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Note

Gas–liquid chromatographic determination of the optical isomers of fenfluramine and norfenfluramine in biological samples

S. CACCIA and A. JORI

Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea, 62-20157 Milan (Italy)

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The separation and determination of optically active amines in biological samples is difficult, and normal separation methods are ineffective because the enantiomers have the same physical properties. However, it is important to achieve their separation and determination because they have different pharmacological[1,2] and biochemical[3,4] effects. A stereoselective metabolism has also been reported for various drugs[5,6].

We report here a method for the determination of the optical isomers of fenfluramine, an anorectic agent, and its de-ethylated metabolite in biological samples after administration of racemic fenfluramine. Very small amounts of the enantiomers studied were determined by using an optically active reagent to form volatile diastereoisomers, which were resolved by gas–liquid chromatography (GLC).

N-Trifluoroacetyl-l-prolyl chloride (TPC) is an optically active reagent of this type[7], and is especially useful for resolution of racemic amines[8–10]. However, small amounts of amine TPC derivatives cannot be measured because these derivatives show a low sensitivity to the electron-capture detector (ECD). We found, however, that substitution of the pentafluoropropionyl group[11] for the trifluoroacetyl group attached to the l-proline yields fenfluramine and norfenfluramine diastereoisomers which are highly sensitive to the ECD. This procedure will enable us to investigate the distribution of the two isomers and their possible interaction.

MATERIALS AND METHODS

Standards and reagents

(+)- and (−)-fenfluramine·HCl and (+)-and (−)-norfenfluramine·HCl were kindly supplied by Servier Labs., Orléans, France. Amantadine·HCl (De Angeli, S.p.A., Milan, Italy) was used as an internal marker. Other reagents were acetone, formic acid, *n*-heptane, thionyl chloride, and l-proline (Carlo Erba, Milan, Italy); toluene (Merck Sharp & Dohme, Rahway, N.J., U.S.A.); and N-pentafluoropropionyl anhydride (Fluka, Milan, Italy).

N-Pentafluoropropionyl-l-prolyl chloride was prepared according to the method described by Wells[10] for N-trifluoroacetyl-l-prolyl chloride. Briefly, 25 mg of l-proline were dissolved in 200 μl of pentafluoropropionyl anhydride at 0°. The excess of anhydride was evaporated under nitrogen and the residue was refluxed for

15 min in 100 μl of thionyl chloride. The excess of thionyl chloride was evaporated under nitrogen and the residue was dissolved in 10 ml of toluene.

Apparatus

A Carlo Erba Fractovap 2150 gas chromatograph with a ^{63}Ni ECD was used. The chromatographic column was a glass tube 2 m long and 4 mm I.D. packed with 80–100-mesh Chromosorb W with 3% OV-225 (Carlo Erba). The column temperature was 190°, the detector temperature 275° and the carrier gas was nitrogen at a flow-rate of 50 ml/min.

For mass spectrometry (MS), 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 sample was introduced by a gas chromatographic procedure under the same conditions as described above.

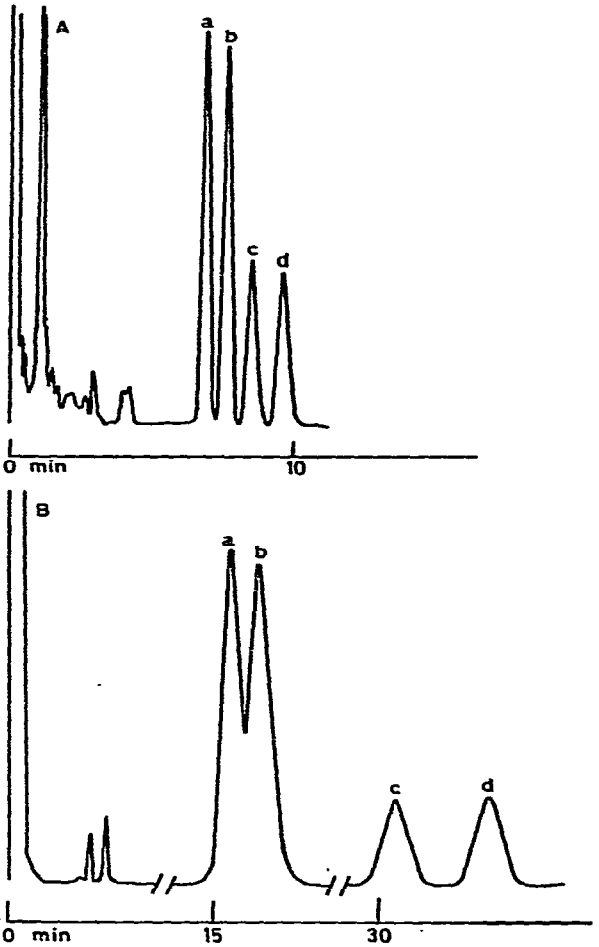

Fig. 1. Chromatograms of the resolution of fenfluramine and norfenfluramine isomers obtained with OV-225 (A) and OV-17- (B) phases. Peaks: a, (−)-norfenfluramine; b, (+)-norfenfluramine; c, (−)-fenfluramine; d, (+)-fenfluramine.

Animals

Female Charles River rats (160 ± 10 g) were treated with racemic fenfluramine (15 mg/kg, i.v.) and killed 1 h after treatment. The brains were immediately removed, frozen with dry-ice and stored at −20° until assay. Plasma was separated from whole heparinized blood by centrifugation and stored at −20°.

Extraction procedure

To 0.1–0.5 ml of water, plasma or red blood cells, various amounts of fenfluramine and norfenfluramine (30–300 ng) were added, together with 250 ng of amantadine·HCl as internal marker, 0.2 ml of 5 *N* sodium hydroxide solution, distilled water to 1.5 ml and 3 ml of toluene.

The test-tubes were shaken mechanically for 15 min, then centrifuged, and 2.5 ml of the contents were transferred into a second test-tube to which 10 μl of amino acid reagent were added. After 30 min, the solution was briefly shaken with 5 ml of 0.1 *N* sodium hydroxide solution, centrifuged and 1 μl was injected into the gas chromatographic column. Brains were homogenized (6 ml/g) in cold acetone–1 *N* formic acid (85:15, v/v). After centrifugation for 15 min at 4°, the supernatant was mechanically shaken twice with *n*-heptane–chloroform (4:1). The organic phase was discarded and the aqueous phase was used for amine extraction as described above.

RESULTS AND DISCUSSION

The reaction of the optical isomers of fenfluramine and norfenfluramine with N-pentafluoropropionyl-1-prolyl chloride yields diastereoisomers which have good resolution and high electron affinity. Fig. 1 compares the gas chromatograms for the separation of the isomer derivatives studied on the highly polar OV-225 (A) and on mildly polar OV-17 (B) at 190°. The resolution of diastereoisomers is better, with a significantly lower retention time, on the highly polar phase (A). The calibration graphs for (+)- and (−)-fenfluramine (a) and (+)- and (−)-norfenfluramine (b) added

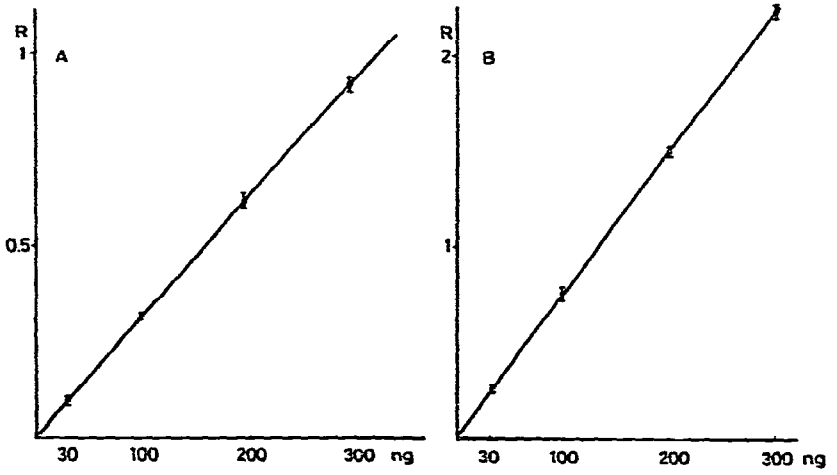

Fig. 2. Calibration graphs for fenfluramine (a) and norfenfluramine (b) from a brain extract. The curves of the (+)- and (−)-isomers are coincident for both compounds.

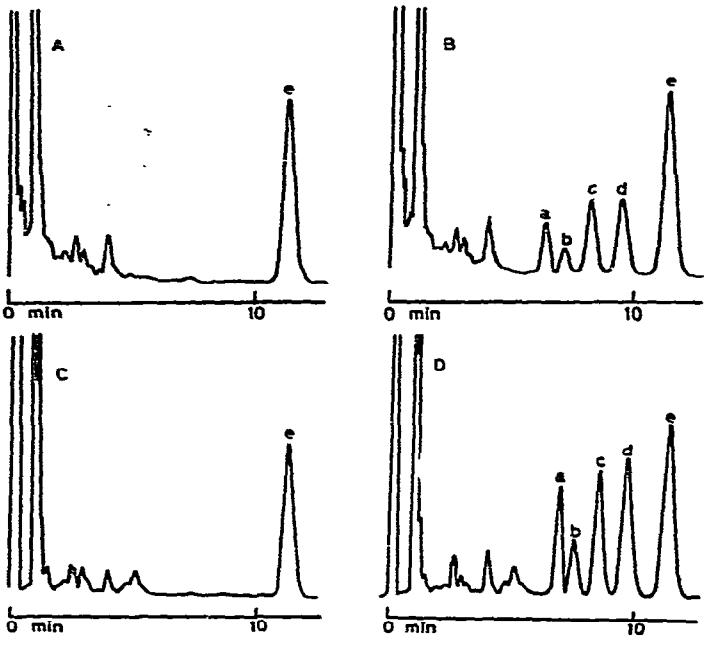

Fig. 3. Chromatograms of biological extracts (plasma = B, red blood cells = D) obtained from rats treated with fenfluramine·HCl (15 mg/kg, i.v.) 1 h before death and from the corresponding blanks (A and C). Peaks: a, (−)-norfenfluramine; b, (+)-norfenfluramine; c, (−)-fenfluramine; d, (+)-fenfluramine; e, amantadine (internal marker).

to brain homogenate are illustrated in Fig. 2. For each compound, the graphs of the two diastereoisomers were completely coincident. Calibration graphs for plasma and red blood cells are not reported because of their similarity to those of the brain homogenate. The graphs obtained by plotting the ratio of the areas of the compounds to that of the internal marker against known amounts of the compounds are linear in the range from 30 to 300 ng per sample. The minimum detectable amount per injection is 10 pg and the recovery from biological samples was 95 ± 2%.

Fig. 3 shows typical chromatograms of extracts from plasma (B) and red

TABLE I

CONCENTRATIONS OF (+)- AND (−)-FENFLURAMINE AND (+)- AND (−)-NORFENFLURAMINE AFTER ADMINISTRATION OF (±)-FENFLURAMINE TO RATS

(±)-Fenfluramine·HCl was given intravenously at a dose of 15 mg/kg 1 h before death. Each value is the mean of four determinations.

Compound determined	*Brain (μg/g ± S.E.)*	*Plasma (μg/ml ± S.E.)*	*Packed red blood cells (μg/ml ± S.E.)*
(+)-Fenfluramine	13.7 ± 1.5	0.438 ± 0.030	1.025 ± 0.1
(−)-Fenfluramine	11.5 ± 1.6	0.359 ± 0.030	0.746 ± 0.06
(+)-Norfenfluramine	1.4 ± 0.1	0.038 ± 0.005	0.126 ± 0.01
(−)-Norfenfluramine	3.2 ± 0.4	0.087 ± 0.009	0.196 ± 0.01

blood cells (D) obtained from a rat treated 1 h before death with racemic fenfluramine hydrochloride (15 mg/kg, i.v.) and the corresponding blanks (A and C).

The identity of the GLC peaks after reaction of optical isomers of fenfluramine and norfenfluramine with the chiral reagent were checked by means of GLC–MS. The analysis was confirmed that the peaks correspond to the diastereosiomeric pairs of (N-pentafluoropropionyl-1-prolyl)-*d,l*-fenfluramine and *d,l*-norfenfluramine.

The results of the distribution of (+)- and (−)-fenfluramine and (+)- and (−)-norfenfluramine in brain, plasma and red blood cells of rats killed 1 h after treatment with racemic fenfluramine (15 mg/kg, i.v.) are reported in Table I.

CONCLUSION

We have developed an analytical method for the determination of the optical isomers of fenfluramine and their metabolites after administration of racemic fenfluramine. The sensitivity and specificity of the method are compatible with pharmacokinetic studies.

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