Journal of Chromatography, 84 (1973) 355–360
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 6850

A SPECIFIC GAS CHROMATOGRAPHIC METHOD FOR THE DETECTION OF *p*-HYDROXYAMPHETAMINE AND *p*-HYDROXYNOREPHEDRINE IN BRAIN TISSUE

G. BELVEDERE, S. CACCIA, A. FRIGERIO and A. JORI
Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan (Italy)
(Received May 9th, 1973)

SUMMARY

A gas chromatographic method for the determination of *p*-hydroxyamphetamine and *p*-hydroxynorephedrine in rat brain tissue is described. The sensitivity and specificity of this method allows the quantitation of these metabolites when present in the brain after administration of amphetamine.

INTRODUCTION

It is well known that *d*-amphetamine is converted into *p*-hydroxyamphetamine (*p*-OHA) by a metabolic microsomal enzymatic reaction in several animal species[1]. Since *p*-OHA is a substrate for dopamine-β-hydroxylase, it may be transformed into *p*-hydroxynorephedrine (*p*-OHNE)[2,3]. It has been suggested[1,4,5] that these two metabolites play a role in the catecholamine depletion exerted by amphetamine in both the heart and the brain. They have been also implicated in the onset of amphetamine tolerance during chronic treatments[5,6].

In previous work, these two metabolites have been determined by utilizing tritium-labelled amphetamine[6,7]. In this paper, we describe an alternative sensitive and specific gas chromatographic method for the determination of *p*-OHA and *p*-OHNE.

With the method described, the measurement of brain amphetamine can be performed in the same sample according to a previously described procedure[8].

EXPERIMENTAL

Standards and reagents

The following substances were used: *d*-amphetamine sulphate (Recordati S.p.A., Milan, Italy); *p*-OHA·HBr (SKF Laboratories, Philadelphia, Pa., U.S.A.); *p*-OHNE·HCl (kindly supplied by Dr. E. Costa, Washington, D.C., U.S.A.); *p*-nitroanisole (Carlo Erba, Milan, Italy); acetone, chloroform, formic acid, ethyl acetate and benzene (Carlo Erba, gas chromatographic grade); heptane (Rudy Pont, Milan, Italy); and trifluoroacetyl anhydride (Fluka, Milan, Italy).

Gas–liquid chromatography (GLC)

A Carlo Erba Fractovap G-1 gas chromatograph with a ^{63}Ni electron capture detector (ECD) was used.

The GLC conditions were as follows. The column was a glass tube, 2 m long and 4 mm I.D., packed with 3% OV-17 on 100–120 mesh Chromosorb Q (Applied Science Lab., State College, Pa., U.S.A.). For *p*-OHA and *p*-OHNE, the column temperature was 150°, the injection port temperature 210° and the detector temperature 250°, and the carrier gas (nitrogen) flow-rate was 40 ml/min. For amphetamine, the column temperature was 210°, the injection port temperature 230° and the detector temperature 250°, and the carrier gas (nitrogen) flow-rate was 40 ml/min.

Mass spectrometry (MS)

An LKB Model 9000 mass spectrometer equipped with a gas chromatograph was used.

The GLC conditions were as follows. The column was a glass tube, 2 m long and 2 mm I.D., packed with 3% OV-17 on 100–120 mesh Gas-Chrom Q (Applied Science Lab.). The column temperature was 160° and the injection port temperature 200°, and the carrier gas (helium) flow-rate was 30 ml/min. The mass spectrometer was set to the following conditions: separator temperature 290°, ion source temperature 290°, trap current 60 μA, electron energy 70 eV and multiplier voltage 3.5 kV.

Extraction procedure

Rat brains were homogenized (6 ml/g) in a cold mixture of acetone–1 *N* formic acid (85:15, v/v), and various amounts of amphetamine (between 2 and 5 μg) and *p*-OHA and *p*-OHNE (between 50 and 150 ng) were added. After centrifugation for 5 min at 4°, the supernatant liquid was acidified and mechanically shaken twice with heptane–chloroform (4:1). The organic phase was discarded and the aqueous phase was diluted with distilled water to 1.5 ml and used for the amine extraction.

A volume of 0.1–0.5 ml was used for the amphetamine determination according to the method described by Anggård *et al.*[8]. The remaining aqueous phase was made strongly acidic ($pH < 2$) with formic acid (85%) and washed with 3 ml of benzene. The acidic phase was made basic with 5 *N* NaOH (pH 10) and washed with 3 ml of benzene; then 0.5 g of NaCl was added and the mixture was extracted twice with 2 ml of ethyl acetate by shaking for 10 min. Aqueous samples containing various amounts of *p*-OHA and *p*-OHNE were extracted in a similar manner to obtain standard graphs.

The organic extract was evaporated under a stream of nitrogen, 50 μl of ethyl acetate and 30 μl of trifluoroacetyl anhydride (TFA) were added and the samples heated at 65° for 10 min. After the reaction, the samples were again evaporated to dryness under a stream of nitrogen; 300 μl of ethyl acetate containing *p*-nitroanisole (*p*-NO_2A) (1 ng/μl) as internal marker were added to the dry residue and 1 μl was injected into the gas chromatographic column.

Animal treatment

Five female Charles River rats (180 ± 10 g) were treated with *d*-amphetamine sulphate (15 mg/kg, i.p.) and sacrificed 2 h after the treatment. The brains were im-

mediately removed, frozen with dry-ice and stored at —20° until the determinations were carried out. The determinations were performed as described above.

RESULTS AND DISCUSSION

Fig. 1 shows the gas chromatograms of an extract (a) from water (standard sample), (b) from tissue (blank sample) and (c) from tissue with *p*-OHA and *p*-OHNE added (internal standard sample). *p*-NO_2A was used as the internal marker because of its suitable retention time.

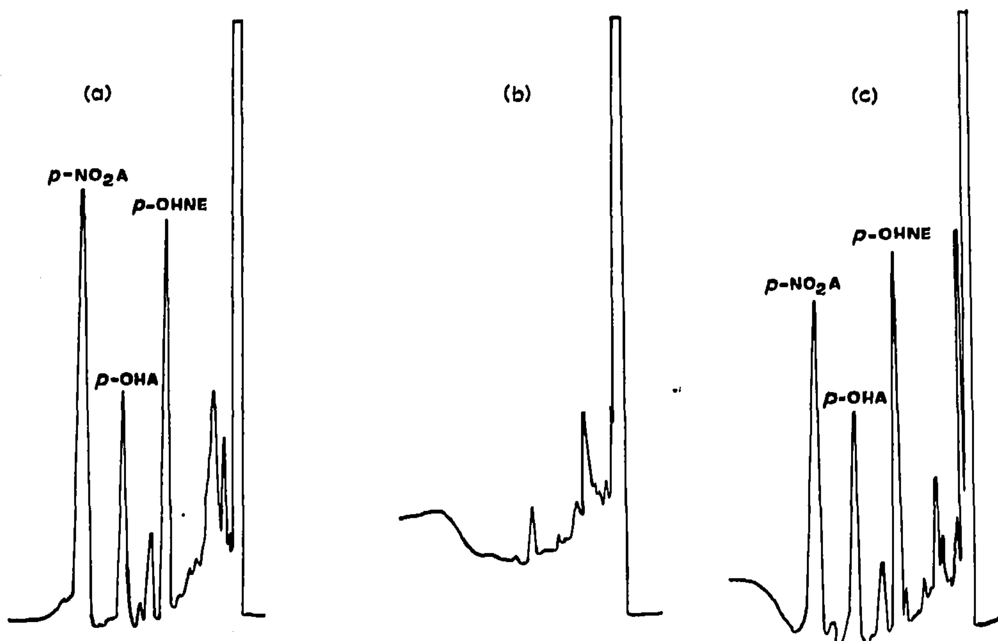

Fig. 1. Gas chromatogram of the trifluoroacetyl derivatives of *p*-hydroxynorephedrine (*p*-OHNE) and *p*-hydroxyamphetamine (*p*-OHA) extracted from water (a) and from brain tissue (c). (b) is the brain tissue blank. *p*-NO_2A = *p*-nitroanisole (internal marker).

Fig. 2 shows the calibration graphs for *p*-OHNE (a) and *p*-OHA (b), obtained by plotting the ratio of the areas of the peaks of the compounds to that of the internal marker against the concentrations of the compounds injected. The graphs are linear in the range between 50 and 250 pg per microlitre of solution injected. The recovery of the *p*-OHA and *p*-OHNE added to the brain was 88 $\pm$ 2%.

Fig. 3 shows the gas chromatogram of a brain extract obtained by a rat treated 2 h before death with *d*-amphetamine sulphate (15 mg/kg, i.p.). The retention time of the specific peaks compared with that of the internal standards (Fig. 1) accounts for the actual presence of the two metabolites in the extract of amphetamine-treated rat brain.

The identities of the GLC peaks after reaction of *p*-OHA and *p*-OHNE with TFA were checked by means of GLC–MS. The mass spectrum of *p*-OHA-TFA (Fig. 4) does not show the molecular ion but the entry of two TFA groups in the molecule

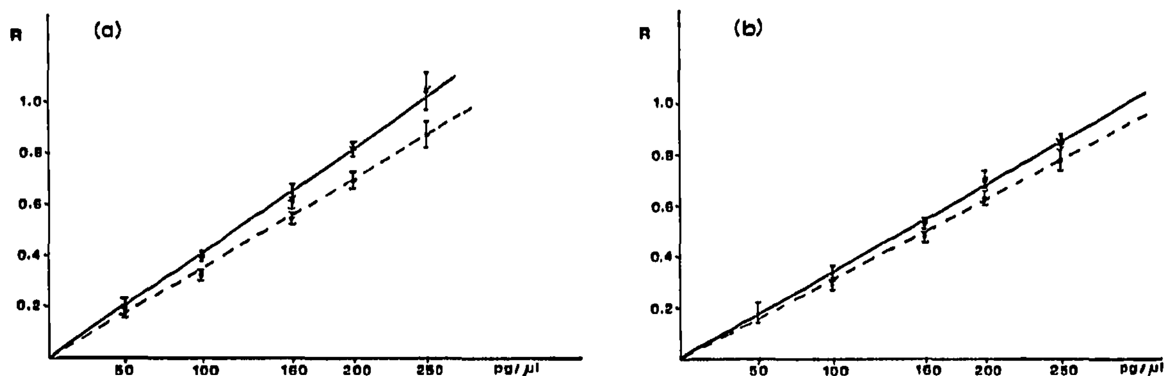

Fig. 2. Standard calibration graphs of *p*-hydroxynorephedrine (*p*-OHNE) (a) and *p*-hydroxyamphetamine (*p*-OHA) (b). The ratio (R) of the peak areas of the compounds to that of the internal marker (*p*-nitroanisole) is plotted against the concentrations of the compounds injected. Solid lines, *p*-OHNE and *p*-OHA extracted from water; broken lines, *p*-OHNE and *p*-OHA extracted from brain homogenate.

can be postulated from the presence of the ion at *m/e* 230 due to the loss of the radical $\cdot OCOCF_3$ from the molecular ion. Another fact in favour of this hypothesis is the presence of the ion at *m/e* 203, which is obtained by fission of the *β*-bond with respect to the aromatic ring of the molecule by loss of the radical $CH_3\dot{C}HNHCOCF_3$; this fragment can also retain a positive charge, giving rise to the base peak at *m/e* 140.

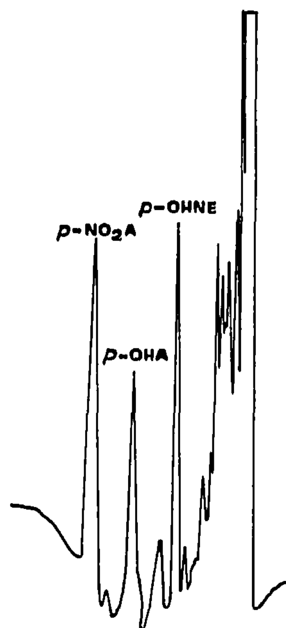

Fig. 3. Gas chromatogram of rat brain tissue 2 h after treatment with *d*-amphetamine sulphate (15 mg/kg, i.p.). *p*-OHNE = *p*-hydroxynorephedrine; *p*-OHA = *p*-hydroxyamphetamine; *p*-NO_2A = *p*-nitroanisole (internal marker).

The behaviour of the *p*-OHNE-TFA is very similar; in this case also the mass spectrum (Fig. 4) does not show the molecular ion, but the presence of a fragment at *m/e* 342 indicates that three TFA groups enter the molecule. This hypothesis is also supported by the presence of the ion at *m/e* 315 due to the loss of the radical $CH_3\dot{C}HNHCOCF_3$ from the molecular ion. This fragment retains a positive charge and gives rise to the base peak at *m/e* 140 in the same manner as that from *p*-OHA-TFA.

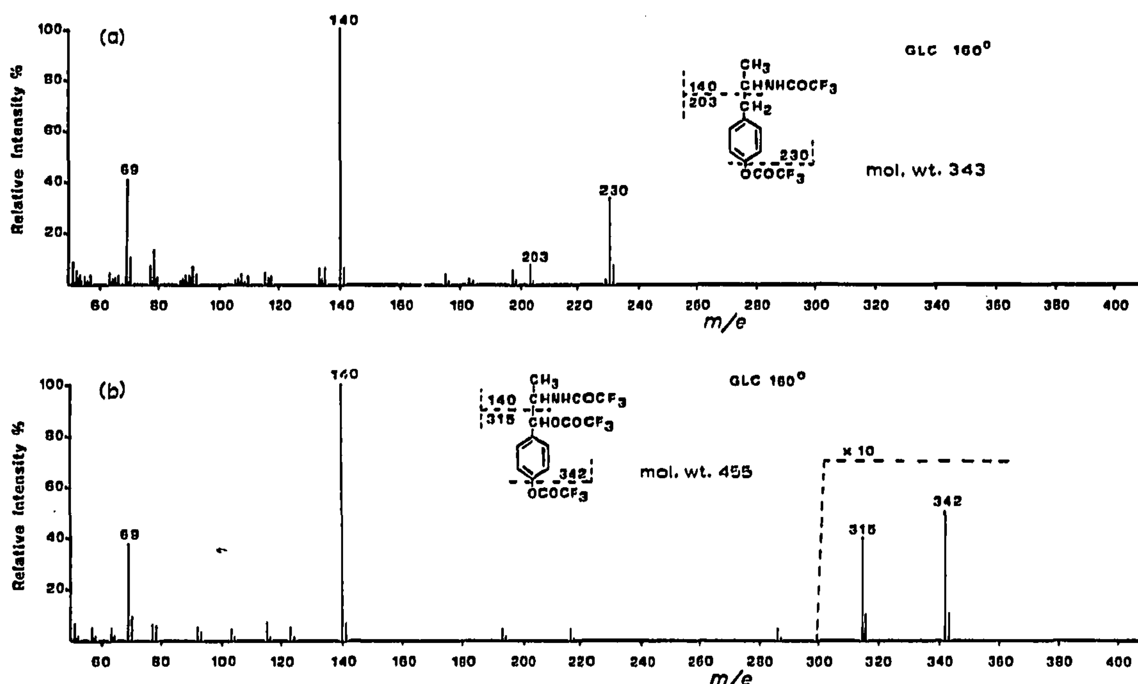

Fig. 4. Mass spectra of trifluoroacetyl derivatives of *p*-hydroxyamphetamine (a) and *p*-hydroxynorephedrine (b).

The concentrations of amphetamine and its metabolites in the brain 2 h after treating rats with amphetamine are reported in Table I. The amphetamine levels are in agreement with those previously reported[9], and the levels of *p*-OHA and *p*-OHNE are in agreement with the concentrations obtained by Clay *et al.*[7].

TABLE I

CONCENTRATION OF AMPHETAMINE AND ITS METABOLITES IN RAT BRAIN 2 h AFTER TREATMENT WITH *d*-AMPHETAMINE SULPHATE (15 mg/kg, i.p.)

Compound determined	*Brain level** (*ng/g* ± *standard error*)
Amphetamine	7010 ± 20
p-Hydroxyamphetamine	68 ± 8
p-Hydroxynorephedrine	77 ± 3

* Each figure is the average of five determinations.

ACKNOWLEDGEMENTS

This study was supported by a grant from the G. L. Pfeiffer Foundation, New York, U.S.A. The technical help of Miss M. Riunno is gratefully acknowledged.

REFERENCES

1 J. Axelrod, in E. Costa and S. Garattini (Editors), *International Symposium on Amphetamines and Related Compounds*, Raven Press, New York, 1970, p. 207.
2 M. Goldstein and B. Anagnoste, *Biochim. Biophys. Acta*, 107 (1965) 166.
3 A. Sjoerdsma and W. von Studnitz, *Brit. J. Pharmacol. Chemother.*, 20 (1963) 278.
4 B. B. Brodie, A. K. Cho and G. L. Gessa, in E. Costa and S. Garattini (Editors), *International Symposium on Amphetamines and Related Compounds*, Raven Press, New York, 1970, p. 217.
5 E. Costa and A. Groppetti, in E. Costa and S. Garattini (Editors), *International Symposium on Amphetamines and Related Compounds*, Raven Press, New York, 1970, p. 231.
6 A. Groppetti and E. Costa, *Life Sci.*, 8, Pt. I (1969) 653.
7 G. A. Clay, A. K. Cho and M. Roberfroid, *Biochem. Pharmacol.*, 20 (1971) 1821.
8 E. Anggård, L.-M. Gunne and F. Niklasson, *Scand. J. Clin. Lab. Invest.*, 26 (1970) 137.
9 A. Jori, E. Dolfini, G. Tognoni and S. Garattini, *J. Pharm. Pharmacol.*, (1973) in press.