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Simultaneous determination of centbutindole and its hydroxy metabolite in serum by high-performance liquid chromatography[a]

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ABSTRACT

A high-performance liquid chromatographic assay has been developed and validated for the determination of centbutindole and its hydroxy metabolite in serum. The method involves extraction of serum samples with diethyl ether at pH > 8, back-extraction into 0.5 *M* hydrochloric acid and finally again with diethyl ether after addition of 2 *M* potassium hydroxide. Separation was accomplished by reversed-phase high-performance liquid chromatography on a cyano column with an acetonitrile–phosphate buffer system. The recovery of centbutindole and its metabolite was always greater than 80%. Calibration curves were linear over the concentration range 0.25–5 ng/ml for centbutindole and 0.05–1 ng/ml for the hydroxy metabolite. Although the lower limit of detection was 0.1 ng/ml for centbuntindole and 0.02 ng/ml for the hydroxy metabolite, the reliable limits of quantitation were 0.25 and 0.05 ng/ml, respectively, using 4 ml of serum.

INTRODUCTION

Centbutindole, 2-γ-[(*p*-fluorobenzoyl)propyl]-1,2,3,4,6,7,12,12a-octahydropyrazino[2′,1′:6,1]pyrido[3,4-b]indole, I, Fig. 1), is a new neuroleptic agent related to the butyrophenone class and, like other neuroleptics, it is also a dopamine antagonist [1–3]. The *l*-isomer of centbutindole is many times more active than the *d*-isomer [4]. In experimental and clinical studies, centbutindole has been found to exert pronounced neuroleptic acitivity in doses lower than other available drugs and clinically it is safe, well tolerated and has fewer side-effects [5].

A high-performance liquid chromatographic (HPLC) method has been reported [6] for the analysis of centbutindole in tablet formulation. However, to date there is no published report on assay of centbutindole in biological samples. In

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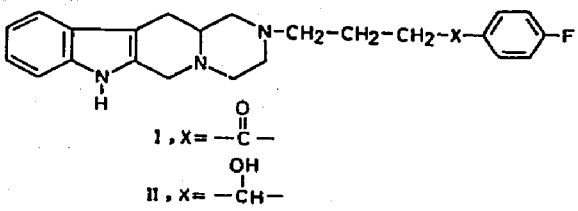

Fig. 1. Structures of centbutindole (I) and its hydroxymetabolite (II).

the present study an HPLC method for the simultaneous determination of centbutindole and its hydroxy metabolite using a reversed-phase cyano column and fluorescence detector has been developed.

EXPERIMENTAL

Reagents and solvents

Pure reference standards of centbutindole (I) and its hydroxy metabolite, 2-γ-[hydroxy-δ-(4-fluorophenyl)butyl]-1,2,3,4,6,7,12,12a-octahydropyrazino-[2′,1′:6,1]pyrido[3,4-b]indole (II), were prepared in this division. Potassium dihydrogenphosphate, (Sarabhai Chemicals, Baroda, India), orthophosphoric acid (Glaxo, Bombay, India) and potassium hydroxide (S.D. Fine Chemicals, Bombay, India) were of analytical grade and were used without further purification. HPLC-grade acetonitrile was procured from S.D. Fine Chemicals. Triple-distilled water from all glass apparatus was prepared and used as solvent. All glassware was washed with detergent, rinsed thoroughly with triple-distilled water and then dried prior to use.

HPLC apparatus and chromatographic conditions

The HPLC instrument consisted of Kontron 600 pump (Kontron Electro Lab., London, UK), a Rheodyne Model 7125 injector with a 20-μl loop (Berkeley, CA, USA) and a Shimadzu RF-530 variable-wavelength fluorescence detector (Kyoto, Japan). Separation was done on a 10 cm × 4.6 mm I.D. cyano cartridge column, 5 μm particle size, preceded by a 2-cm precolumn (Pierce Chemical, Rockford, IL, USA). Column effluent was monitored at an excitation wavelength of 280 nm and an emission wavelength of 350 nm. Chromatograms were recorded and integrated by Nelson software (Nelson Analytical, Cupertino, CA, USA) on a PC/XT computer.

The mobile phase consisted of acetonitrile and 0.01 *M* potassium dihydrogenphosphate (adjusted to pH 3 with orthophosphoric acid) in the ratio 60:40. It was filtered and degassed before use. Chromatography was performed at ambient temperature at a flow-rate of 1 ml/min.

Stock and standard solution preparation

Stock solutions containing 0.2 mg/ml I and II as free base were prepared in acetonitrile and stored at 4°C without any noticeable degradation. Combined working standards were prepared in mobile phase in the range 40–800 ng/ml I and 10–200 ng/ml II.

Calibration samples

Serum calibration samples were prepared by adding varying volumes of stock solutions of I and II in acetonitrile to 4 ml of serum. The amounts added corresponded to concentrations ranging from 0.25 to 5 ng/ml I and 0.05 to 1 ng/ml II. The standards were vortex-mixed briefly and stored at −20°C until used for analysis.

Extraction

To 4-ml serum samples containing I and II were added 2 ml of water and 200 μl of 2 *M* potassium hydroxide in a 20-ml (150 mm × 5 mm) glass test tube. The constituents were extracted three times, each with 5 ml of diethyl ether. During each extraction, tubes were vortex-mixed for 15 s followed by centrifugation for 5 min at 500 *g*. The ether layer was transferred to a (125 mm × 10 mm) glass test tube, after snap freezing the aqueous serum layer in liquid nitrogen. The combined ether layer was evaporated under a nitrogen stream at 35°C to about 1 ml and then back-extracted with 1 ml of 0.5 *M* hydrochloric acid. The aqueous layer was basified with 300 μl of 2 *M* potassium hydroxide and finally extracted once with 5 ml of diethyl ether. The organic layer was separated and evaporated as above. The residue was reconstituted in 30 μl of mobile phase and analysed by HPLC using a 20-μl injection loop.

Accuracy and precision

I and II were added to serum ($n = 6$) and the concentrations were determined against their corresponding standard calibration curves. The accuracy of the method was calculated based on the difference between the mean calculated and added concentrations, while precision was evaluated by calculating the inter-day coefficients of variation (C.V.).

RESULTS AND DISCUSSIONS

Chromatography

A cyano column was used to separate compounds I and II in this study. These compounds were not eluted from commonly used reversed-phase columns such as C_8, C_{18}, phenyl or nitro columns. A decrease in the percentage of acetonitrile in the mobile phase resulted in peak broadening as well as an increase in the retention time of these compounds. Similarly, an increase in ionic strength of phosphate buffer not only decreased the retention time but also increased peak sharp-

ness. A mobile phase containing acetonitrile and 0.01 *M* potassium dihydrogenphosphate in the ratio 60:40 was found to be optimal for the proper resolution of compounds I and II from endogenous serum substances.

Selectivity and specificity

Fig. 2 shows typical chromatograms of an extract of drug-free human serum (left), serum standard spiked with 2.5 and 0.5 ng/ml I and II (middle), and a serum sample from a volunteer treated with I (right). The retention times of I and II were approximately 10 and 8.6 min, respectively. No interfering peaks were detected in control human serum samples or the clinical samples from volunteers, who had received I orally. Serum components eluting prior to that of I and II did not interfere. Based on a signal-to-noise ratio of 3, the detection limit of the assay from 4 ml of serum was 0.1 and 0.02 ng/ml for I and II. However, the lower limit of quantitation was set at 0.25 and 0.05 ng/ml (C.V. $<14\%$). This method provided adequate sensitivity and specificity for monitoring serum levels of I and II.

Extraction efficiency

A double liquid extraction with diethyl ether was necessary to get rid of the interfering substances in serum. This was necessary because of the large volume (4 ml) of serum being extracted and reconstituted in a very small volume (30 μl), resulting in about 130-fold concentration of extracted components. The extraction recoveries of I and II were determined at three (high, medium and low) levels

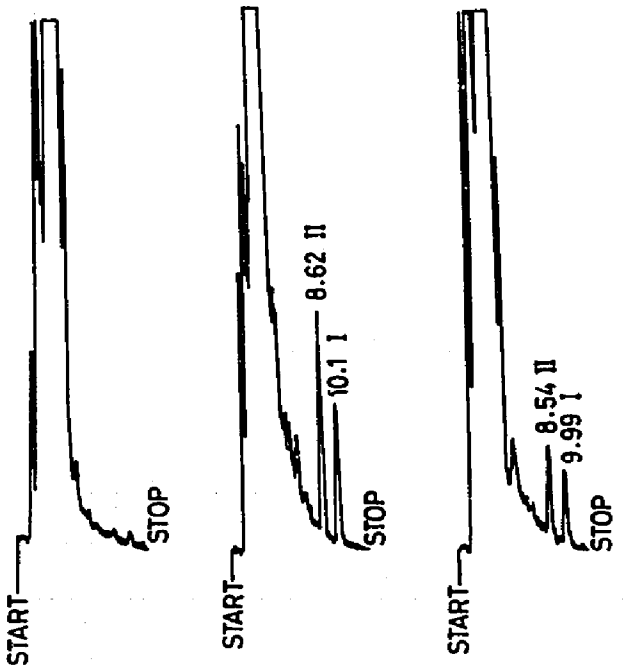

Fig. 2. Chromatograms of (left) drug-free serum, (middle) serum standard spiked with 2.5 ng/ml I and 0.5 ng/ml II and (right) serum of volunteer treated with I.

TABLE I

ABSOLUTE RECOVERIES OF I AND II FROM SPIKED SERUM SAMPLES (n = 5)

Drug	Concentration (ng/ml)	Recovery (%)	Coefficient of variation (%)
I	0.25	81.2	12.3
	1.00	84.6	9.5
	5.00	85.6	4.8
II	0.05	80.8	13.2
	0.20	86.3	6.4
	1.00	82.7	7.7

of calibration by comparing the peak areas obtained after direct injection of standard solution with those obtained after the whole extraction procedure. The absolute recoveries of I and II were greater than 80% and independent of their concentration (Table I).

Linearity and reproducibility

The calibration curves were linear over the concentration range 0.25–5 ng/ml I and 0.05-l ng/ml II with correlation coefficients (r) of 0.994 and 0.992, respectively, with minimal intercept (Table II). The reproducibility and accuracy of the method were determined by processing spiked serum samples at three concentrations with respect to calibration curves run each day. Six samples were analysed for each concentration. The within-day coefficient of variation ranged from 8.7 to 13.9% for I and from 3.6 to 12.8% for II. The day-to-day variations of samples analysed on nine different days over a period of one month were 7.7–13.8% for I and 4.2–10.4% for II (Tables III and IV).

The inter-day assay variation was also estimated by comparing the linear regression slopes of nine standard curves. Over a period of one month the slope averaged 12 849 and 97 124 (C.V. 15.7 and 11.5%), respectively, for I and II. The

TABLE II

STANDARD CURVE REPRODUCIBILITY FOR I AND II

	Standard curve for I			Standard curve for II		
	Slope	Intercept	r	Slope	Intercept	r
Mean	12 849	1237	0.996	97 142	1957	0.998
C.V. (%)	15.7	50.86	0.67	11.53	71.73	0.23

TABLE III

PRECISION AND ACCURACY FOR I

Spiked concentration (ng/ml)	Concentration found (mean ± S.D.) ng/ml)	Coefficient of variation (%)	Accuracy (% of mean deviation)
Within-day (n = 6)			
0.25	0.23 ± 0.03	13.9	−8.8
1.00	1.04 ± 0.126	12.1	4.6
5.00	4.83 ± 0.421	8.7	−3.4
Day-to-day (n = 9)			
0.25	0.26 ± 0.036	13.8	5.2
1.00	0.97 ± 0.113	11.6	−3.0
5.00	5.28 ± 0.410	7.7	5.7

TABLE IV

PRECISION AND ACCURACY FOR II

Spiked concentration (pg/ml)	Concentration found (mean ± S.D.) (pg/ml)	Coefficient of variation (%)	Accuracy (% of mean deviation)
Within-day (n = 6)			
50	53 ± 6.80	12.8	6
200	192 ± 16.5	8.5	−4
1000	1036 ± 36.8	3.6	3.6
Day-to-day (n = 9)			
50	52 ± 5.4	10.4	4
200	206 ± 18.3	8.9	3
1000	927 ± 41.2	4.2	−2.8

TABLE V

SERUM CONCENTRATIONS OF CENTBUTINDOLE IN A HUMAN VOLUNTEER AFTER A SINGLE 3-mg ORAL DOSE

Time (h)	Concentration (ng/ml)
0.5	1.0
1	1.2
2	2.74
6	4.06
12	2.98
36	0.62
48	0.33

accuracy of the method expressed as the mean deviation of all concentrations from the theoretical value ranged from −8.8 to 5.7 and from −4 to 6 for I and II, respectively. These results demonstrate that the method is reproducible and accurate.

Application of method in clinical pharmacokinetics

The assay method described herein was applied to study single-dose pharmacokinetics of centbutindole in normal healthy volunteers. The method was sensitive enough to follow centbutindole up to 48 h after a single 3-mg oral dose (Table V). The detailed pharmacokinetics will be published elsewhere.

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