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Note

Reversed-phase liquid chromatographic method with amperometric detection for the determination of the dopamine agonist 2-(N-propyl-N-2-thienylethylamino)-5-hydroxytetralin (N-0437) in human plasma and urine

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The drug 2-(N-propyl-N-2-thienylethylamino)-5-hydroxytetralin hydrochloride (N-0437, I) was developed and synthesized by Horn et al. [1]. Being a strong D2 agonist, the compound has potential applications in the treatment of Parkinson's disease [2–4]. Initially, a radioreceptor assay was developed, which specifically determined the (−)-enantiomer, but which can be used in studies on the racemate if enantioselective kinetics do not occur or are not taken into account [5]. Recently, a high-performance liquid chromatographic (HPLC) method with coulometric detection was developed for determination of the plasma drug concentration in animals, with a quantitation limit of ca. 30 ng/ml [6]. Owing to the high potency and expected first-pass effect of the drug [7], it was felt that a more sensitive method was required for human studies. Therefore, we investigated the potential of amperometric detection, which generally provides lower detection limits than "coulometric" detection, as well as the possibilities for preconcentration and clean-up of the biological fluids by column extraction.

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EXPERIMENTAL

Chemicals and reagents

Racemic 2-(N-propyl-N-2-thienylethylamino)-5-hydroxytetralin hydrochloride (I) and 2-(N-propyl-N-2-*p*-fluorophenylethylamino)-5-hydroxytetralin hydrochloride as internal standard (I.S.) were synthesized by Horn et al. [1]. The structures are given in Fig. 1. Compound I was found to be at least 99% pure and the I.S. at least 98% pure, by means of HPLC and thin-layer chromatography (TLC). Tritiated I was obtained from Amersham (Little Chalfont, U.K.) with a specific activity of 80.6 Ci/mmol and a radiochemical purity of at least 95%, checked by HPLC. The tritium label was present in the propyl side-chain. 1-Octanesulphonic acid (sodium salt) was supplied by Kodak (Rochester, NY, U.S.A.) and methanol (HPLC grade) by Baker (Deventer, The Netherlands). All other chemicals were analytical-reagent grade and obtained from Merck (Darmstadt, F.R.G.). Throughout the study deionized water was used (Milli-Q purification system, Millipore, Bedford, MA, U.S.A.).

Standard solutions

Stock solutions of 1 mg/ml I and 1 mg/ml I.S. were prepared in Milli-Q water and stored at −20 °C. After one year no degradation was observed. Appropriate dilutions of the drug and the I.S. were made daily in drug-free plasma or urine to provide concentrations of 0.20–100 ng/ml.

Glassware

Screw-capped centrifuge tubes (Sovirel® 18, Quickfit, SA, Epernon France) were cleaned with a 2% solution of Extran MA 01 alkaline (Merck). The tubes were rinsed with distilled water and cleaned again in a mixture of 80 ml of hydrogen peroxide (36%), 300 ml of hydrochloric acid (36%) and 120 ml of distilled water by standing overnight. The tubes were rinsed again with distilled water and dried at 105 °C.

Chromatography

The chromatographic system consisted of a Model SP 8800 HPLC pump

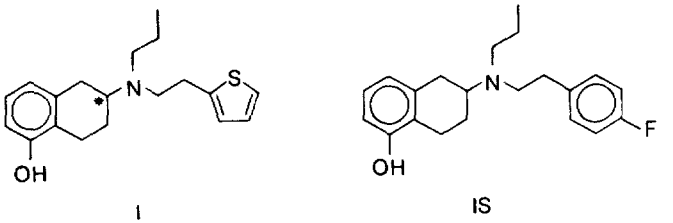

Fig. 1. Structures of I and the internal standard (IS) used in this study. The asterisk marks the position of the asymmetric carbon atom.

(Spectra Physics, San Jose, CA, U.S.A.) and a Model C6W injection system with a 100-μl loop, (Valco Instruments, Houston, TX, U.S.A.). Detection was performed with an amperometric detector, Model AMOR, equipped with a glassy carbon electrode (Spark Holland, Emmen, The Netherlands). Peak heights were recorded with a 3396A reporting integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

The separation was performed on a 150 mm$\times$3.8 mm I.D. column packed with 4-μm Nova Pak C_{18} (Waters, Milford, MA, U.S.A.). A flow restrictor (Spectra Physics) was placed between the pump and the injection valve, to minimize the impact of pump pulsations on the chromatograms. HPLC system connections, downstream of the injector, were via 0.13 mm I.D. stainless-steel tubings. The connection between the column and the detector was via 0.25 mm I.D. PTFE tubing.

The isocratic mobile phase used for the separations contained 50 m*M* sodium phosphate (pH 5.5), 35% acetonitrile and 2.5 m*M* 1-octanesulphonic acid. Sodium chloride, 5 m*M*, was added to the mobile phase for stabilization of the AgCl in situ reference electrode. The eluent was filtered through a 0.20-μm type RC membrane filter (Schleicher and Schuell, Dassel, F.R.G.), then degassed in an ultrasonic bath for 15 min. During the measurements the eluent was continuously de-aerated with helium. The flow-rate was 1.00 ml/min at ambient temperature (ca. 22°C). The detector was thermostatted at 25°C with a circulating water-bath (Wilton, De Meern, The Netherlands), to minimize fluctuations in background current.

When not in use, the chromatographic system and the detector were continuously flushed with eluent at a flow-rate of 0.2 ml/min. The glassy carbon electrode was cleaned weekly with acetone. When acetone cleaning did not improve the response, the electrode was polished and cleaned according to the manual, as this phenomenon indicates too much surface pollution.

Sample preparation

To 1.0 ml of plasma or urine, 100 μl of water containing the I.S. were added and the mixture was vortex-mixed for 1 min. After this, 900 μl of water were added and the vortex-mixing was repeated. When spiking a sample, 100 μl of water containing the required amount of I and/or I.S. were added to 1.0 ml of bodyfluid, and the above procedure was applied.

Bond Elut® extraction columns, type Si (silica gel), bed volume 2.8 ml (Analytichem International, Harbor City, CA, U.S.A.), were used in combination with a Vac Elut vacuum control station. Each column was pretreated by successive washings with 5 ml of methanol, 5 ml of water, 5 ml of dichloromethane and 5 ml of water. After removal of the final washing, 1.9 ml of the diluted sample were transferred to the washed column and a slight vacuum was applied (80 kPa). Under these conditions it takes nearly 2 min to elute the sample. The columns were rinsed with 4 ml of water under 80 kPa. During the wash-

ings, care was taken that the column did not run dry and that it remained submersed at all times. The columns were placed in a plastic tube and centrifuged for 3 min at 635 *g*, to remove any remaining droplets of water. Elution of the absorbed I and I.S. was achieved by passing 7.5 ml of dichloromethane through the column under a pressure of 80 kPa. The eluate was collected in a 10-ml Pyrex glass tube (Sovirel), after which 25 μl of acidified methanol were added (40 m*M* acetic acid in methanol). The sample was vortex-mixed and the organic layer was evaporated to dryness within 30 min, under a stream of nitrogen at 20°C. The residue was reconstituted in 250 μl of eluent by ultrasonication for 1 min to achieve complete dissolution, and 100 μl of the sample were injected into the HPLC column.

Recovery

The absolute recovery of the extraction procedure was determined using tritiated I. Approximately 170 pg, corresponding to 0.04 μCi of tritiated I, were spiked in plasma or urine, with or without 100 ng/ml I and 100 ng/ml I.S. bodyfluid. Comparable experiments were also done with 4 μCi of tritiated I. Fractions of 1 ml were collected in glass counting vials (Packard, Groningen, The Netherlands). The radioactivity was measured after adding 6 ml of Aqualuma Plus (Lumac, Oud-Beijerland, The Netherlands) in an LS 1800 Beckman scintillation counter (Beckman Industrial, La Habra, CA, U.S.A.). Quenching was corrected by the H-number method.

Validation procedure

Assays were validated in plasma and urine as follows. Six and four pools of the analyte were prepared for plasma and urine, respectively, by dissolving weighed amounts of I in known volumes of drug-free plasma or urine. On each of three days at least two replicate samples from each pool were extracted and analysed. Concentrations were determined by means of a standard curve prepared freshly on the day of analysis. An appropriate standard curve was prepared for each set of samples. Peak-height data were collected with the integrator and analyte/I.S. peak-height ratios were calculated; the calibration curve was obtained by linear regression. Peak heights provided better results than peak areas, especially at lower concentrations.

RESULTS AND DISCUSSION

In electrochemical detection (ED), the response depends on the applied voltage. The current–voltage curves of I and I.S. are shown in Fig. 2A. Both compounds started to show an electrochemical response at an applied voltage of 0.5 V. The responses reached a plateau at 1.0 V, but the signal-to-noise ratios showed maxima from 0.7 to 0.8 V. Therefore, 0.75 V was selected as the optimal voltage, because at higher voltages interferences from the bodyfluids were ob-

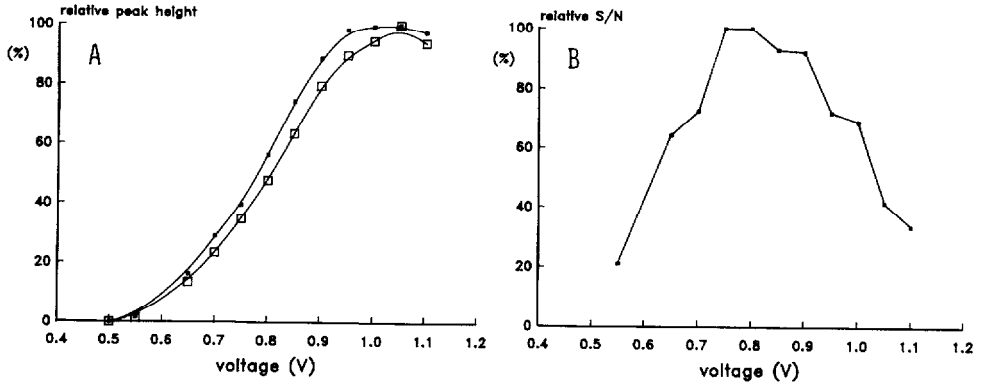

Fig. 2. (A) Relationship between applied oxidation potential and detector response for I (■) and I.S. (□). Data were obtained under the chromatographic conditions described in the text and are expressed as percentage values relative to the maximum response obtained for the respective compounds. (B) Relationship between applied oxidation potential and signal-to-noise ratio.

served. The relationship between the applied oxidation potential and signal-to-noise ratio is shown in Fig. 2B.

In prior studies we evaluated two types of electrochemical detector. The coulometric Coulochem detector (ESA, Bedford, MA, U.S.A.) resulted in high background currents and noise and therefore poor detection limits. Similar results were described by Forzy et al. [8]. The amperometric detector (AMOR), equipped with a glassy carbon electrode, showed a low background current and baseline noise and was therefore better able to detect small amounts of the drug. The detection limit for the described system was 5 pg of I, based on a signal-to-noise ratio of 3.

The absolute recovery determined using tritiated I, with or without 100 ng/ml I and 100 ng/ml I.S., was greater than 98% in all cases. This indicates that small or large amounts of the drug and I.S. have no influence on the recovery, and that inappropriate displacement of the drug from the Si column does not occur. Fig. 3 shows a radioactive profile of the extraction. The activities found in fractions 2, 3 and 4 are caused by the radioactive impurities in tritiated I.

The dichloromethane fraction was collected in Pyrex glass tubes. The cleaning as described was necessary to eliminate impurities that were left after cleaning only with Extran. These impurities gave an electrochemical response and thus interfered with the chromatograms.

Validation in plasma

The validation of the methodology for analysis in plasma was divided in two parts to create sufficiently comparable conditions for the drug and I.S. One part dealt with high concentrations (5–75 ng/ml I with 10 ng/ml I.S.) and one with low concentrations (0.2–20 ng/ml I with 2.5 ng/ml I.S.). The equations

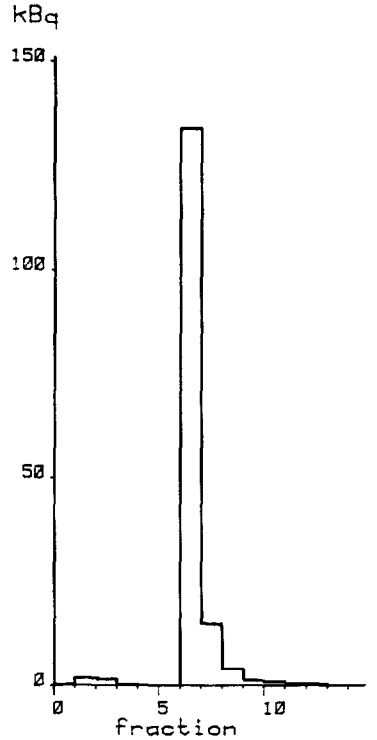

Fig. 3. Radioactive profile of the extraction, using 1 ml of plasma spiked with 4 μCi of tritiated I.

for the regression lines and correlation coefficients were $y=0.658x+0.013$, $r^2=0.998$ for the low concentrations and $y=0.152x-0.154$, $r^2=0.999$ for the high concentrations. The absolute recoveries were 91.93% (S.D. 5.7%, $n=6$) for I and 79.59% (S.D. 9.1%, $n=6$) for the I.S., determined at 2, 10 and 50 ng/ml. The limit of quantitation was 0.3 ng/ml plasma, which corresponds to ca. 1 pmol/ml. The accuracy and precision data are presented in Table I. Typical chromatographic profiles obtained from blank and spiked human plasma are shown in Fig. 4. The peaks with retention times of 5 and 14 min are caused by impurities found in dichloromethane.

Validation in urine

For the calibration curves concentrations of I ranged from 0.75 to 100 ng/ml of urine, and the concentration of the I.S. was 10 ng/ml. The equation for the regression line and correlation coefficient was $y=0.195x+0.081$, $r^2=0.999$. The absolute recoveries were 90.29% (S.D. 10.1%, $n=6$) for I and 83.18% (S.D. 7.6%, $n=5$) for the I.S., determined at 2, 10 and 50 ng/ml. The limit of quantitation was at least 1.5 ng/ml of urine, which is ca. 5 pmol/ml. Table II shows

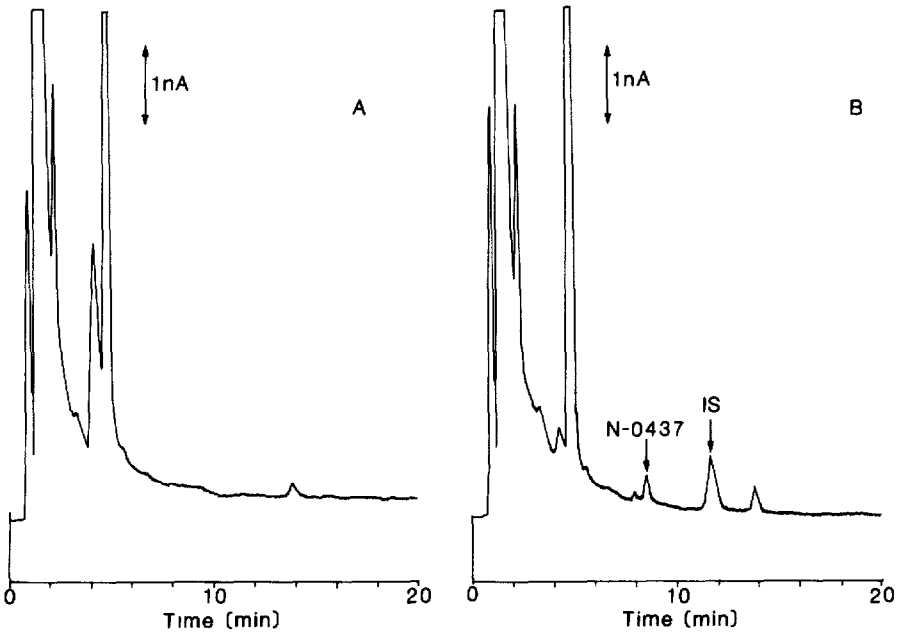

Fig. 4. Analysis of I in human plasma. (A) Blank plasma extract; (B) plasma spiked with 500 pg/ml I (N-0437) and 2.5 ng/ml I.S.

TABLE I

ACCURACY AND PRECISION DATA FOR I IN PLASMA

Added (ng/ml)	Found, (mean ± S.D.[a], $n=2$) (ng/ml)	Overall, (mean ± S.D., $n=6$) (ng/ml)	Within-day R.S.D.[b] ($n=6$) (%)	Between-day R.S.D.[c] ($n=6$) (%)
0.30	0.25 ± 0.034	0.30 ± 0.063	10.73	21.00
0.75	0.79 ± 0.249	0.70 ± 0.049	2.42	7.00
1.50	1.27 ± 0.021	1.38 ± 0.098	5.74	7.10
4.50	4.30 ± 0.04	4.53 ± 0.21	0.81	4.64
15.00	15.60 ± 0.52	15.01 ± 0.34	2.14	2.27
45.00	49.31 ± 3.11	47.64 ± 2.47	4.00	5.18

[a]Mean value on a single day.
[b](S.D./X)100%; S.D. $=\sqrt{[(n_1-1)\text{S.D.}_1^2+(n_2-1)\text{S.D.}_2^2+(n_3-1)\text{S.D.}_3^2]/(n_1+n_2+n_3-3)}$; X = overall mean; n = number of observations [9].
[c]$\frac{\text{S.D. of overall mean concentration}}{\text{overall mean concentration}} \cdot 100\%$.

the accuracy and precision data for I in urine. Typical chromatographic profiles obtained from human blank and spiked urine are shown in Fig. 5.

In summary, this method allows the measurement of the aminotetralin I

TABLE II

ACCURACY AND PRECISION DATA FOR I IN URINE

Added (ng/ml)	Found, (mean ± S.D.[a], $n=3$) (ng/ml)	Overall, (mean ± S.D., $n=9$) (ng/ml)	Within-day R.S.D.[b] ($n=9$) (%)	Between-day R.S.D.[c] ($n=9$) (%)
1.50	1.49 ± 0.15	1.57 ± 0.11	6.21	7.00
4.50	4.69 ± 0.34	4.69 ± 0.18	6.90	3.84
15.00	15.25 ± 0.53	15.28 ± 0.65	4.89	4.25
45.00	46.12 ± 3.61	45.65 ± 2.87	6.97	6.29

[a]Mean value on a single day.

[b](S.D./X)100%; S.D. $= \sqrt{[(n_1-1)\text{S.D.}_1^2 + (n_2-1)\text{S.D.}_2^2 + (n_3-1)\text{S.D.}_3^2]/(n_1+n_2+n_3-3)}$; X = overall mean; n = number of observations [9].

[c]$\frac{\text{S.D. of overall mean concentration}}{\text{overall mean concentration}} \cdot 100\%$.

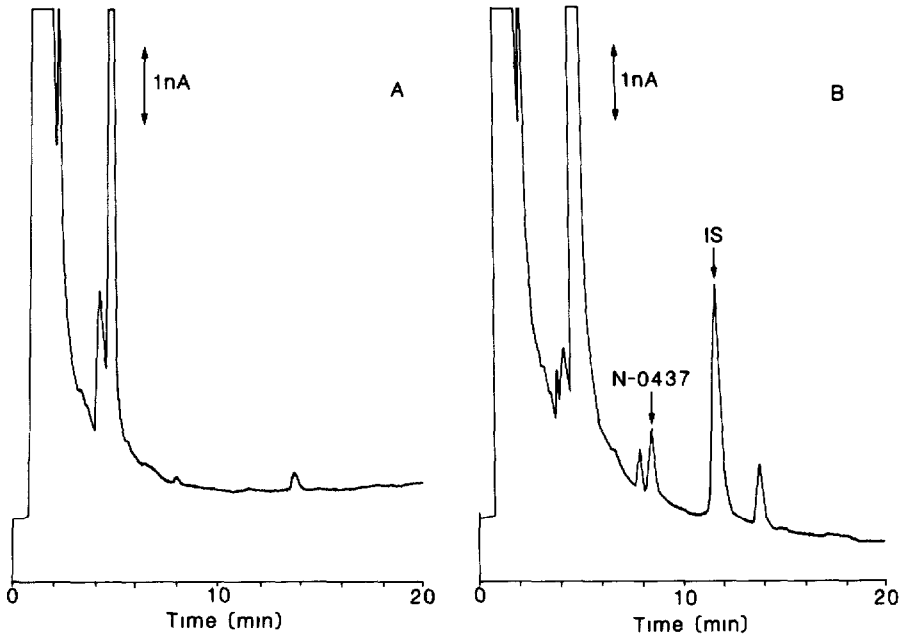

Fig. 5. Analysis of I in human urine. (A) Blank urine extract; (B) urine spiked with 1.5 ng/ml I (N-0437) and 10 ng/ml I.S.

with picomole sensitivity, in human plasma and urine. Variance analysis showed that the concentration and day of analysis had no influence at the relative recovery ($P<0.05$). The within-day R.S.D. did not exceed 11% at any level. The method is now used for determinations of the drug after oral and intra-

venous administration of pharmacologically appropriate doses in humans, as well as in rats, for which the method is equally suitable.

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