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Note

High-performance liquid chromatographic determination of a new benzamide in biological fluids for use in a human pharmacokinetic study

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Pharmacologically, 5-(methylaminosulphonyl)-N-[(1-allyl-2-pyrrolidinyl)-methyl]-2-methoxy-4-aminobenzamide (I) is a member of a series of methoxy-benzamide compounds that interfere in dopaminergic nerve transmissions and are used in therapy as a result of this property, particularly in psychiatric disorders as with sulpiride [1—3].

The benzamide I possesses certain pharmacological characteristics of sulpiride, but these are accentuated. Binding to different types of dopaminergic receptors is even more dissociated, and is its antagonism of certain behavioural effects provoked by apomorphine.

In this present work, a high-performance liquid chromatographic (HPLC) method with UV detection was developed for the determination of I in body fluids. The proposed technique which is, selective, reliable and sensitive, has been used in human pharmacokinetic studies. Examples of kinetics are given.

EXPERIMENTAL

Materials and reagents

5-(Methylaminosulphonyl)-N-[(1-allyl-2-pyrrolidinyl)methyl]-2-methoxy-4-aminobenzamide (RIV 2093, I) and the internal standard, metoclopramide, were obtained from Delagrange (Paris, France) and were used as 0.001, 0.01 and 0.1 g/l solutions for I and 0.01 and 0.1 g/l solutions for metoclopramide in glass-distilled water; these solutions were prepared by dilution of a 1.00 g/l aqueous stock solution. Chloroform was of HPLC grade (Merck, Darmstadt, F.R.G.). Methanol was doubly distilled in an all-glass apparatus before use.

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Sodium hyroxide, ammonium acetate, glycine and sodium chloride were all of analytical-reagent grade (Merck). Sodium hydroxide and ammonium acetate were used as 0.1 *M* solutions in glass-distilled water. Buffer of pH 8.9 was prepared to contain 0.1 *M* glycine in 0.1 *M* sodium chloride (88.5 ml) and 0.1 *M* sodium hydroxide (11.5 ml).

High-performance liquid chromatography

HPLC was performed using a Spectra-Physics SP 8100 instrument with a Valco syringe-loading valve fitted with a 50-μl sample loop, a Spectra-Physics SP 8110 automatic sample injection system and a stainless-steel column (25 cm × 4.6 mm I.D.) packed with C_{18}-bonded LiChrosorb (10 μm). The column effluent was monitored with a Schoeffel Model SF 770 variable-wavelength UV detector operated at 226 nm. The mobile phase was 0.1 *M* ammonium acetate—methanol (20:80) and was helium-degassed during use. Methanol and 0.1 *M* ammonium acetate solution were filtered through a membrane filter (0.45 μm).

The oven temperature was 50°C and the flow-rate was 0.8 ml/min, which corresponds to a pressure of approximatively 40 bar. The signal was recorded and the peak areas determined with a Spectra-Physics SP 4100 computing integrator (chart speed 1 cm/min). Determinations of amounts were made automatically by comparison of the peak areas of the samples with those given by known concentrations of standards.

Sample preparation

Plasma (1—4 ml) or urine (0.5—10 ml) was pipetted into a 50-ml glass centrifuge tube and the volume adjusted to 4 ml (plasma) or 10 ml (urine) with water. Internal standard solution (0.4 ml at 0.01 g/l for plasma or 0.75 ml at 0.1 g/l for urine), buffer (pH 8.9) (0.5 ml) and sodium hydroxide solution (0.2 ml) were added. The pH was adjusted to 8.9 by addition of 0.1 *M* sodium hydroxide when necessary. After extraction with 20 ml of chloroform for 20 min and centrifugation at 9950 *g* for 10 min, the tubes were kept at −20°C for 1 h. The organic phase was retained and evaporated to dryness at room temperature.

Before HPLC analysis, the mobile phase (0.2 ml for plasma and 1 ml for urine) was added. After ultrasonic mixing, the samples were filtered through a membrane filter (0.2 μm) and 50 μl of this solution were injected into the column.

Instrument calibration

Calibration graphs for control plasma or urine (4 ml) were prepared using concentrations of 0.05, 0.125, 0.250, 0.500 and 1.250 μg/ml for plasma and 1.25, 2.50, 6.25, 12.5 and 25 μg/ml for urine. The procedure was carried out in the same way for the samples of plasma and urine.

Data analysis

The ratio of the peak area of benzamide to that of the internal standard was used as the assay parameter. Peak area ratios were plotted against analyte concentration and calibration graphs obtained from least-squares regression analysis of the data.

RESULTS

Retention times

Retention times were 4.84 min (k' = 0.679) for I and 6.28 min (k' = 1.166) for the internal standard (Fig. 1). There were no interfering peaks in the control plasma and urine samples at the retention times of the above-mentioned compounds.

Linearity

In plasma, the peak area ratio of benzamide to the internal standard varied linearly with concentration over the range used. The calibration graphs passed through the origin (intercept not significantly different from zero: −0.00425 ± 0.00751). An excellent correlation coefficient from the linear regression analysis was obtained [$r^2 = (0.99993 \pm 6.32) \cdot 10^{-5}$; $n = 12$] and the calculated slope of the line was 2.17 ± 0.0168 ng^{-1} ml.

For the urine samples, the reliability of the assay was of the same order, with a slope of $(0.0250 \pm 8.74) \cdot 10^{-4}$ ng^{-1} ml, an intercept of 0.0165 ± 0.0144 and a correlation coefficient of $(0.99980 \pm 2.16) \cdot 10^{-4}$ (n = 12).

Reproducibility

The inter-assay coefficients of variation (C.V.) measured from analyses (n = 12) of standard solutions prepared in plasma and urine containing I at concentrations of 0.05—1.25 μg/ml for plasma and 1.25—25 μg/ml for urine are presented in Table I.

The intra-assay C.V. measured from replicate analyses (n = 10) of two standard solutions prepared in urine containing I at either 2.5 or 25 μg/ml were 0.68 and 1.85%, respectively.

The reproducibility of the chromatographic method was determined with

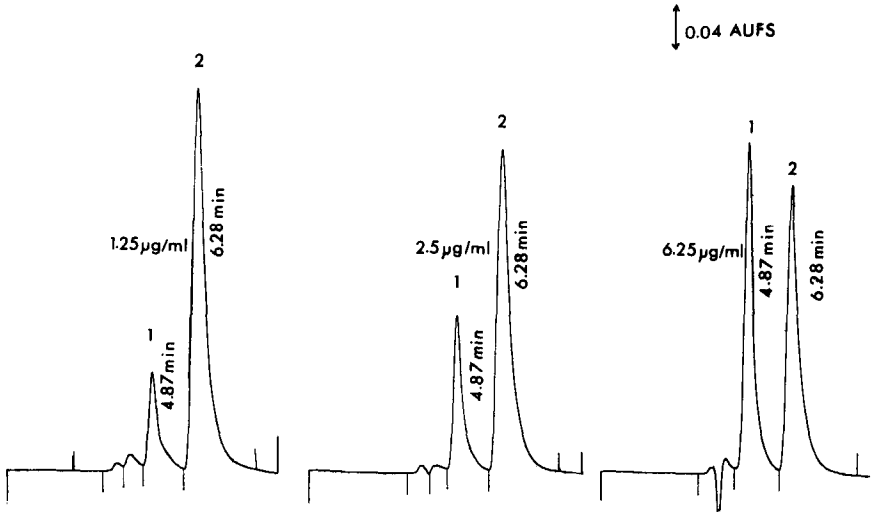

Fig. 1. Typical chromatograms obtained after injection on a LiChrosorb RP-18 column of 50 μl of the chloroform extracts of 4 ml of urine spiked with I (1.25, 2.50 and 6.25 μg/ml). Peaks: 1 = I; 2 = metoclopramide. For chromatographic conditions, see text.

TABLE I

INTER-ASSAY REPRODUCIBILITY OF THE ASSAY

n = 12 at each concentration.

Plasma concentration (μg/ml)	C.V. (%)	Urine concentration (μg/ml)	C.V. (%)
0.050	1.86	1.25	4.96
0.125	4.66	2.50	3.66
0.250	1.62	6.25	2.49
0.500	1.49	12.50	2.74
1.250	0.692	25.00	3.66

TABLE II

ACCURACY OF THE METHOD

Theoretical concentration (μg per 4 ml)	Experimental concentration (μg per 4 ml)	Percent of the dose	Relative error (%)
20	20, 22, 20, 19.9, 20	102.00	+2
40	40.8, 40.6, 40.5, 40.3, 40.5	101.25	+1.25
70	70.3, 70, 71, 70.5, 70	100.57	+0.57

two solutions of I prepared in urine at concentrations of 6.25 and 25 μg/ml. After extraction, each sample was injected in replicate (n = 10). The coefficients of variation were 0.266 and 0.456%, respectively.

Accuracy

The precision of the method was determined on three samples of urine spiked with I at concentrations of 5, 10 and 17.5 μg/ml. Each sample was determined in replicate (n = 5). Results expressed as a percentage of the theoretical concentrations, with the relative errors, are presented in Table II.

Extraction efficiencies

For both I and metoclopramide, the extraction efficiencies were determined by extracting plasma or urine prior to the addition of the internal standard (I.S.). Peak area ratios of I to metoclopramide for I and of metoclopramide to I for metoclopramide were compared with methanol standards containing appropriate concentrations of I and metoclopramide. They were 99% for I (I.S. = metoclopramide) and 90% for metoclopramide (I.S. = I).

Limit of detection

The limit of detection of I, which represents a signal-to-noise ratio of 2:1, was 12 ng/ml extracted from 4 ml of plasma; this concentration was derived from experimental values.

Application

The method was applied to the measurement of plasma and urine concentra-

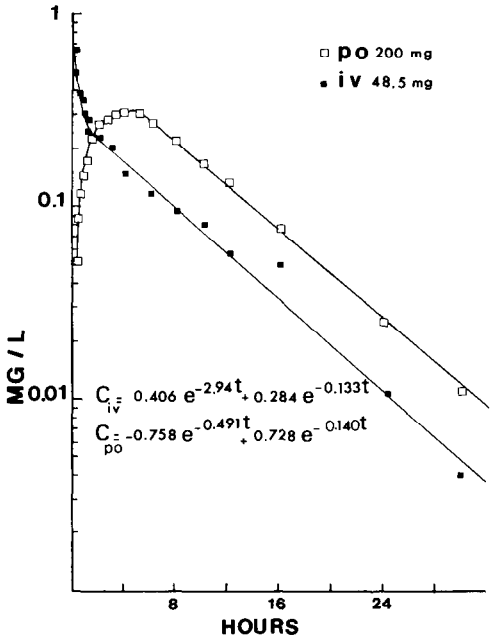

Fig. 2. Plasma levels of I following intravenous and oral administrations to a healthy volunteer. The exponential parameters were calculated using the "extended least-squares" method, MSDOS PHARM program [4, 5].

tions of the unchanged drug in twelve healthy subjects. The kinetics after intravenous (50 mg) and oral (200 mg) administration can be followed for up to 36 h (Fig. 2).

After intravenous administration, the plasma concentration versus time and urinary excretion rate versus time plots were consistent with an open two-compartment body model. The apparent half-lives of λ_1 distribution and λ_2 elimination phases were 0.18 and 4.8 h, respectively. The mean residence time was about 7 h. The steady-state volume of distribution was 2.3 l/kg and the total clearance was 22 l/h; 57% of the administered dose was recovered unchanged in urine.

After oral administration (tablet), the absorption was rapid, with an apparent half-life of 1 h. Pharmacokinetic parameters determined either from plasma or urinary data were almost identical; 23% of unchanged drug was recovered in urine and the bioavailability was about 40%.

Each subject was given a 50-mg oral dose every 8 h for eighteen doses. The doses were given at approximately 7 a.m., 3 p.m. and 11 p.m. daily. The pharmacokinetic parameters determined after oral administration (200 mg) were used for simulation after multiple dosing. The maximum and the minimum steady-state plasma levels were very close to the predicted values (Fig. 3). On day 7, a single oral dose of 200 mg was administered and the pharmacokinetic parameters were determined. The apparent half-life of the terminal phase increased from 5 h (first oral dose of 200 mg) to 9 h after this last dose.

An enzymatic saturable process or an accumulation in a deep compartment after multiple dosing explains the increase in the half-life.

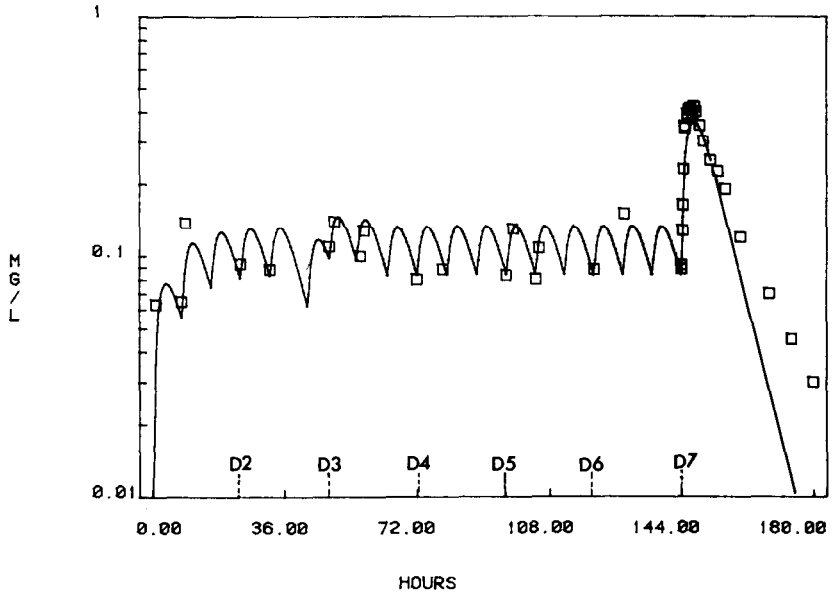

Fig. 3. Steady-state plasma levels and post-steady-state decline of I in one volunteer who received a 50-mg tablet of I three times a day for six days and a final 200-mg dose. Plasma levels were always determined just before the ingestion of the first and the second doses and 1.5 h after these periods on days 1, 3 and 5. The cumulated curve was simulated from the single dose (200 mg) study data using the IGPHARM program [6].

CONCLUSION

The HPLC technique proposed for the determination of I is selective, reliable and sensitive and has been used in a pharmacokinetic study in humans.

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