, Japan) on PC (Compaq Deskpro). Chromatographic separations were performed on a C_{18} reversed phase column (Shperi-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 vortex mixer (Max-

imix-1 Type 16700 mixer, Thermolyne, Iowa, USA), ultrasonic bath (Bransonic, Danbury, CT, USA), a model C-30 centrifuge (Remi, India) and a model SVC-200H speed vac concentrator (Savant, NY, USA) were used for sample preparation.

2.3. Chromatographic conditions

Optimum separation between **I**, **II** and IS from the endogenous components of rat serum was achieved using isocratic elution. The mobile phase consisted of acetonitrile, methanol and 50 mM potassium dihydrogen orthophosphate buffer (pH 3.5) in the ratio of 35:25:40 (%v/v). Mobile phase solvents were filtered and degassed before use. The mobile phase was pumped at a flow rate of 1 ml/min.

2.4. Standard solutions

Mixed stock solution of **I** (100 μg/ml) and **II** (50 μg/ml) was prepared by dissolving 10 and 5 mg of the respective compounds in 100 ml of methanol. Stock solution of IS (67/127, 200 μg/ml) was prepared by dissolving 10 mg in 50 ml acetonitrile. Working stock of IS (20 μg/ml) was prepared by appropriate dilution of stock solution. Subsequent mixed working stock of **I** (4 μg/ml) and **II** (2 μg/ml) was prepared by appropriate dilution of the mixed stock solution. Further dilutions were prepared from mixed working stocks in methanol in the range of 12.5–400 ng/ml for **I** and 6.25–200 ng/ml for **II**. This analytical standard curve was employed for determination of recovery of **I** and **II** from serum. Calibration and quantity control (QC) samples of **I** and **II** were prepared by spiking a fixed volume of pooled drug-free rat serum with appropriate volume of the mixed working stock solution to get 1.25–40 ng/ml concentrations for **I**, and 0.625–20 ng/ml for **II**. All spiked serum samples were prepared ensuring that the volume ratio of organic phase added was less than 2%. Calibration and Q.C. standards were stored at −60 °C until analysis.

2.5. Extraction procedure

To 1 ml serum (drug free, spiked or test) was added 20 μl of I.S (20 μg/ml), 100 μl potassium hydroxide (1 M), and 4 ml ether in a 15 ml, clean 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 clean tube by freezing the aqueous layer in liquid nitrogen. Extraction was repeated with another 4 ml aliquot of ether. The combined ether extract was evaporated under reduced pressure in Speed Vac Concentrator (Savant Instrument, Farmingdale, NY, USA). The residue was acidified with 250 μl of 0.5 *N* hydrochloric acid and washed with 2 ml *n*-hexane. The acid layer was basified with 200 μl of 1 M potassium hydroxide solution and extracted with 2×3 ml ether. The combined ether extract was evaporated under reduced pressure in Speed Vac Concentrator. The residue was reconstituted in 100 μl menthol for loading onto the HPLC system.

2.6. Validation

2.6.1. Lowest limit of detection (LOD) and quantitation (LOQ), reproducibility and repeatability

The detection limit of the HPLC assay (LOD) of **I** and **II** after sample clean-up was estimated in serum corresponding to three times baseline noise ($S/N > 3$). The lowest limit of quantitation was (LOQ) defined as the concentration of the sample, which was quantified, with less than 20% deviation. Triplicate injections of the mobile phase standards in the standard curve range were made in a single batch and replicate injections were used to assess the reproducibility and precision in terms of retention times and peak response.

2.6.2. Calibration curve: model selection and verification

Standard curves drawn from three different batches were pooled and both pooled and individual data sets were used to select the calibration model. Microsoft Excel software [6] was used to plot the calibration curve in serum by linear regression with different weights ($1/x$, $1/x^2$, $1/\sqrt{x}$).

The unknown concentrations in serum were calculated from the calibration curves.

2.6.3. Recovery

Recoveries of **I** and **II** from spiked serum samples were calculated by comparing the peak areas at low, medium and high concentration levels with standard curve obtained by analyzing the corresponding standard dilutions in methanol, injected directly.

2.6.4. Accuracy and precision

Accuracy and precision of the assay method were studied at low (lowest limit of quantitation, LOQ), 2.5 ng/ml for **I** and 1.25 ng/ml for **II**; medium (5 ng/ml for **I** and 2.5 ng/ml for **II**); and high (40 ng/ml for **I** and 20 ng/ml for **II**) concentration levels. Triplicate serum samples at each of the three concentration levels were processed and analysed in each run and three such batches were accessed. Variations in accuracy and precision were expressed as % bias and relative standard deviation (%R.S.D.), respectively [7]. Acceptance limits of $\pm 20\%$ at the LOQ and $\pm 15\%$ at other concentrations in calibration range were used for the validation [8]. The effect of the freeze-thaw cycles (three cycles) as well as the stability in serum samples on storage at -30 °C upto 6 months was monitored.

3. Results and discussion

3.1. Optimisation of sample clean-up and chromatographic conditions

With the chromatographic conditions reported by Paliwal et al. [9], **II** and **I** were not sufficiently resolved for quantitation. Moreover, the analytes **I** and **II** eluted along the down slope of solvent front at 3.7 and 4.2 min, respectively, along with endogenous substances of serum. Hence, modifications were necessary in the chromatographic condition that could give better selectivity, and baseline resolution among **I** and **II**. Thus, molarity of buffer, the ratios of organic modifiers and type of column material were optimized. A C_{18} column was used to achieve good separation of **I** and **II**. Instead of a nitrile column used earlier, elution of the analytes from 220 mm C_{18} column helped in better baseline resolution, with an increase in retention times, thus improving the selectively of **I** and its metabolite **II**.

The effects of molar strength and nature of buffer in the mobile phase response of both analytes was also studied [10]. Buffers like potassium dihydrogen orthophosphate and ammonium acetate were used for the preparation of mobile phase. However, there was no significant shift in the retention times of **I** and **II** when ammonium acetate was used instead of phosphate buffer. However, there was an increase in the sharpness of the eluting analytes along with the interfering endogenous substances. A change in the molar strength of phosphate buffer from 25 to 50 mM increased the sensitivity of both **I** and **II**. Varying proportions of several organic modifiers like acetonitrile, methanol, tetahydrofuran (THF) and *N*, *N*′-dimethyloctylamine (DMOA) on the elution behaviour of **I** and **II** were also studied. An increasing proportion of methanol in the mobile phase led to the slight decrease in fluorescence with a corresponding increase in the retention times of both **I** and **II**. Incorporation of 2% of THF to the mobile phase led to the quenching of fluorescence of both **I** and **II** by 50%. During optimisation of mobile phase composition, the best result in terms of resolution, and run time were obtained using mobile phase comprising of acetonitrile: methanol: KH_2PO_4 buffer (pH 3.5, 50 mM) in the ratio of 35:25:40 (% v/v). The HPLC system reproducibility was assessed by using the replicate injections both in terms of retention times and peak responses of the individual compounds. The results showed that the variations were within acceptable limits of $P > 0.05$ in one way ANOVA. Comparison of the peak responses for analytes of **I** and **II** over three runs in a single day showed that% R.S.D. was not more than 3.00 over the whole range of standard curve for **I** and **II**.

Sample clean up technique was also optimised to get rid of the interfering endogenous substances without sacrificing the recoveries of **I** and **II**. A proper selection of extraction solvent was essential for yielding maximum recoveries. Keeping in

the mind the log $P(3.72 \pm 0.033)$ and pK_a (6.9 ± 0.095), various extraction solvents *n*-Hexane, ethyl acetate, ether and their admixtures were tried. Extraction solvents (*n*-Hexane, ethyl acetate) other than ether were incapable of producing consistent repeatability in extraction recoveries for **I** and **II**. Single extraction of 1 ml drug free serum with ether was not satisfactory in achieving good extraction recovery. A double extraction of the matrix with the same solvent considerably increased the recoveries of **I** and **II** but simultaneously it also increased the interferences in their region. **I** and **II** being weak bases, addition of 0.5 N HCl converted them into salts. Since salts have an inherent capability of existing as charged species, they will have appreciable solubility in polar solvents like water and little solubility in non-polar solvents. Organic solvents likes *n*-Hexane and ethyl acetate were tried to remove the endogenous compounds in the serum extract. Ethyl acetate being polar solvent was capable of removing most of the endogenous substances, but in addition extracted both **I** and **II**, however, the low solubility of the analytes in hexane was exploited, and thus hexane was used in back extracting the endogenous interfering peaks in serum. A slight trace of a polar interference in the region of **II** was persistent even after back extraction with hexane. Thus, a compromise was made with assay sensitivity and the sample injection loop was decreased from 50 to 20 μl. 67/127 was used as the I. S. which was monitored at a wavelength of 270 nm on UV-VIS spectrophotometer. The recovery of I. S. was consistent and greater than 85% in the employed extraction system. The extraction procedure and the chromatographic conditions were suitable for the quantitative analysis of **I**, **II**, and I. S with an LOD of 1.25 ng/ml for **I** and 0.625 ng/ml for **II**. The serum endogenous substances did not interfere with the elution of any of the analytes (Fig. 2). The retention times of the analytes **I**, **II** and I. S. were 5.7, 8.3, and 9.5 min, respectively. Fig. 2 illustrates the chromatograms of drug free serum analytical standard containing **I** and **II** and spiked serum with **I**, **II** and I. S. The calibration model was selected based on the individual calibration data by linear regression with

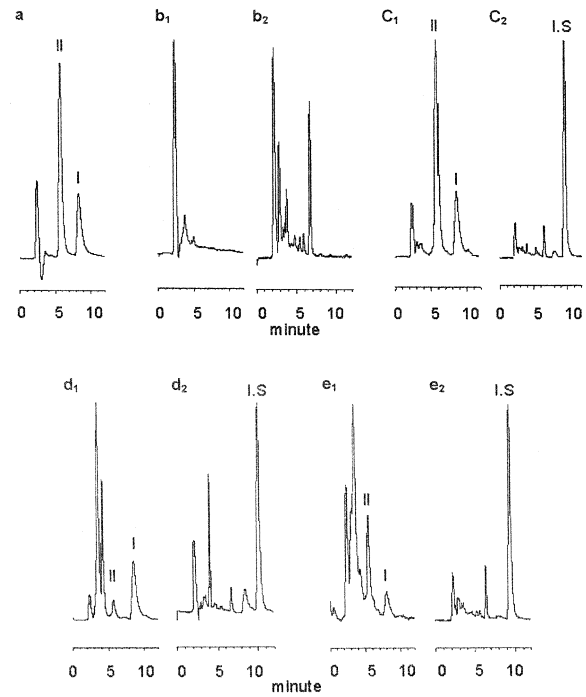

Fig. 2. Representative chromatograms of (a) analytical standard containing 400 ng/ml of **I** and 20 ng/ml of **II**, (b_1, b_2) drug free serum, (c_1, c_2) calibration standard containing 40 ng/ml of **I** and 20 ng/ml of **II**, (d_1, d_2) rat serum sample taken 0.5 h, and (e_1, e_2) 10 h postdose of 4 mg/kg of I. The subscripts 1, and 2 in parenthesis indicate the corresponding detectors (RF-10A, and UV–VIS, respectively) used to monitor the analysis.

or without intercept and weighting factors ($1/x$, $1/x^2$ and $1/\sqrt{x}$). The best-fit line was determined by least square regression analysis with a weighing factor $1/x^2$ and found to have $Y = 6498\ X - 52222$ and $Y = 31160\ X - 57147$ for **I** and II, respectively ($r^2 = 0.999$). Where, y is peak area, x is concentration in ng/ml and r^2 is the correlation coefficient. The RSD values for the slope and intercept of **I** and **II** were less than 1%. The concentrations of unknown serum samples were read from the calibration curve in serum. The analysis of the validation samples in four batches also confirmed the suitably of the model and hence this equation was used to calculate recovery, accuracy and precision of both the analytes. The LOQ was found to be 2.5 ng/ml for **I** and 1.25 ng/ml for **II**.

Table 1
Absolute recoveries of **I** and **II** from spiked rat serum

Concentration (ng/ml)			Recovery (%)	CV (%)
Analyte	Theoretical	Observed		
I	2.5	2.26	90.4 ± 7.7	8.5
II	1.25	0.98	78.6 ± 2.1	2.7
I	5	4.59	91.9 ± 10.3	11.2
II	2.5	2.07	82.8 ± 4.2	5.1
I	40	36.2	90.5 ± 6.4	7.0
II	20	16.8	84.1 ± 9.6	10.2

3.2. Recovery

The recoveries of **I** and **II** from spiked serum samples were calculated by comparing peak areas at low, medium and high concentration levels with those obtained from the analysis of corresponding standard dilutions in mobile phase injected directly. The recoveries of **I** and **II** from serum were 79–92% (Table 1).

3.3. Accuracy and precision

Table 2 summarises within and between run precision accuracy for the determination of **I** and **II** in fortified rat serum. Within and between run% R.S.D. (precision) was less than ± 10% at low, medium and high concentrations. The accuracy of the method expressed as % bias for within between run was less than ± 10% at low, medium and high concentrations.

3.4. Stability

The analytes showed less than 1% deviation from the nominal concentration at both high and low concentration levels upto three freeze-thaw cycles. There was no change in the concentration observed for the analytes at − 30 °C upto 6 months.

4. Application

A solution formulation containing 1 mg/ml of **I** was prepared in acetate buffer (pH 4.5) containing 20% polyethylene glycol to increase its aqueous solubility. The formulation was administered p.o. to the rats at a dose level of 4 mg/kg. Serial blood samples were collected post oral dose and serum was harvested and stored at − 60 °C until analysis. Typical representative chromatograms of blank and treated sample are shown in Fig. 2. The chromatograms did not exhibit any interference from endogenous components of samples in the regions of elution of both **I** and **II**. A serum concentration–time profile for **I** and its metabolite **II** after a single oral dose of 4 mg/kg in rats is shown in Figs. 3 and 4. The method was sensitive enough to follow up **I** and **II** upto 24 h post-dose.

Table 2
Accuracy and precision of **I** and **II** from Spiked rat serum

Analyte		Spiked control (ng/ml)					
		Low		Medium		High	
		I	**II**	**I**	**II**	**I**	**II**
Theoretical (ng/ml)		2.5	1.25	5	2.5	40	20
Mean (ng/ml)		2.46	1.23	5.16	2.48	40.57	20.9
% Bias	Intra-batch	−1.35	−2.66	1.54	−0.69	1.87	8.18
	Inter-batch	−0.52	−2.16	2.62	−1.67	1.77	5.82
% RSD	Intra-batch	6.32	1.78	8.88	3.92	3.17	3.63
	Inter-batch	0.59	8.74	7.52	1.99	10.01	10.96

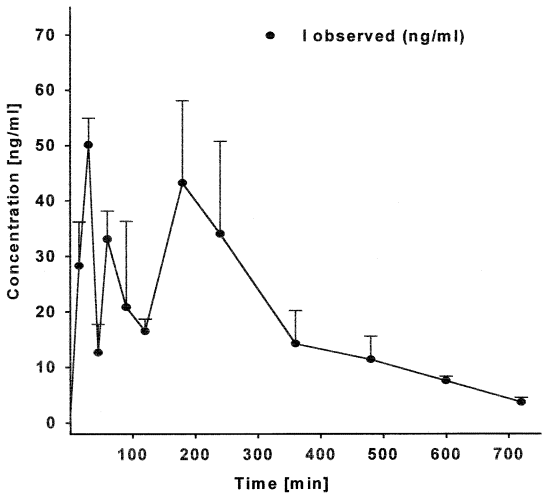

Fig. 3. Concentration–time profile of **I** after single oral dose of 4 mg/kg in rats.

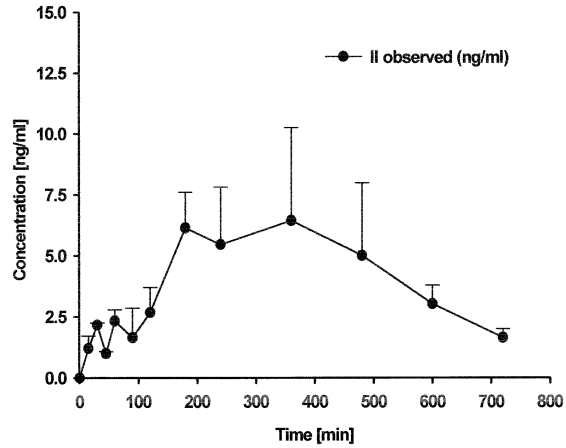

Fig. 4. Concentration–time profile of **II** after single oral dose of 4 mg/kg in rats.

5. Conclusions

Optimum separation of the analytes was achieved by isocratic elution. This separation was developed in rat serum for the first time for the quantification of **I** and **II**. Variations in recoveries of **I** and **II** were below $\pm 20\%$ at LOQ and $\pm 15\%$ at all other concentrations. Stability studies of **I** and **II** in rat serum were not felt necessary as samples were not stored for long periods of time. On sample collection, fixed volumes of serum was transferred to individual tubes and stored at -60 °C until analysis. It was insured that the samples not subjected to frequent freeze-thaw cycles prior to analysis. Thus, the new assay method was developed and validated found suitable for the detection of both **I** and its metabolite(s) observed in serum with adequate precision and accuracy.

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