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Note

Assay of prolintane in plasma by capillary gas chromatography with nitrogen-selective detection

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Prolintane (Fig. 1) has been used as a stimulating antidepressant drug since 1962. In this communication a method for the assay of prolintane in plasma is described, which is much more sensitive than the previously published methods using thin-layer chromatography (TLC) [1, 2] and gas chromatography (GC) with packed columns and flame ionization detection (FID) [3—5]. These procedures were insufficient for measuring plasma levels after therapeutic doses, the basis of pharmacokinetic studies in man. Recently the use of a capillary column together with a nitrogen-selective detector has proved to be a very sensitive and thus suitable instrument for such an analysis [6, 13].

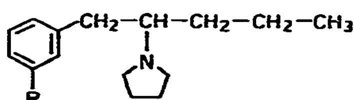

Fig. 1. Structural formulae of prolintane (R = H) and internal standard (R = CH_3).

EXPERIMENTAL

Reagents and chemicals

Prolintane [(*R*,*S*)-1-phenyl-2-(N-pyrolidyl)-pentane] and the internal standard [(*R*,*S*)-1-(3-tolyl)-2-(N-pyrolidyl)-pentane] were of analytical grade. ^{14}C-Labelled prolintane (specific activity 12.6 μCi/mg ≡ 4.62 × 10^5 dps mg^{-1}) was synthesized in the isotope laboratory of the Biochemical Department of Dr. Karl Thomae GmbH. It was labelled in the carbon-2 position of the pentane chain.

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n-Hexane and ethyl acetate were from Mallinckrodt (Wesel, G.F.R.) and were Nanograde quality, Nos. 4159 and 3427, respectively. Hydrochloric acid and sodium hydroxide were purchased from Merck (Darmstadt, G.F.R.); they were p.a. quality, Nos. 319 and 6498, respectively. Thin-layer chromatography was performed with silica-gel plates (Merck, No. 5715) in the solvents cyclohexane—diethylamine—benzene (75:20:15, v/v) and benzene—dioxane—aqueous ammonia (60:35:1, upper phase).

Apparatus

The gas chromatograph used was a Hewlett-Packard 5840 equipped with an N—P detector Model 18848A and an autosampler No. 7672. The column was a WCOT, 25 m × 0.3 mm I.D., Duranglass, the stationary phase Carbowax 20M, purchased from Perkin-Elmer (Überlingen, G.F.R.) and Bebjack (Kissing, G.F.R.). Operating conditions were: injector temperature 230°C, detector temperature 230°C. After injection in the splitless mode, the split was opened 1 min later and the temperature program was started. The column temperature was programmed from 70°C to 170°C at 10°C/min and then 15 min isothermal.

Gas flow-rates were: carrier (helium) 2 ml/min, hydrogen 3 ml/min, air 50 ml/min, auxiliary gas (helium) 35 ml/min. The helium was purified by an Oxisorb kit (Messer-Griesheim, Frankfurt, G.F.R.) to improve the service life of the column. The evaporator used was a Vortex-Evaporator (Searle-Buchler, Fort Lee, NJ, U.S.A.).

Analytical procedures

Extraction. Blood was sampled via heparinized syringes and centrifuged. The plasma obtained was stored frozen at −30°C in glass tubes. It was thawed at room temperature and 2-ml portions were pipetted into 25-ml centrifuge tubes. Then 200 µl of water containing 183.4 ng of the internal standard (as the citrate ≡ 100 ng of base) were added, followed by 2 ml of 0.2 *N* NaOH and 10 ml of *n*-hexane. The samples were mixed for 15 min on a shaking machine.

After centrifuging, the plasma phase was frozen at −20°C. The organic phase was transferred to another tube and extracted with 1 ml of 0.2 *N* HCl. The water phase was frozen, and the organic layer discarded. After thawing the water phase was made alkaline with 1.5 ml of 0.2 *N* NaOH and extracted with *n*-hexane. The hexane phase was concentrated in silanized tubes in the evaporator and reconstituted with 50 µl of ethyl acetate and transferred to the autosampler; 2 µl were injected into the chromatograph.

Preparation of the calibration curve. The calibration curve was constructed by preparing a stock solution containing 1.168 µg/ml prolintane hydrochloride in plasma. After incubation (2 h, 37°C) this plasma was diluted with non-spiked plasma to obtain a concentration range of 10—100 ng/ml. By this procedure we hoped to attain an equilibration of prolintane to plasma proteins. These standard samples were analysed by the same procedure as described above.

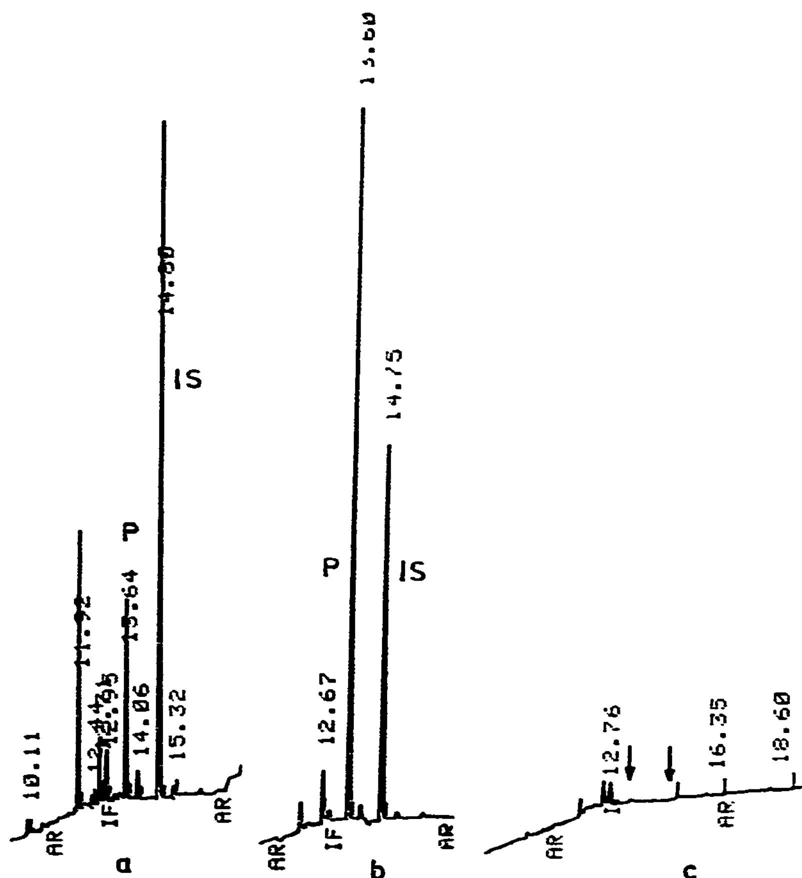

Fig. 2. Chromatograms showing human plasma levels of prolintane at concentrations of (a) 7.7 ng/ml, (b) 52.8 ng/ml, and (c) blank. P = Prolintane, IS = internal standard.

RESULTS AND DISCUSSION

The use of an N-FID instead of an FID increases the selectivity. In Fig. 2 the gas chromatograms of extracts from plasma compared with the plasma blank are shown. The blanks are very low (< 1 ng/ml) and nearly constant for all volunteers. The detection limit of the assay is about 1 ng/ml, the detection limit of the pure substance lower than 50 pg. The detection limit is determined by the variation of the blanks [7] and not by the detection limit of the system, as we inject only 1/25th of the extract.

The back-extraction is necessary for two reasons. First, we attain selectivity, as interfering non-basic compounds are removed. Without back-extraction we have a detection limit of about 5 ng/ml. Secondly, this procedure is necessary to improve the life of the Carbowax column, as with each injection the column would be coated with about 100 μg of triglycerides and 100 μg of cholesterol.

The calibration curve is linear between 10 and 100 ng of prolintane per ml (the range of therapeutic doses). It is described by the equation

$y = bx + a$, where $a = -0.021$, $b = 3.09 \times 10^2$, and $r = 0.9989$.

TABLE I

REPRODUCIBILITY OF PROLINTANE DETERMINATION ON THE SAME DAY (A) AND ON DIFFERENT DAYS (B)

	Plasma level (ng/ml)	Mean peak ratio	No. of determinations	S.D. (%)
A	10	0.268	5	4.5
	25	0.719	5	2.1
	100	3.07	5	2.7
B	20	0.58	4	8.5
	100	3.07	4	8.7

The reproducibility of the method was studied by preparing plasma samples containing different amounts of prolintane and analysing them on different days. The results are shown in Table I.

By liquid scintillation counting, the recovery of ^{14}C-labelled prolintane was found to be 69.7% ± 2.2% (n = 5) at a concentration of 50 ng/ml and 72.6% ± 2.8% (n = 5) at a concentration of 200 ng/ml. During this experiment 100 ng of internal standard were present to reproduce the same conditions as for the construction of the calibration curve. This relatively low recovery for the whole procedure is due to the fact that we used only single extraction steps. But as we used a very similar internal standard the variation of the recovery does not influence the precision of the assay. The use of silanized glass is strongly recommended to minimize losses caused by adsorption. This is of special importance as we used hexane without an alcohol. The use of hexane gives the lowest blank (cf. selectivity) and has an appropriate extraction efficacy for prolintane (Table II).

TABLE II

PARTITION RATIOS FOR PROLINTANE BETWEEN BUFFERS AND DIFFERENT SOLVENTS

	Partition ratio (C_{org}/C_{aq})		
	Hexane	Ethyl acetate	Toluene
NaOH 0.1 N	>100	26	>100
Phosphate buffer (pH 6.9)	3.5	3.0	8.0
HCl 0.1 N	0.02	0.06	0.05

The selectivity of the method was tested by two experiments. First, we applied 10 mg/kg [^{14}C]prolintane to six rats (200 g) and killed them 1 min, 3 min, 10 min after application. The plasma was extracted according to our procedure and the extracts analysed by TLC in different solvent systems. The hexane extract contained only the parent compound, quite different to extract obtained with other extraction solvents (ethyl acetate, chloroform). This means that polar metabolites are not extracted by our procedure. For legal reasons this

experiment with the labelled compound could only be done in animals. Secondly, identification of the compounds eluting from the gas chromatograph was further carried out by GC—mass spectrometry (MS) (Finnigan 3300 with computer system 6015). The mass spectrum of prolintane shows no molecular peak. The base peak is at m/z 126. The spectrum at the retention time of prolintane showed no interference with other substances.

For routine pharmacokinetic analysis the combination of glass capillary GC with N-FID is superior to glass capillary GC—MS as it is more robust against contamination from endogenous substances. In this special case the usable selected ion monitoring trace is at m/z 126. This means that it is easily disturbed by fragmentation ions arising from endogenous material. This fact was emphasized recently in the literature [8].

The method is useful for serial analyses. As the extraction steps are performed in centrifuge tubes and the phase separation is done by freezing out, 40 samples can be analysed a day by one person. The advantages of phase separation via centrifugation and freezing out (the latter step was first described by Diekmann [9] are as follows: (1) All samples can be handled simultaneously (depending on equipment). (2) There are never problems with the formation of emulsions. The small gelatinous phase between the organic and water phase (containing lipids) is solid too. (3) The water content of the organic phase is much smaller at −20°C than at 20°C, thus no drying is necessary. (4) No additional glassware such as separation funnels and pipettes (adsorption, contamination) are used to separate the water phase from the organic phase. Using an autosampler, the long time needed for an analysis run (30 min) is of minor importance as day and night operation is possible.

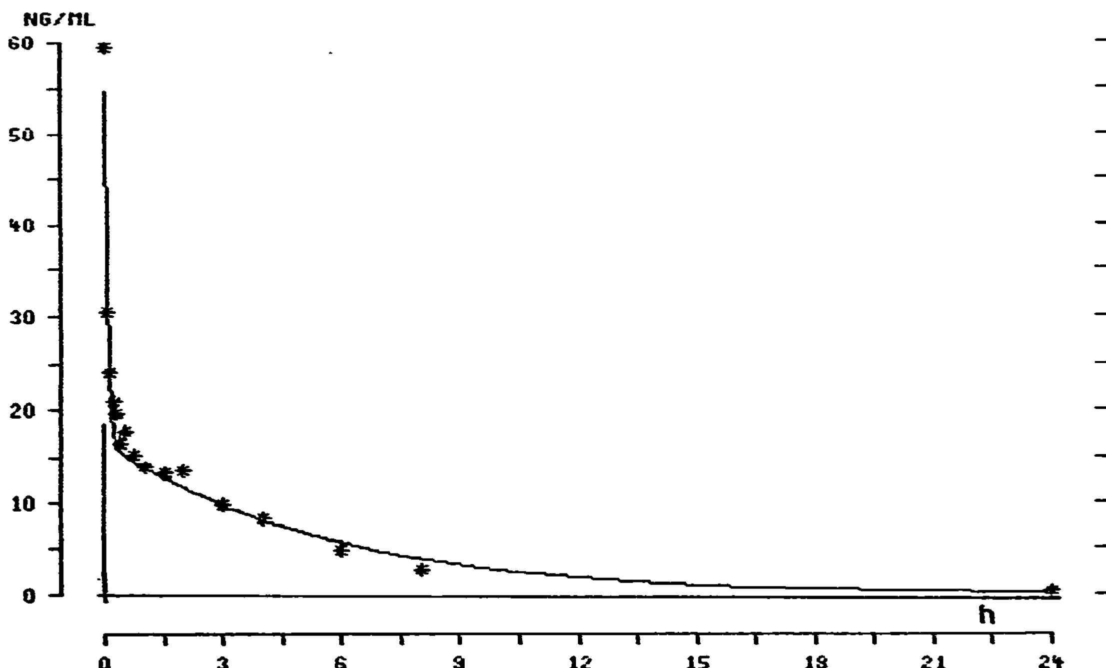

Fig. 3. Plasma level of prolintane in human subjects (n = 5) following intravenous administration of 0.15 mg/kg by a 45-sec infusion. *, Experimental values; —, computer-fitted curve.

APPLICATION

Fig. 3 shows the mean plasma level of five human volunteers after a dose of 0.15 mg/kg prolintane by a short (45-sec) infusion. The values measured are fitted with a two-compartment model with one side-compartment "Thomae Topfit" [10]. The terminal elimination half-life is about 4.5 h under controlled urinary pH and is determined by the back-diffusion of prolintane from the tissues. Compared to earlier studies [5], in which only total radioactivity was measured, we find that prolintane is rapidly metabolised. The kinetics of prolintane are characterised by a large first-pass effect [11]. This can be demonstrated easily by the intravenous experiment, from the total clearance Cl_{tot} [12], which in this case is 1.6 l/min. This clearance is of the magnitude of the hepatic blood-flow.

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