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Note

Preparative high-performance liquid chromatographic method for the 2-hydroxy metabolite of carpipramine, excreted by isolated perfused liver

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Carpipramine (Fig. 1) is a benzo[*b,f*]azepine derivative synthesized by Nakanishi et al. in 1970 [1] (Fig. 1). Its pharmacological properties have been studied in animals [2] and humans [3]. Carpipramine (Prazinil®) is used in the treatment of schizophrenia and reduced psychomotor activity [4], of asthenia in neurotic patients [5] and of deficiency syndromes following withdrawal in alcoholics and drug addicts.

Recently, metabolic studies in humans and animals [6] demonstrated an

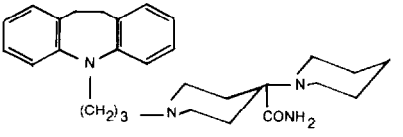

Fig. 1. Structure of carpipramine.

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active biotransformation of carpipramine after oral administration. About twenty metabolites were isolated from urine and faeces, and each of them was structurally identified. 2-Hydroxycarpipramine is of major interest, as the main metabolite present in humans as well as in the three animal species studied (rat, rabbit, dog). Thus, it is of interest to prepare it in order to study its reactivity on immunological procedure for drug monitoring. Pharmacological studies have shown that the hydroxylated metabolite of carpipramine, like 2-hydroxydesipramine, could exhibit some therapeutic activity [7,8].

EXPERIMENTAL

Reagents

Carpipramine dihydrochloride (MW 446.64) and 2-hydroxycarpipramine (RP 46238, Lot PIF 2117M) were supplied by Specia (Paris, France). All dosages are expressed relative to carpipramine base. Reagents and solvents were of analytical grade from Prolabo (Paris, France). Enzyme hydrolysis of biological media was carried out with *Helix pomatia* crude solution from I.B.F. (Villeneuve la Garenne, France) containing 10^4 Fishmann U/ml β-glucuronidase and 10^6 Roy U/ml sulphatase.

Animals

Male Sprague–Dawley rats (300 g) and male New Zealand White rabbits (1.5 kg) were purchased from Charles River (Saint Aubin les Elbeuf, France.)

Carpipramine (50 mg/kg), as an aqueous solution, was administered orally on an empty stomach. Urine and faeces were collected and pooled 24 and 48 h after dosing.

Liver perfusion

The animals were anaesthetized with pentobarbital (40 mg/kg) and treated with heparin (50 U/kg intravenously). Then, the vena portae, the inferior vena cava and the bile duct were cannulated. The liver was removed from the animal and connected to the perfusion apparatus. The liver was perfused as 37°C with 150 ml of Brauer medium: glucose, 180 mg; NaCl, 542 mg; $NaHCO_3$, 38 mg; KCl, 25 mg; $CaCl_2$, 7.2 mg; Na_2HPO_4 (0.1 *M*), 6 ml; KH_2PO_4 (0.1 *M*), 1 ml; H_2O, 60 ml; pH 7.4; albumin (25 g l^{-1}) [9]. The system was oxygenated by O_2–CO_2 (95:5, v/v), and the perfusion flow-rate was adjusted to 4 ml min^{-1} per gram of liver. The pH values before and after the passage through the liver in the outflowing medium were measured continuously. After the pre-perfusion period of 45 min, carpipramine (5 mg dissolved in 5 ml of methanol–water, 5:95) was added to the perfusion reservoir. The samples of perfusate and bile were collected for 120 min, until the end of the perfusion.

Extraction from biological media

Sample of urine (100 ml) and faeces (100 g) were extracted according to Bieder et al. [6]. The bile (5 ml) was adjusted to pH 5.0 with acetic acid. *Helix pomatia* crude solution (1 ml) was added, and the mixture was maintained for 48 h at 37°C. The hydrolysed bile was adjusted to pH 12.0 with 1.0 *M* NaOH, and extracted with 20 ml of chloroform–2-propanol (95:5, v/v). The organic phase was washed with 20 ml of water, and the solvent was evaporated to dryness in vacuo to give purified extracts, before and after hydrolysis with *Helix pomatia* crude solution.

The perfused liquid (35 ml) was extracted after protein precipitation with 50 ml of ethanol–acetone (50:50, v/v). Then, after filtration, the solvent was evaporated to dryness in vacuo to give crude extracts, before and after hydrolysis. The extract was treated with 10 ml of water, adjusted to pH 12.0 with 1.0 *M* NaOH and extracted with chloroform–2-propanol (95:5, v/v). The solvent was washed with water (20 ml), filtered and then evaporated to dryness in vacuo to obtain a final purified extract.

Thin-layer chromatography (TLC)

The extracts were dissolved in 5 ml of chloroform–methanol (80:20, v/v) and chromatographed on Merck silica plates (HF 254) in one of the following mixtures of chloroform–methanol–ammonia ($d=0.89$): (a) 90:9:1 (v/v); (b) 95:4.5:0.5 (v/v); (c) 85:13.5:1.5 (v/v); (d) 97:2.7:0.3 (v/v).

After development and drying of the plates, the compounds were detected by examination under UV light at 254 and 366 nm, or by spraying with the iron perchloride–perchloric acid reagent: solution A, 0.5% (w/v) $FeCl_3$ in perchloric acid diluted with an equal volume of distilled water; solution B, 2% (w/v) sodium nitrite in distilled water.

The chromatogram was sprayed with solution A, then with solution B, and the resultant colours were recorded. It was then heated at 110°C for 5 min and the spots were examined under UV light at 254 and 366 nm.

Analytical high-performance liquid chromatography (HPLC)

Gradient elution was necessary because of the considerable differences in the polarity of the metabolites of carpipramine. HPLC was performed with a gradient apparatus with a Gilson dual-piston pump (Model 802) (Villiers le Bel, France), a Rheodyne 7120 injection valve (Cotati, CA, U.S.A.) with a 100-μl loop, a LiChrosorb (150 mm×4 mm I.D.) Si 60 5-μm column (Merck-Clevenot, Nogent sur Marne, France), with UV detection at 260 nm using an LDC spectromonitor III (Riviera Beach, FL, U.S.A.) and a Kipp and Zonen BD8 recorder (Rotterdam, The Netherlands).

The linear gradient elution profile was monitored by an Apple II computer (Computer International, Neuilly sur Seine, France). The elution time was 115 min and the flow-rate 1 ml/min. The mobile phases used were: (1) chlo-

roform–methanol–ammonia ($d=0.89$) (99.5:0.475:0.0025, v/v) and (2) chloroform–methanol–ammonia ($d=0.89$) (80:19:1, v/v).

Preparative HPLC

HPLC was performed with the same apparatus used for the analytical study, but with a 15 cm × 0.95 cm I.D. steel column. The 2-hydroxycarpipramine was then purified by HPLC using an isocratic elution method with a mobile phases of chloroform–methanol–ammonia ($d=0.89$) (85:13.5:1.5, v/v) at a flow-rate of 3 ml/min. 2-Hydroxycarpipramine isolated by this method was submitted to analytical TLC in the solvents described above to confirm its purity.

Structural analysis

IR spectroscopy, ^{1}H NMR spectroscopy and mass spectrometry were performed as previously described by Bieder et al. [6], who identified twenty metabolites of carpipramine. In the present work D (carpipramine) = V, J = IX, M (2-hydroxycarpipramine) = XII, O = XIV, Q = XVII, R = XVIII, S = XX.

RESULTS AND DISCUSSION

After extraction of the oxidative metabolites of carpipramine from biological media, TLC was used to localize the major metabolite, namely 2-hydroxycarpipramine. The comparison of the R_F value with those of the reference compounds allowed its identification. 2-Hydroxycarpipramine had an R_F value of 0.54 in the mobile phase chloroform–methanol–ammonia ($d=0.89$) (85:13.5:1.5, v/v), inhibited the TLC plate fluorescence at 254 nm and gave an orange spot with the perchloric acid reagent, which is characteristic of the phenol group. Carpipramine had an R_F value of 0.69 in the same solvent system and presented a blue spot [6].

The HPLC study was carried out to enable quantitative estimation of the 2-hydroxycarpipramine in the biological media, and to determine the optimum conditions for isolating the hydroxylated derivative in sufficient amounts. An example of a chromatogram obtained after extraction of a urine sample is presented in Fig. 2, and it shows that carpipramine (D) and 2-hydroxycarpipramine (M) have retention times of 28 and 32 min, respectively.

The metabolism of carpipramine is an important aspect of its biological behaviour because previous bioavailability studies in animals [10] and in humans [11] showed that the amount of unchanged carpipramine excreted is low and that the metabolites represent the major form of the drug. The metabolism leads to the formation of a number of hydroxylated derivatives, particularly in rats and rabbits, previously chosen for their hydroxylation capacity [12].

The aim was to produce 2-hydroxycarpipramine, so three different assays were performed successively on perfused rat livers with three doses of carpipramine (5, 10 and 20 mg). The optimal ratio of metabolite to parent drug was

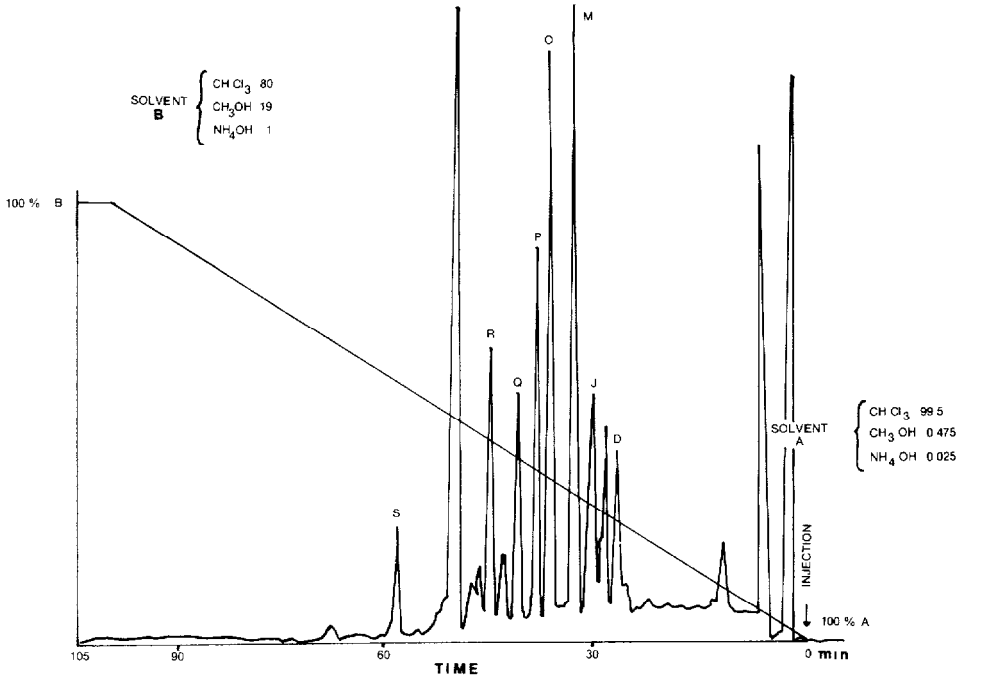

Fig. 2. HPLC of urine extracts with gradient elution.

obtained with the 5 mg dosage per liver. The extraction conditions yielded most of the hydroxylated metabolites, as confirmed by TLC examination of the bile aqueous phase residue.

The gradient elution profile used in the present HPLC procedure was derived from te conditions previously described [6]. Modifications were made to the mobile phases and the gradient profile in order to obtain the best separation of 2-hydroxycarpipramine from the other metabolites. For preparative purposes we also chose isocratic HPLC conditions to isolate the 2-hydroxycarpipramine in sufficient amounts: 10 mg, 95% pure.

During liver perfusion, carpipramine like imipramine [13] disappeared rapidly from the perfusate. Every metabolite detected in urine and faeces of rats and rabbits treated with carpipramine in vivo was observed in the bile collected during 2 h of perfusion. Perfusate collected and extracted (before and after hydrolysis) lacks any metabolites in detectable amounts. A large proportion of free 2-hydroxycarpipramine was obtained after enzymic hydrolysis of the bile. The liver is able to oxidize carpipramine into phenols and to conjugate them into glucuronides or sulphates. Thus we have confirmed, by this technique, that carpipramine undergoes hepatic oxidative metabolism.

The liver perfusion technique is a good method for following the metabolic fate of a xenobiotic. Besides in vitro systems (cell cultures, subcellular frac-

tions), the perfused organ is similar enough to the in vivo situation for rapidly produced metabolites of therapeutic interest to be studied.

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