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Note

Gas–liquid chromatographic determination of 4′-hydroxynomifensine in biological samples

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The metabolic pathways of nomifensine, a non-tricyclic antidepressant[1], include hydroxylation of the phenyl ring with the formation of 4′-hydroxynomifensine[2]. This metabolite is active in various pharmacological and biochemical tests, suggesting it might contribute to the pharmacological and clinical effects of nomifensine[3,4].

A number of analytical methods have been described for the determination of nomifensine[5–8]. In this note we describe a gas chromatographic method for 4′-hydroxynomifensine in plasma and brain. The procedure has been applied to investigate the kinetics of 4′-hydroxynomifensine in rats.

MATERIALS AND METHODS

4′-Hydroxynomifensine and nomifensine hydrogenmaleate were obtained from Hoechst (Frankfurt/M, G.F.R.). Pentafluoropropionic anhydride was obtained from Fluka (Milan, Italy). Other reagents were: formic acid, heptane, chloroform (Carlo Erba, Milan) Italy); and benzene (Pestanal grade, Hoechst).

Apparatus

A Fractovap 2150 gas chromatograph (Carlo Erba) with a ^{63}Ni electron capture detector was used. The column was a glass tube (3 m × 4 mm I.D.) packed with Supelcoport (100–120 mesh) with 3% OV-25 as the liquid phase (Pierce, Rockford, Ill., U.S.A.). Column temperature was 240°, detector temperature 250° and injector port temperature 250°. Carrier gas was nitrogen at a flow-rate of 50 ml/min.

For mass spectrometry, a mass spectrometer combined with a gas chromatograph (LKB 9000) was used under the following conditions: energy of the ionization beam, 70 eV; ion source temperature, 250°; accelerating voltage, 3.5 kV; and trap current, 100 μA. The gas chromatograph was operated under the same conditions as above.

Animals

Female CD-COBS rats (Charles River, Como, Italy), average weight 180 ± 10 g, were used.

Extraction from plasma. To 0.1–1.0 ml of heparin-treated plasma, 50 μl of a

methanolic solution of nomifensine (1 µg/ml) and 0.5 *M* phosphate buffer pH 8.8 were added to a final volume of 2.5 ml, followed by 10 ml of benzene. The samples were mechanically shaken, centrifuged and the organic phase was transferred to test-tubes containing 1.5 ml of 1 *N* formic acid, which were then shaken for 15 min. After centrifugation, the organic phase was discarded and the acidic aqueous phase was made alkaline (pH 8.8) and re-extracted with 10 ml of benzene. After centrifugation, the benzene phase was evaporated to dryness.

Extraction from brain. Brain was homogenized (6 ml/g) in cold acetone–1 *N* formic acid (85:15), and centrifuged for 15 min at 4°; the supernatant was shaken twice with *n*-heptane–chloroform (4:1). The organic phase was discarded and the aqueous phase was used for drug extraction as described for plasma.

Derivative formation

The dry residue was dissolved in 0.5 ml of benzene, 100 µl of pentafluoropropionic anhydride were added and the samples were heated at 60° for 1 h. After the reaction, the samples were shaken with 1 ml of 1 *M* phosphate buffer pH 6, centrifuged and 1 µl of the benzene phase was injected into the gas chromatographic column.

Internal standard curves

Drug-free plasma and brain samples with known amounts (5–100 ng) of 4'-hydroxynomifensine were analyzed concurrently with each set of unknown samples. Concentrations of 4'-hydroxynomifensine in the unknown samples were obtained from the ratio of the peak areas to the internal standard curves.

RESULTS AND DISCUSSION

Specificity

Fig. 1 presents typical chromatograms of extracts from (A) homogenized brain of rats treated with 4'-hydroxynomifensine (10 mg/kg, i.p.), from (B) homogenized brain to which 50 ng of the compound and the internal standard were added and from (C) drug-free homogenized brain. The extract from untreated brain does not show peaks that could interfere with analysis of 4'-hydroxynomifensine. The pentafluoropropionyl derivatives of 4'-hydroxynomifensine and nomifensine give symmetrical peaks, well separated, with retention times of 3.5 and 4.5 min respectively. Specificity of the analysis was confirmed when unknown brain samples of rats given 4'-hydroxynomifensine were analyzed by combined gas–liquid chromatography–mass spectrometry (GLC–MS). The mass spectrum obtained from analysis of the GLC peak (Fig. 2) was identical to that obtained after injection of 4'-hydroxynomifensine pentafluoropropionate.

Calibration curves and sensitivity of the method

Calibration curves were constructed by adding known amounts of 4'-hydroxynomifensine to plasma and homogenized brain of untreated rats and processing these samples as described above. In these experimental conditions the ratio of the peak area of 4'-hydroxynomifensine pentafluoropropionate to that of the internal standard was linear in the range 0.01–0.2 ng per injection. 0.01 ng per injection (2 µl)

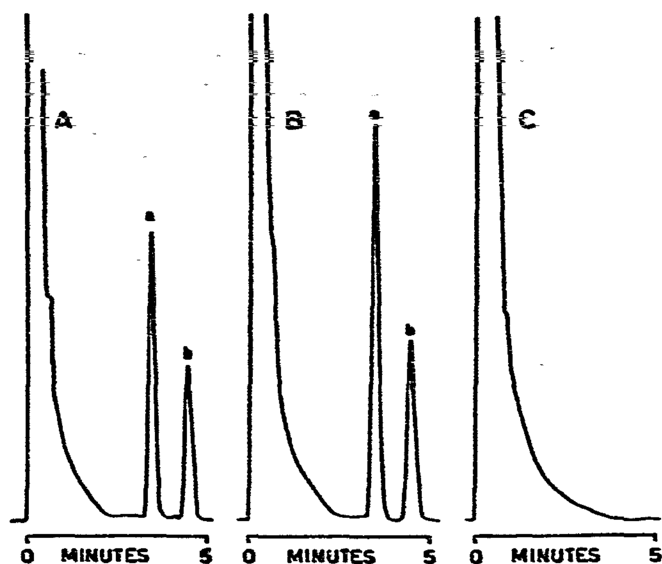

Fig. 1. Gas chromatograms of brain extracts, from rats injected with 4'-hydroxynomifensine (A), from brain to which 50 ng of 4'-hydroxynomifensine (a) and nomifensine (b) were added (B) and from drug-free homogenized brain (C).

was the lower detection limit, corresponding to 5 ng per ml of plasma or per g of brain tissue.

Recovery

At pH 8.8, 4'-hydroxynomifensine was well extracted with benzene with a recovery of 95%. Back extraction into a small volume of 1 *N* formic acid gives a very clear blank chromatogram but decreases overall recovery. In the 10–100 ng range the mean overall recovery from control plasma was 81.0 ± 4.8% and from control brain homogenate 76.0 ± 7.5%.

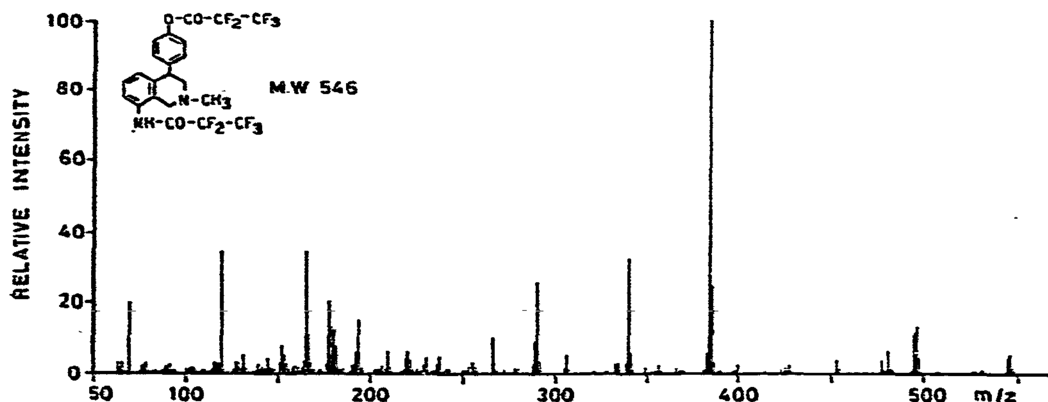

Fig. 2. Mass spectrum of 4'-hydroxynomifensine pentafluoropropionate.

Precision

To determine the within-assay and between-assay precision of the method, 4'-hydroxynomifensine (10 and 100 ng) was added to drug-free plasma, which was divided and stored at −20°. Five samples of each concentration were determined simultaneously and ten at different times. The results are reported in Table I.

TABLE I

WITHIN-RUN AND RUN-TO-RUN PRECISION IN DETERMINATION OF PLASMA 4'-HYDROXYNOMIFENSINE (4HN)

Precision	*n*	*4HN (ng/ml) added*	*Found*	*Coefficient of variation (%)*
Within-run	5	10	8.3	3.0
		100	81.2	3.3
Run-to-run	10	10	7.8	6.9
		100	81.8	7.8

Animal studies

Metabolic studies of the oral administration of [^{14}C]nomifensine have shown that 4'-hydroxynomifensine present in man and monkey urine accounts for *ca.* 7% of the administered nomifensine; the metabolite could not be identified in urine of other animal species[2]. Expriments in this laboratory after intraperitoneal administration of nomifensine (10 and 20 mg/kg) to rats confirm that the metabolite is not detectable in plasma and brain (<5 ng/ml or g) within 8 h of administration.

In order to collect information on the kinetic profile of nomifensine metabolite, female rats were treated intraperitoneally with 4'-hydroxynomifensine (10 mg/kg)

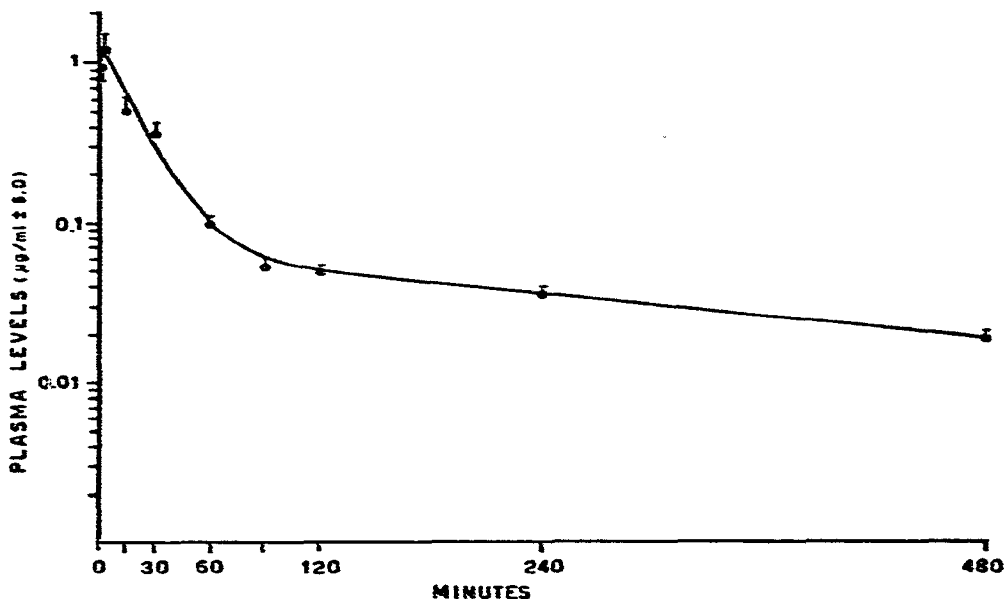

Fig. 3. Curve of plasma level *vs.* time for 4'-hydroxynomifensine after intraperitoneal injection (10 mg/kg) to rats. Each point is the mean from five animals.

and plasma and brain were analyzed as described. Fig. 3 shows the curve of plasma concentration *vs.* time for the compound. 4′-Hydroxynomifensine was rapidly adsorbed, rising to a peak at 5 min (1.18 ± 0.27 μg/ml). Plasma concentrations showed a biphasic decline thereafter, with an initial phase lasting 90 min, followed by a second slower phase with a half-life of 264 min. Table II reports the concentrations of 4′-hydroxynomifensine in four rat brain areas (striatum, brainstem, hippocampus, telencephalon). No substantial differences were noted in 4′-hydroxynomifensine kinetics. Brain peak concentrations were reached after 15 min with levels five times higher than in plasma. Half-lives ($T_{\frac{1}{2}}$) and areas under the curves (AUC) were also comparable in all the brain areas considered (Table III).

TABLE II

LEVELS OF 4′-HYDROXYNOMIFENSINE (μg/g ± S.D.) IN RAT BRAIN AREAS

Each value is the mean from five rats.

Time (min)	*Striatum*	*Brainstem*	*Hippocampus*	*Telencephalon*
1	0.16 ± 0.04	0.17 ± 0.05	0.16 ± 0.06	0.18 ± 0.05
5	1.08 ± 0.34	1.24 ± 0.58	0.92 ± 0.23	1.17 ± 0.14
15	2.10 ± 0.47	2.05 ± 0.33	2.03 ± 0.17	2.60 ± 0.83
30	1.53 ± 0.35	1.62 ± 0.12	1.66 ± 0.21	1.94 ± 0.32
60	0.79 ± 0.16	0.60 ± 0.12	0.61 ± 0.12	0.62 ± 0.16
90	0.36 ± 0.02	0.34 ± 0.06	0.32 ± 0.07	0.40 ± 0.06
120	0.30 ± 0.05	0.30 ± 0.11	0.25 ± 0.08	0.31 ± 0.14
240	0.24 ± 0.09	0.21 ± 0.05	0.20 ± 0.09	0.18 ± 0.04
480	0.13 ± 0.03	0.11 ± 0.04	0.11 ± 0.03	0.13 ± 0.04

TABLE III

AREAS UNDER THE CURVE (AUC) AND β HALF-LIFES ($T_{\frac{1}{2}}$) OF 4′-HYDROXYNOMIFENSINE

AUC and β $T_{\frac{1}{2}}$ were calculated assuming a two-compartment model for oral administration. Each value is the mean ± S.D. from five groups of rats randomized before calculation of the kinetic parameters.

	AUC (*μg/ml or g × min*)	$T_{\frac{1}{2}}$ (*min*)
Plasma	43.74 ± 12.89	264.23 ± 22.65
Striatum	242.86 ± 43.23	288.31 ± 81.12
Brainstem	210.30 ± 25.25	294.80 ± 49.97
Hippocampus	201.85 ± 29.75	300.40 ± 64.20
Telecephalon	221.52 ± 25.60	277.11 ± 60.72

CONCLUSION

The present gas chromatographic method appears to be sufficiently specific and sensitive for kinetic studies with 4′-hydroxynomifensine, and might prove useful in kinetic studies after administration of nomifensine in animal species in which the active metabolite is formed in significant amounts. Our studies show that in rats 4′-hydroxynomifensine is not detectable in plasma or brain and therefore cannot play a role in the pharmacological effects of nomifensine in the animal species considered.

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REFERENCES

1 I. Hoffmann, *Arzneim.-Forsch.*, 23 (1973) 45.
2 W. Heptner, I. Hornke, F. Cavagna, H.-W. Fehlhaber, W. Rupp and H. P. Neubauer, *Arzneim.-Forsch.*, 28 (1978) 58.
3 H. Kruse, I. Hoffmann, H. J. Gerhards, M. Leven and U. Schacht, *Psychopharmacology*, 51 (1977) 117.
4 B. Costall and R. J. Naylor, *Brit. J. Clin. Pharmacol.*, 4 (1977) 89S.
5 L. Vereczkey, G. Bianchetti, V. Rovei and A. Frigerio, *J. Chromatogr.*, 116 (1976) 451.
6 E. Bailey, M. Fenoughty and L. Richardson, *J. Chromatogr.*, 131 (1977) 347.
7 J. Chamberlain and H. M. Hill, *Brit. J. Clin. Pharmacol.*, 4 (1977) 117S.
8 W. Heptner, M. J. Badian, S. Baudner, O. E. Christ, H. M. Fraser, W. Rupp, K. E. Weimer and H. Wissman, *Brit. J. Clin. Pharmacol.*, 4 (1977) 123S.