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DETERMINATION OF METHAMPHETAMINE AND ITS METABOLITES IN RAT TISSUES BY GAS CHROMATOGRAPHY WITH A NITROGEN–PHOSPHORUS DETECTOR

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SUMMARY

A method for the determination of methamphetamine and its metabolites in tissues of the rat receiving methamphetamine was developed using gas chromatography with nitrogen–phosphorus detection. The extraction procedure, volatility of various derivatives, the acylation procedure, mass spectra of various derivatives and the recovery of methamphetamine and its metabolites in rat tissues are reported. The detection limits of pentafluorobenzoyl derivatives of methamphetamine and amphetamine, and heptafluorobutyryl derivatives of *p*-hydroxymethamphetamine and *p*-hydroxyamphetamine, were *ca.* 0.1 and 0.15 ng, respectively. This method could be used to determine concentrations as low as 10–15 ng/g of methamphetamine and its metabolites; recoveries from the rat brain, liver and serum were 94–106, 103–115 and 94–96%, respectively.

INTRODUCTION

In recent years, the abuse of methamphetamine has markedly increased, and has become a serious problem in Japan. Therefore the development of a simple, rapid and sensitive assay for methamphetamine is required. Various methods for the determination of methamphetamine and related amines in biological materials have been reported, including gas chromatography (GC)[1–3], gas chromatography with electron-capture detection (GC–ECD)[4–6], gas chromatography–mass spectrometry (GC–MS)[7–10], high-performance liquid chromatography[11,12] and immunoassay[13–15].

Caldwell *et al.*[16] showed that metabolic reactions of methamphetamine were *p*-hydroxylation, *β*-hydroxylation, demethylation and deamination, and that great species differences were present in the amounts of these metabolites. Conventionally, metabolites of methamphetamine in biological materials have been determined by using radiolabelled methamphetamine[16,17], GC–MS[18] and GC–ECD[19]. However, there have been no reports of a simple and sensitive method for the GC determination of methamphetamine and its metabolites in tissue samples.

The availability of a nitrogen–phosphorus detector has made it possible to

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analyze compounds containing nitrogen and phosphorus at a highly sensitive level. In a previous paper, a rapid and highly sensitive method was reported for determination of methamphetamine and its metabolites in rat urine using GC–nitrogen–phosphorus detection (NPD)[20]. However, this method could not be applied to homogenized tissue samples because of a number of extraneous peaks recorded by GC–NPD.

Consequently, this paper describes a simple and highly sensitive method for determination of methamphetamine and its metabolites in rat tissues using GC–NPD.

EXPERIMENTAL

Apparatus

GC analysis was carried out with a Shimazu GC-7AG gas chromatograph equipped with nitrogen–phosphorus and flame ionization detectors. The columns were: A (1 m × 3 mm I.D.), glass, packed with 2% Thermon-3000 on Chromosorb W AW DMCS (80–100 mesh); B (2 m × 3 mm I.D.), glass, packed with 3% OV-17 on Gas-Chrom Q (80–100 mesh) and C (0.5 m × 3 mm I.D.), glass, packed with 1% OV-17 on Gas-Chrom Q (80–100 mesh). The temperature of columns A, B and C was programmed at 10°C/min from 200 to 250°C, at 5°C/min from 140 to 180°C and at 10°C/min from 120 to 150°C, respectively. The carrier gas (helium) flow-rate was 50 ml/min. The chart speed was 5 mm/min.

Mass spectrometric analysis was carried out a JEOL JMS D-300 mass spectrometer connected to the gas chromatograph equipped with column B. The temperature was programmed at 10°C/min from 100 to 300°C; injection port temperature 280°C, separator temperature 250°C and ionizing voltage 200 eV in the chemical ionization (CI) and 70 eV in the electron impact (EI) mode. Isobutane was used as a reagent gas. The carrier gas (helium) flow-rate was 30 ml/min.

Reagents

Methamphetamine hydrochloride was obtained from Dainippon Pharmaceutical Co. (Osaka, Japan). *p*-Hydroxyamphetamine hydrobromide was donated by Smith Kline and French Laboratories (Philadelphia, PA, U.S.A.). *p*-Hydroxymethamphetamine hydrochloride was prepared from *p*-methoxyphenylacetone by the method of Buzas and Dufour[21]. Trifluoroacetic and heptafluorobutyric anhydride were supplied by Pierce Chemical Co. (Rockford, IL, U.S.A.). Pentafluorobenzoyl chloride was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). β-Glucuronidase was supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade available commercially.

Animals

Male albino rats (Wistar strain, 300–330 g) were injected intraperitoneally with 10 mg methamphetamine per kg and killed 2 h later. The brain, liver, kidney and spleen were immediately removed, and kept frozen at −20°C until analysis. Whole blood collected in a 10-ml centrifuge-tube was centrifuged for 10 min at 700 *g*, and the serum was stored at −20°C until analysis.

Extraction of methamphetamine and its metabolites from tissues

The tissues (0.3–1.0 g) were homogenized in 20 ml of cold acetone–1 *M* formic acid[19] (5:1, v/v) and then centrifuged at 10,000 *g* for 15 min. The supernatant was shaken twice with 20 ml of diethyl ether. The ether layer was discarded, and the methamphetamine and its metabolites were extracted from the aqueous layer according to procedures A and B described below.

Procedure A (assay of methamphetamine and amphetamine on column A). The above-mentioned layer (1 ml) or the serum (1 ml) was transferred to a screw-cap round bottom centrifuge-tube, and 1 ml of distilled water, 0.5 ml of 10 *M* sodium hydroxide and 6 ml of hexane were added. The tube was shaken for 10 min and centrifuged for 5 min at 700 *g*. Five ml of the hexane layer were transferred to a 10-ml screw-cup round bottom centrifuge-tube and back-extracted with 2 ml of 0.1 *M* hydrochloric acid. After centrifugation, the hexane was aspirated. Then 0.5 ml of 10 *M* sodium hydroxide were added to the aqueous layer, which was re-extracted with 6 ml of hexane. The contents were mixed for 10 min, and then centrifuged. A 5-ml volume of the hexane layer was transferred to a 10-ml screw-cap round bottom centrifuge-tube, and 1 ml of pentafluorobenzoyl chloride (1 μmol/ml in pentane) was added. The sample was heated at 60°C for 20 min. After the reaction, the solvent was almost completely evaporated under a stream of nitrogen at 40°C. The residue was dissolved in acetone and an aliquot (1–2 μl) was injected into the gas chromatograph.

Procedure B (assay of p-hydroxymethamphetamine and p-hydroxyamphetamine on column B). The above-mentioned aqueous layer (1 ml) was transferred to a 10-ml screw-cap round bottom centrifuge-tube, and 1 ml of distilled water and 0.2 ml of 20% Na_2CO_3 were added. The aqueous layer was washed three times with 6 ml of heptane. After centrifugation for 10 min at 700 *g*, the aqueous layer was saturated with NaCl and then extracted with 6 ml of ethyl acetate. After centrifugation, 5 ml of the ethyl acetate layer were transferred to a 10-ml screw-cap round bottom centrifuge-tube. The organic layer was concentrated to 0.2–0.4 ml under a stream of nitrogen at 40°C. Heptafluorobutyric anhydride (0.1 ml) was added, and then heated at 60°C for 40 min. The solvent was almost completely evaporated under a stream of nitrogen at 40°C. The subsequent procedure was the same as in procedure A.

Procedure C (assay of total p-hydroxymethamphetamine and p-hydroxyamphetamine on column B). The tissues (0.3–1.0 g) were homogenized with 15 ml of 0.2 *M* sodium acetate–acetic acid buffer (pH 3.8) and then centrifuged at 10,000 *g* for 15 min. The supernatant was incubated with β-glucuronidase (1.6 IU) at 37°C for 24 h. After centrifugation for 10 min at 700 *g*, the supernatant was adjusted to pH 9.5 with 20% Na_2CO_3, saturated with sodium chloride and extracted with 20 ml of ethyl acetate. After centrifugation, 17 ml of the ethyl acetate layer were transferred to another tube and then concentrated to *ca.* 6 ml under a stream of nitrogen at 40°C. The organic layer was back-extracted with 2 ml of 0.1 *M* hydrochloric acid and centrifuged. The hydrochloric acid layer was washed once with 6 ml of heptane, made alkaline with 0.2 ml of 20% Na_2CO_3 and then washed three times with 6 ml of heptane. The subsequent procedure was as in procedure B.

Extraction conditions for methamphetamine and its metabolites

A mixture of 1 ml of a standard solution of methamphetamine and its metabolites (1 μg in distilled water) and 1 ml of distilled water was adjusted to pH 12 with

TABLE I

EFFICIENCIES OF SOLVENTS AND ALKALINE CONDITIONS FOR THE EXTRACTION OF METHAMPHETAMINE AND ITS METABOLITES

The values are expressed as peak height (cm) per ng. n.d. = Not detectable.

Compound	*Solvent and alkaline conditions*									
	Hexane		*Heptane*		*Diethyl ether*		*Ethyl acetate*		*CI**	
	pH 9.5	*pH 12*	*pH 9.5*	*pH 12*	*pH 9.5*	*pH 12*	*pH 9.5*	*pH 12*	*pH 9.5*	*pH 12*
Methamphetamine	3.0	3.3	3.4	3.0	3.6	3.8	3.4	3.5	4.7	5.2
Amphetamine	3.7	3.9	4.0	3.6	4.2	4.4	4.0	4.1	5.3	5.5
p-Hydroxymethamphetamine	n.d.	n.d.	n.d.	n.d.	1.1	n.d.	3.6	2.9	4.8	n.d.
p-Hydroxyamphetamine	n.d.	n.d.	n.d.	n.d.	1.1	n.d.	3.7	2.7	4.6	0.1

* CI = Chloroform–isopropanol (3:1, v/v).

10 *M* sodium hydroxide or to pH 9.5 with 20% Na_2CO_3 and saturated with NaCl. The sample was extracted with 6 ml of hexane, heptane, diethyl ether, ethyl acetate or chloroform–isopropanol (3:1, v/v). After centrifugation for 10 min at 700 *g*, 5 ml of the organic solvent layer were transferred to a 10-ml screw-cap round bottom centrifuge-tube, and 20 μl of acetic acid were added. The extract was evaporated just to dryness under nitrogen at 40°C. The residue was dissolved in 0.2 ml of ethyl acetate, and 0.1 ml of heptafluorobutyric anhydride was added. The sample was heated at 60°C for 40 min, and evaporated under nitrogen until became a wet. The residue was dissolved in acetone and an aliquot (2 μl) was injected into the GC column C.

RESULTS AND DISCUSSION

Extraction conditions for methamphetamine and its metabolites

As shown in Table I, chloroform–isopropanol (3:1, v/v) had a higher efficiency for extraction of methamphetamine and its metabolites from water at pH 9.5 than the other solvents. However, a number of extraneous peaks were observed from the chloroform–isopropanol extract of the blank liver under the experimental conditions. Hexane had a high efficiency for extraction of methamphetamine and amphetamine, and the extract from blank liver had fewer extraneous peaks than the other extracts. *p*-Hydroxymethamphetamine and *p*-hydroxyamphetamine were efficiently extracted by ethyl acetate, and the chromatogram of this extract from the blank liver showed only a few extraneous peaks. These results indicated that methamphetamine and amphetamine were efficiently extracted with hexane under strong alkaline conditions (10 *M* sodium hydroxide), and *p*-hydroxymethamphetamine and *p*-hydroxyamphetamine were efficiently extracted with ethyl acetate at pH 9.5, adjusted with Na_2CO_3, and saturated with NaCl.

Volatility of various derivatives of methamphetamine and its metabolites

In order to test the volatility of derivatives of amines, three kinds of derivatives of methamphetamine and its metabolites derivatized by trifluoroacetic, heptafluorobutyric anhydrides, and pentafluorobenzoyl chloride were prepared at 60°C. After reaction (for 40 min), the samples were evaporated under a stream of nitrogen at 40°C until they were just dry (0 min). Then the same procedure were repeated but the nitrogen was passed for 15 min after the samples had dried up (0 min). Methamphetamine and amphetamine derivatives were analyzed using column A, and *p*-hydroxymethamphetamine and *p*-hydroxyamphetamine derivatives using column B.

O'Brien *et al.*[1] previously reported that, under a stream of nitrogen at room temperature, 50% of free methamphetamine and amphetamine were lost even if the sample was analyzed immediately after reaching dryness. It has also been reported[20] that 40% of free methamphetamine and amphetamine were lost from the extract mixed with acetic acid (20 μl) when nitrogen was passed for 5 min after reaching dryness. About 3–22% *p*-hydroxymethamphetamine and *p*-hydroxyamphetamine were lost when nitrogen was passed for 15 min after reaching dryness. These data suggest that much attention should be paid to the volatility of free methamphetamine and amphetamine in evaporation procedures.

As shown in Table II, the perfluoroacylated derivatives of methamphetamine and amphetamine are more volatile than the free amines, and their degree of volatilization is greater than that of *p*-hydroxymethamphetamine and *p*-hydroxyamphetamine. However, the pentafluorobenzoyl derivatives of methamphetamine and amphetamine are highly stable. Based on these results, pentafluorobenzoyl derivatives were used for the analysis of methamphetamine and amphetamine, and heptafluorobutyryl derivatives for *p*-hydroxymethamphetamine and *p*-hydroxyamphetamine.

Mass spectra of pentafluorobenzoyl and heptafluorobutyryl derivatives of methamphetamine and its metabolites

The EI and CI mass spectra of pentafluorobenzoyl derivatives of methamphetamine and amphetamine, and heptafluorobutyryl derivatives of *p*-hydroxymethamphetamine and *p*-hydroxyamphetamine, are shown in Figs. 1 and 2, respectively. The EI mass spectra of the pentafluorobenzoyl derivatives gave no molecular ions, but a base peak at *m/e* 195 due to the pentafluorobenzoyloxonium ion. The spectra gave other ions at *m/e* 167 due to loss of a neutral CO molecule from that ion (*m/e* 195), at *m/e* 252, 238 and 118 due to cleavage of the C–C bond at the β-position and of the C–N bond at the α-position (Fig. 1-1, -2). These EI mass spectral coincide with those reported by Delbeke and Debackere[6]. The EI mass spectra of the heptafluorobutyryl derivatives gave no molecular ions, but a base peak at *m/e* 254 and 240, other peaks at *m/e* 303 and 330 due to β- and α-cleavage and at *m/e* 169 due to the heptafluorobutyryl ion (Fig. 1-3, -4). The CI mass spectra of the pentafluorobenzoyl derivatives of methamphetamine and amphetamine, and the heptafluorobutyryl derivatives of *p*-hydroxymethamphetamine and *p*-hydroxyamphetamine, gave the quasi-molecular ions at *m/e* 344, 330, 558 and 544, respectively (Fig. 2).

These results indicated that the pentafluorobenzoyl derivatives of methamphetamine and amphetamine were N-monobenzylated, and heptafluorobutyryl derivatives of *p*-hydroxymethamphetamine and *p*-hydroxyamphetamine were N- and O-diacylated, respectively.

TABLE II

LOSS OF VARIOUS DERIVATIVES OF METHAMPHETAMINE AND ITS METABOLITES UNDER A STREAM OF NITROGEN AT 40°C

The percentage values were calculated by:

$$\frac{\text{peak height (0 min)} - \text{peak height (15 min)}}{\text{peak height (0 min)}} \times 100$$

The amines (1 μg) were incubated with trifluoroacetic anhydride (0.1 ml), heptafluorobutyric anhydride (0.1 ml) or pentafluorobenzoyl chloride (1 μmol) at 60°C for 40 min.

Compound	*Derivative*		
	Trifluoroacetyl	*Heptafluorobutyryl*	*Pentafluorobenzoyl*
Methamphetamine	69.8	79.1	0
Amphetamine	53.7	65.5	0
p-Hydroxymethamphetamine	18.7	26.4	–
p-Hydroxyamphetamine	12.8	8.6	–

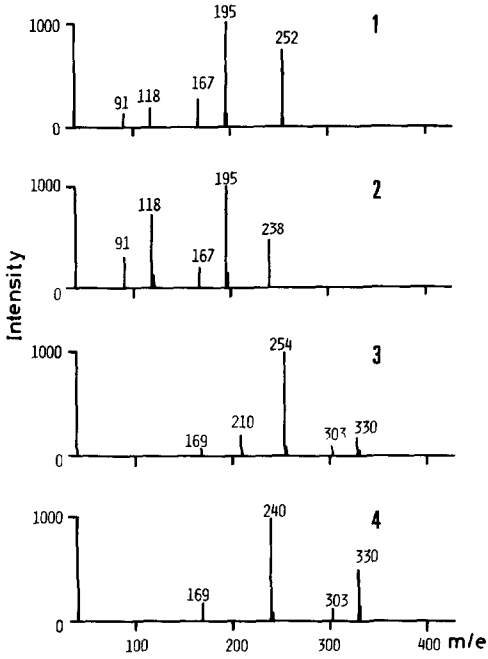

Fig. 1. EI mass spectra of pentafluorobenzoyl derivatives of methamphetamine (1) and amphetamine (2), and heptafluorobutyryl derivatives of *p*-hydroxymethamphetamine (3) and *p*-hydroxyamphetamine (4).

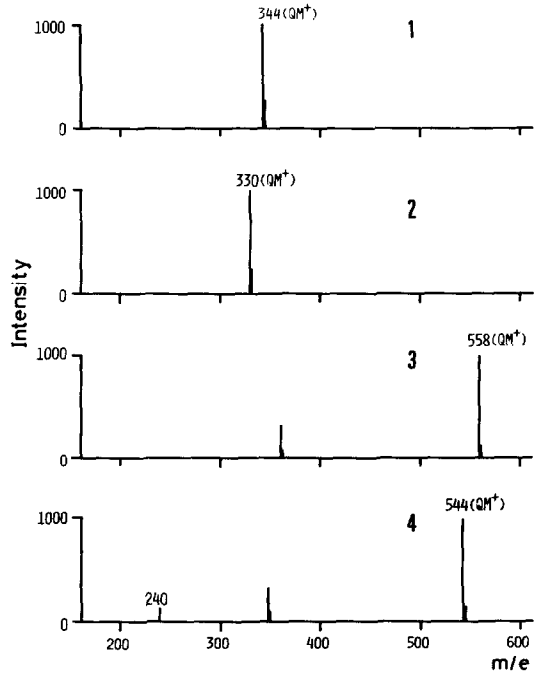

Fig. 2. CI mass spectra of pentafluorobenzoyl and heptafluorobutyryl derivatives. Details as in Fig. 1.

Effect of reaction time on the formation of the pentafluorobenzoyl and heptafluorobutyryl derivatives of methamphetamine and its metabolites

In previous papers it was reported that pentafluorobenzoyl derivatization of methamphetamine and amphetamine was completed within 5 min at 60°C[5], and heptafluorobutyryl derivatization of *p*-hydroxymethamphetamine and *p*-hydroxyamphetamine within 15–30 min at 60°C[20]. Based on these results, the following experimental conditions for derivatization were chosen: methamphetamine and amphetamine, reaction time 20 min, temperature 60°C; *p*-hydroxymethamphetamine and *p*-hydroxyamphetamine, reaction time 40 min, temperature 60°C.

Calibration curves

Calibration curves for methamphetamine and its metabolites were obtained by plotting the peak heights of the compounds against the concentrations injected. As shown in Fig. 3, the various graphs gave good linearity within the range 1–4 ng of methamphetamine and its metabolites.

Recovery from brain, liver and serum

Gas chromatograms of pentafluorobenzoyl derivatives of methamphetamine and amphetamine, and heptafluorobutyryl derivatives of *p*-hydroxyamphetamine and *p*-hydroxymethamphetamine from the extract of liver using procedures A and B are shown in Figs. 4 and 5, respectively. In the gas chromatograms of the blank liver obtained using procedures A and B there were no extraneous peaks (Figs. 4c and 5c). Gas chromatograms of extracts from control human liver using procedures A and B also showed no extraneous peaks (data not shown), neither did gas chromatograms of extracts from the blank rat liver.

The results of the recovery experiments of methamphetamine and its metabolites added to the brain, liver and serum are shown in Table III. The average recoveries of methamphetamine, amphetamine, *p*-hydroxymethamphetamine and *p*-hydroxyamphetamine were 93.5–107.0, 95.7–115.4, 94.7–102.6 and 97.5–102.5%, respectively. Satisfactory recoveries of methamphetamine and its metabolites added to

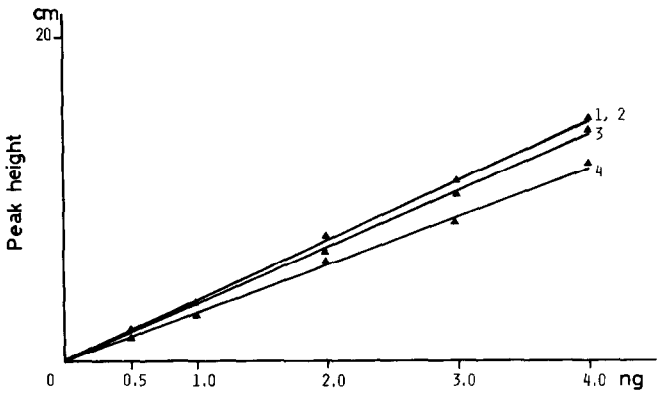

Fig. 3. Calibration curves of pentafluorobenzoyl derivatives of methamphetamine (1) and amphetamine (2), and heptafluorobutyryl derivatives of *p*-hydroxymethamphetamine (3) and *p*-hydroxyamphetamine (4).

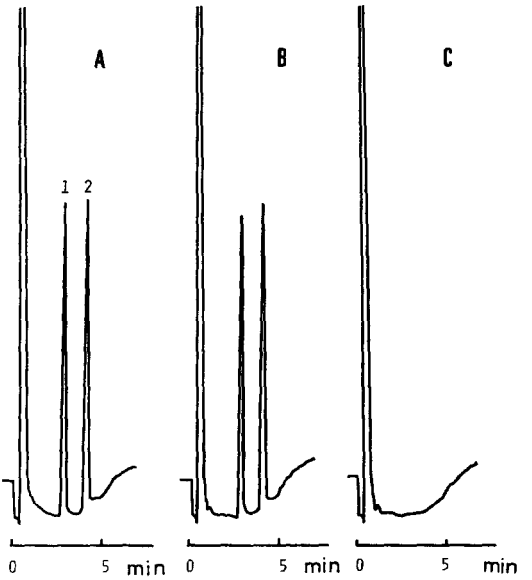

Fig. 4. Gas chromatograms of (A) 2 ng of pentafluorobenzoyl derivatives of methamphetamine (1) and amphetamine (2) extracted from water using procedure A, (B) an extract from the liver containing 1.0 μg of methamphetamine and amphetamine and (C) an extract from the blank liver. Retention times for peaks 1 and 2 on the column A were 2.6 and 3.9 min, respectively.

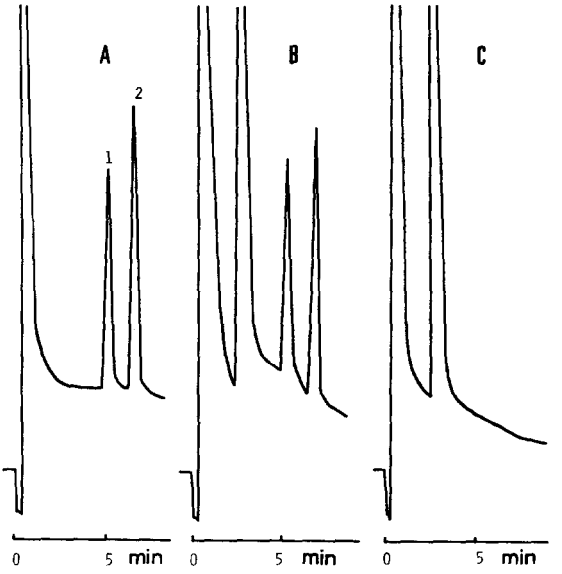

Fig. 5. Gas chromatograms of (A) 2 ng of heptafluorobutyryl derivatives of *p*-hydroxyamphetamine (1) and *p*-hydroxymethamphetamine (2) extracted from water using procedure B, (B) an extract from the liver containing 1.0 μg of *p*-hydroxymethamphetamine and *p*-hydroxyamphetamine and (C) an extract from the blank liver. Retention times for peaks 1 and 2 on column B were 5.1 and 6.7 min, respectively.

TABLE III

RECOVERIES OF METHAMPHETAMINE AND ITS METABOLITES FROM THE BRAIN, LIVER AND SERUM

Compound	*Recovery (mean ± S.E.) (%)*		
	Brain	*Liver*	*Serum*
Methamphetamine	94.2 ± 7.2	107.0 ± 6.7	93.5 ± 2.3
Amphetamine	105.7 ± 5.8	115.4 ± 5.1	95.7 ± 3.4
p-Hydroxymethamphetamine	94.7 ± 4.8	102.6 ± 1.4	–
p-Hydroxyamphetamine	97.5 ± 2.9	102.5 ± 1.4	–

biological materials proved this method to be applicable for the determination of these compounds in such materials, and this method could be applicable to human tissues.

In previous work, the detection limits of methamphetamine and its metabolites by the GC–ECD method were reported: pentafluorobenzoyl derivatives of methamphetamine and amphetamine, 10–25 pg[5,6]; trichloroacetamide derivative of methamphetamine, 25 pg[4] and trifluoroacetyl derivative of *p*-hydroxymethamphetamine, 50 pg[19]. On the other hand, the detection limits obtained for trifluoroacetyl and heptafluorobutyryl derivatives of methamphetamine and amphetamine and the trifluoroacetyl derivative of *p*-hydroxymethamphetamine by the GC–MS method were 20[8,18], 5[9] and 100 pg[18], respectively. The most comprehensive method for determination of methamphetamine and related amines is GC–FID, and is applied to the routine screening of the amines in urine. The detection limits for these derivatives of methamphetamine and amphetamine using this method were 20–30 ng.

In this study, the detection limits for pentafluorobenzoyl derivatives of methamphetamine and amphetamine were shown to be *ca.* 0.1 ng, and for the heptafluorobutyryl derivatives of *p*-hydroxymethamphetamine and *p*-hydroxyamphetamine, to be 0.15 ng, respectively. These results indicate that a concentration as low as 10–15 ng/g tissue of methamphetamine and its metabolites can easily be detected by GC–NPD.

Detection of the amines in rat tissues

Typical gas chromatograms of the amines from tissues of a rat receiving methamphetamine (10 mg/kg, i.p.) are shown in Fig. 6. Fig. 6A shows the chromatogram of pentafluorobenzoyl derivatives extracted from the liver of the rat using procedure A. Fig. 6B shows heptafluorobutyryl derivatives extracted from hydrolyzed liver of the rat using procedure C and Fig. 6C shows the gas chromatogram of the extract from hydrolyzed liver of the control rat. In Fig. 6C there are many extraneous peaks within 5 min, but no peaks in the area were *p*-hydroxylated metabolites of methamphetamine would appear as in Fig. 6B, even when determined by using an extract concentrated 10-fold as in the case of the control rat liver.

The identification of the GC peaks (a–d) as carried out by means of GC–CI–MS. The CI mass spectra of peaks a, b, c and d showed the quasi-molecular ion at *m/e* 344, 330, 544 and 558, respectively. These compounds were identified as meth-

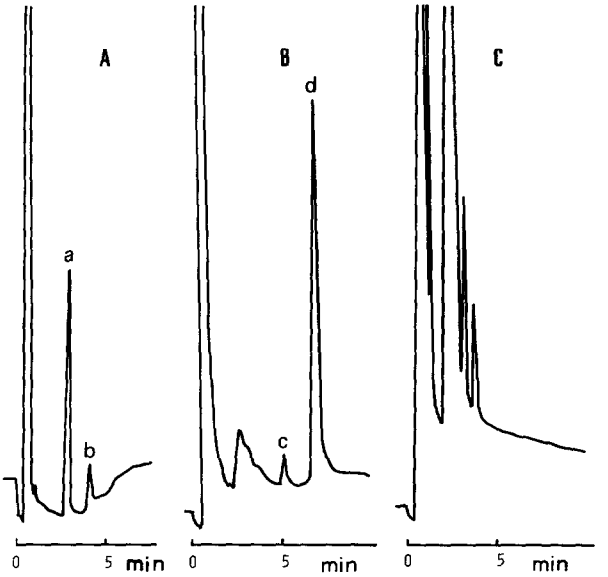

Fig. 6. Gas chromatograms of (A) pentafluorobenzoyl derivatives of an extract from the rat liver procedure A, (B) heptafluorobutyryl derivatives extracted from hydrolyzed aliquots of the rat liver using procedure C after dosing with methamphetamine (10 mg/kg, i.p.) and (C) an extract from hydrolyzed aliquots of the blank liver.

amphetamine, amphetamine, *p*-hydroxyamphetamine and *p*-hydroxymethamphetamine by comparing their mass spectra with those of the authentic compounds.

The concentrations of methamphetamine and its metabolites in rat tissues determined 2 h after intraperitoneal injection of methamphetamine (10 mg/kg) are shown in Table IV. Kuhn and Schanberg[22] reported that the amphetamine concentration in some tissues of the rat was highest in the kidney, and the *p*-hydroxyamphetamine concentration was highest in the liver. In the present study, the methamphetamine concentration was also highest in the kidney. In the liver, the concen-

TABLE IV

CONCENTRATION OF METHAMPHETAMINE AND ITS METABOLITES IN TISSUES (μg/g OR μg/ml)

Two hours after intraperitoneal injection of methamphetamine (10 mg/kg) to male Wistar rats. The values are averages obtained from three rats. n.d. = Not detectable.

Tissue	*Methamphetamine*	*Amphetamine*	*p-Hydroxy-methamphetamine*		*p-Hydroxy-amphetamine*	
			Conjugated	*Free*	*Conjugated*	*Free*
Liver	2.0	0.3	38.8	8.4	5.3	1.7
Kidney	7.2	1.9	0.7	0.4	0.33	0.07
Spleen	3.4	0.6	1.6	0.5	0.4	n.d.
Brain	2.2	0.6	–	0.24	–	0.03
Serum	0.4	0.05	–	n.d.	–	n.d.

tration of *p*-hydroxymethamphetamine was highest, and 20–40 times higher than that of *p*-hydroxymethamphetamine in the spleen and kidney.

In conclusion, the determination of methamphetamine and its metabolites in rat tissues by GC–NPD is so simple and highest sensitive that it is expected that the method will be applicable to the determination of these amines in biological materials.

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