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SIMULTANEOUS DETERMINATION OF ENDOGENOUS NOREPINEPHRINE AND DOPAMINE-β-HYDROXYLASE ACTIVITY IN BIOLOGICAL MATERIALS BY CHEMICAL IONIZATION MASS FRAGMENTOGRAPHY

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SUMMARY

A new clean-up procedure is described for the quantitation of biogenic amines and their metabolites by gas chromatography–chemical ionization mass fragmentography. The procedure is carried out in an acidic, non-aqueous medium. The metabolites as well as the amines are extracted with greater than 92% recovery. Using a CPG-10 column, the procedure is most suitable for the purification of these amines and their metabolites from the extract. It has been applied to the simultaneous determination of endogenous norepinephrine and dopamine-β-hydroxylase (DBH) activity in rat adrenal by use of [$^{2}H_{2}$]dopamine as a substrate and [$^{2}H_{6}$]-norepinephrine as an internal standard. The quantitation limit of the DBH activity is 0.1 pmole/mg of rat adrenal per hour.

INTRODUCTION

In a previous paper[1] we described a specific and sensitive method for the analysis of biogenic amines and their metabolites by gas chromatography–chemical ionization mass fragmentography. Much attention has been focused on the activity of enzymes such as monoamine oxidase, catechol-O-methyltransferase and phenylethanolamine-N-methyltransferase which are related to the metabolism of the biogenic amines. The quantitation of these enzyme activities may be useful for the chemical diagnosis of some diseases.

Recently, the relationship between dopamine-β-hydroxylase and hypertension in a spontaneously hypertensive rat has been investigated[2] and has been extended to man[3]. If the amounts of the endogenous amines and the activities of their related enzymes could be determined simultaneously in the same biological materials, it would help to clarify the physiological roles of the biogenic amines *in vivo*. It is necessary to develop a sensitive and specific method of analysis because the biogenic amines and their related enzymes in biological materials exist in very low concentration. Fortunately, mass spectrometry makes it possible to discriminate between these endogenous amines and the corresponding stable isotope labelled compound. The

isotopically labelled compounds are employed as a substrate for the enzyme assay.

In addition, the establishment of an effective extraction and clean-up procedure for mass fragmentographic analysis is indispensable. The success of the analysis depends upon the development of a suitable procedure. Adsorption chromatography on alumina[4] or Amberlite XAD-2[5] has been conventionally used for clean-up of biogenic amines in biological materials, but it has the disadvantage of not producing reproducible results because of the oxidative decomposition of the amines in neutral or alkaline solution.

This paper describes a new system for the extraction and clean-up procedure of biogenic amines by means of gas chromatography–chemical ionization mass fragmentography, and the simultaneous quantitation of endogenous norepinephrine and dopamine-β-hydroxylase activity in rat adrenal using [$^2H_2$]dopamine as a substrate and [$^2H_6$]norepinephrine as an internal standard.

EXPERIMENTAL

Materials

Dopamine (DA) hydrochloride, norepinephrine (NE) and pentafluoropropionic acid (PFPA) were purchased from Tokyo Kasei, Tokyo, Japan. Pentafluoropropionyl-1,2,4-triazole (PFPT) was synthesized by the method reported previously[6]. Deuterium oxide (^2H_2O), lithium aluminium deuteride ($LiAl^2H_4$) and deuterium chloride (35% ^2HCl in ^2H_2O) were purchased from Merck Sharp & Dohme, Toronto, Canada. A method for the synthesis of [α-$^2H_2$]DA and [$^2H_6$]NE will be reported elsewhere. 5-(4′-Chlorobutyl)picolinic acid was synthesized in our laboratories. [^{14}C]Homovanillic acid (HVA) (40–50 mCi/mmole), [2-^{14}C]vanillylmandelic acid (VMA) (40–50 mCi/mmole), [1-^{14}C]5-hydroxytryptamine (5-HT) (40–50 mCi/mmole) and [1,1′-$^3H_2$]NE (10–15 Ci/mmole) were purchased from the Commissariat à l'Énergie Atomique, Gif-sur-Yvette, France. [7-^{14}C]NE (10 mCi/mmole), indole [β-2-^{14}C]acetic acid (IAA) (40–60 mCi/mmole) and [5-^{14}C]-5-hydroxy-3-indoleacetic acid (5-HIAA) (10 mCi/mmole) were obtained from New England Nuclear, Boston, Mass., U.S.A. Commercially available isobutane (99.99%) and ammonia (99.9%) were used as reagent gases for chemical ionization mass spectrometry. Controlled-pore glass (CPG-10) (120–200 mesh; mean pore diameter, 74.47 Å) was obtained from Electro-Nucleonics, Fairfield, N.J., U.S.A. An artificial cerebrospinal fluid (artificial CSF) was prepared by the method of Bertilsson *et al.*[7]: it contains $NaH_2PO_4 \cdot 2H_2O$ (78 mg), $Na_2HPO_4 \cdot 2H_2O$ (45 mg), $MgCl_2 \cdot H_2O$ (81 mg), $CaCl_2$ (72 mg), KCl (222 mg), NaCl (7.19 g), $NaHCO_3$ (2.10 g) and human serum albumin (250 mg) per 1 l of water.

Sampling of biological materials

Ten millilitres of blood were placed in a centrifuge tube containing two units of heparin per ml, and the heparinized blood was centrifuged at 10,000 *g* for 20 min, keeping the temperature at 4°. The plasma was transferred to a test-tube and stored at —20° before use. Male Wistar rats (200–250 g) were killed by bleeding, and the brains and adrenals were stored immediately at —20° prior to analysis.

Assay for dopamine-β-hydroxylase (DBH)

The principle of this method was based on the gas chromatography–mass spectrometric determination of [$^2H_2$]NE which was formed by DBH using [$^2H_2$]DA as a substrate. The incubation mixture was prepared according to the method of Nagatsu and Udenfriend[3]. Fifteen microlitres of human serum or an aliquot of homogenate of rat adrenal (2 ml of 1 *M* acetate buffer at pH 5.0 per pair of adrenals) was pipetted into a test-tube. To the tube was added 400 μl of water, 200 μl of 1 *M* acetate buffer (pH 5.0) and 200 μl of 0.2 *M* N-ethylmaleimide, and the mixture was shaken vigorously on a vortexmixer, and then 50 μl of 0.2 *M* sodium fumarate, 50 μl of 0.2 *M* ascorbic acid (freshly prepared), 10 μl of 20 m*M* iproniazide, 50 μl of catalase (1500 units) and 1 mg of [$^2H_2$]dopamine hydrochloride were added as a substrate. The reaction mixture was incubated at 37° for 45 min, 1 μg of [$^2H_6$]NE was added as an internal standard to the mixture and the solution was lyophilized rapidly. The assay for DBH was carried out by the quantitation of the resulting [$^2H_2$]NE in the acetonitrile solution obtained through the extraction and clean-up procedures using gas chromatography–chemical ionization mass fragmentography. Blanks consisting of serum or tissue homogenate heated to 95° for 5 min were run in all experiments, as was an internal standard consisting of 1 μg of [$^2H_6$]NE added to the entire reaction mixture.

Extraction and clean-up procedure

The reaction mixture was rapidly frozen in solid carbon dioxide and acetone, and lyophilized (Kyowa TC-20BM, Tokyo, Japan). Four millilitres of acetonitrile and 10 μl of PFPA were added to the lyophilized sample and the mixture was ultrasonicated for 1–2 min to extract biogenic amines quantitatively. After centrifugation at 3000 *g* for 5 min, the supernatant was concentrated to *ca.* 10 μl *in vacuo* at below 30°. The residue was dissolved in 50 μl of PFPT and allowed to stand for 30 min at room temperature. The reaction mixture was loaded carefully on a column of CPG-10 (0.5 × 0.9 cm) which had previously been equilibrated with *n*-hexane. The column was washed with 4 ml of *n*-hexane and then eluted with 4 ml of benzene (containing 2% PFPT). The benzene eluate was used for the analysis. This eluate was evaporated to dryness at 30°. Ten microlitres of acetonitrile solution (containing 2% PFPT) were added to the residue, and then 1 or 2 μl of this solution was injected into a gas chromatograph–mass spectrometer equipped with a chemical ionization source.

Gas chromatography–chemical ionization mass spectrometry

A Shimadzu-LKB 9000 gas chromatograph–mass spectrometer equipped with a chemical ionization source (Shimadzu Seisakusho, Kyoto, Japan) was used. The operating conditions were as follows: Shimadzu MID-PM 9060s multiple-ion detector (MID); gas chromatograph column, 1.5% OV-101 on Chromosorb G HP (80–100 mesh), 2 m × 2.5 mm (Applied Science Labs., State College, Pa., U.S.A.); column temperature, 170°; flash heater temperature, 280°; ion source temperature, 200°; carrier gas (helium) flow-rate, 30 ml/min; ionization potential, 500 eV; accelerating voltage, 3500 V; pressure in analyzer unit, 10^{-6} Torr; pressure in ionization chamber, 0.5–1 Torr; reagent gas, isobutane or ammonia.

RESULTS AND DISCUSSION

Derivatization

In general, it is important to select derivatives suitable for gas chromatography–mass spectrometry, especially in the case of mass fragmentography, because the adsorption and instability of the sample may vary with its physicochemical properties. A number of investigations on the derivatization of biogenic amines and their metabolites have been reported as having solved those problems[9]. Donike[10] has recommended the O-trimethylsilyl-N-trifluoroacetyl derivatives of catecholamines which are more stable than the corresponding perfluoroacyl derivatives. Such derivatives have the disadvantage that the isotope peaks from the silicon atoms affect the ions of the deuterated internal standard. In this study, the pentafluoropropionyl (PFP) derivatives were adopted because fluorine atoms have no isotope peaks and the PFP derivatives are more stable than the corresponding trifluoroacetyl derivatives[11]. Compared with heptafluorobutyryl derivatives, these derivatives minimized the disadvantage that decreasing resolution of the ions and increased fluctuations of the mass marker in a sector-type mass spectrometer are observed at high mass caused by the increase of the molecular weight.

The instability that causes the derivatives to be hydrolyzed easily in a solution of low concentration (10 ng/ml) could be prevented by dissolving them in a solution containing 2% of PFPT. In order to obtain reproducible results this sample solution must be analyzed immediately.

Extraction and clean-up procedure for biogenic amines and their metabolites in biological materials

The procedure is illustrated in the flow sheet in Fig. 1. A mixture of acetonitrile and pentafluoropropionic acid was employed as a solvent system for simultaneous extraction of these amines and their metabolites from biological materials. In this solvent system the amines are converted into highly lipophilic PFPA salts which are soluble in acetonitrile, and these salts as well as their acidic metabolites are extracted easily with this mixture. The PFPA not only serves as a deproteinizing agent, but also prevents the oxidative decomposition of the amines.

Table I shows the recoveries of biogenic amines and their acidic metabolites through this extraction procedure when a 10-ng sample was added to 10 ml of artificial CSF. The results indicate that the metabolites as well as the amines are extracted efficiently with a recovery of greater than 92%.

Through several trials to develop an effective clean-up procedure, it was found that a micro-column of CPG-10 glass beads was the most suitable for purifying these amines and their metabolites, as shown in Fig. 2. Other types of column packings such as silica gel were not applicable to quantitative analysis. Table II shows the recovery of [^{3}H]NE in each step of the clean-up procedure. The results indicate that the [^{3}H]NE is recovered without any loss during this procedure.

The purity of the benzene eluate from the CPG-10 column was checked by mass fragmentography. The ion-cluster technique[12] was employed for the evaluation of the clean-up procedure. An equimolar mixture of NE and its [$^2H_6$]NE was added to the artificial CSF, and extracted and purified through the procedure in Fig. 1. The ions at *m/e* 590 and 596, the base peaks of PFP derivatives of NE and [$^2H_6$]NE,

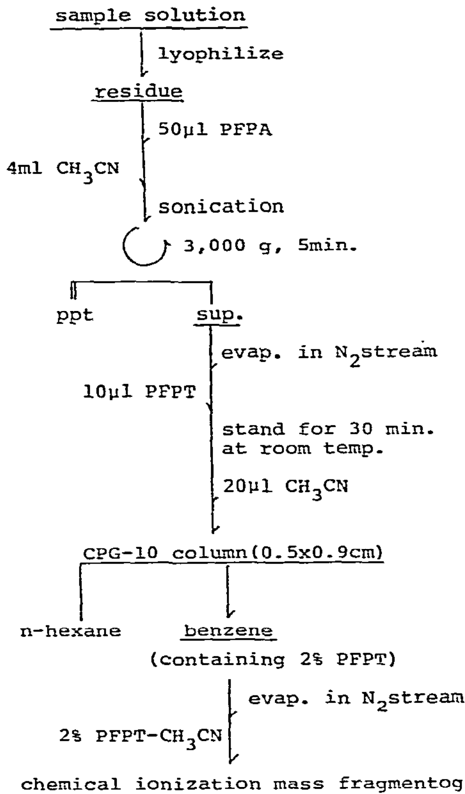

Fig. 1. Extraction, clean-up and derivatization procedure for biogenic amines and their metabolites in biological materials.

TABLE I

EXTRACTION EFFICIENCY OF BIOGENIC AMINES AND THEIR METABOLITES FROM ARTIFICIAL CEREBROSPINAL FLUID

Substance	*Added amount of radio-activity (dpm)*	*Recovered amount of radio-activity (dpm)*	*Recovery (%)*
[^{14}C]NE	154,400	128,310	83.3
[^{14}C]5-HT	160,200	149,310	93.3
[^{14}C]HVA	99,778	99,454	99.6
[^{14}C]VMA	176,658	176,232	99.7
[^{14}C]IAA	175,108	161,700	92.3
[^{14}C]5-HIAA	217,796	211,764	97.2

were monitored. Fig. 3 shows that this technique is useful for purifying the amines in biological materials without interference from endogenous substances. The two ions at *m/e* 590 and 596 are of equal intensity. Furthermore, the retention time of [$^{2}H_{6}$]NE was shorter than that of NE and can be explained in terms of an isotope effect caused by the affinity of the deuterated materials for the column.

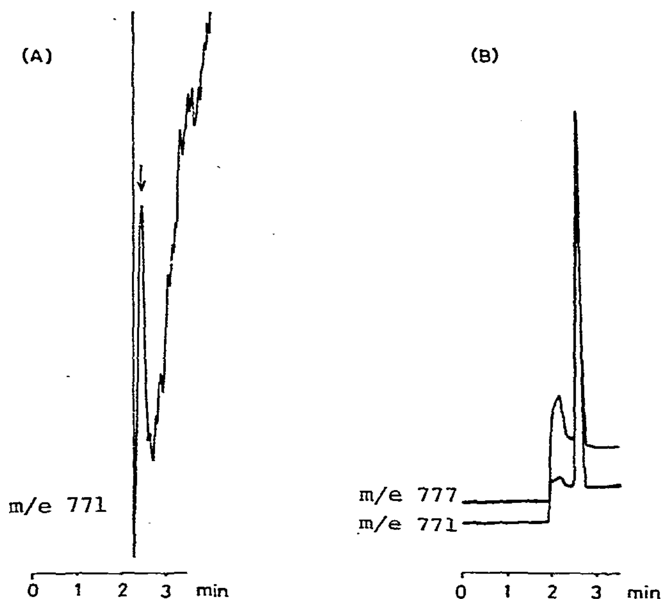

Fig. 2. Mass fragmentograms of NE obtained from the artificial CSF (plus 10 ng of NE and [$^2H_6$]NE) through the extraction and clean-up procedure. Reagent gas NH_3. A, Purification over silica gel (NE); B, purification over CPG-10 (NE and [$^2H_6$]NE).

TABLE II

RECOVERY OF THE ^{3}H RADIOACTIVITY IN EACH STEP OF THE CLEAN-UP PROCEDURE

Fraction			*Recovery (%)*
[^{3}H]NE			100
CH_3CN extraction			100
CPG-10	*n*-hexane fraction	10.2	72.7
	benzene PFPT	62.5	

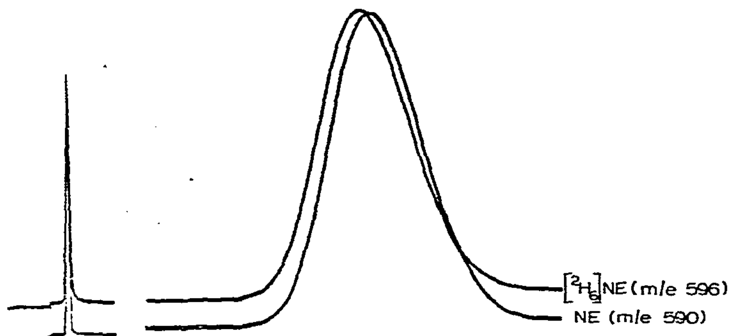

Fig. 3. Mass fragmentograms of NE and [$^2H_6$]NE (addition of 50 ng) obtained from the artificial CSF through the extraction and clean-up procedure. Left-hand side, normal chart speed (1 cm/min); right-hand side, high speed (17 cm/min).

Fig. 4 shows the calibration curve for NE obtained by the clean-up procedure. The peak height ratio exhibits a linear relationship with the concentration of NE in the range 2–10 ng/ml. The quantitation limit of this method is 2 ng/ml.

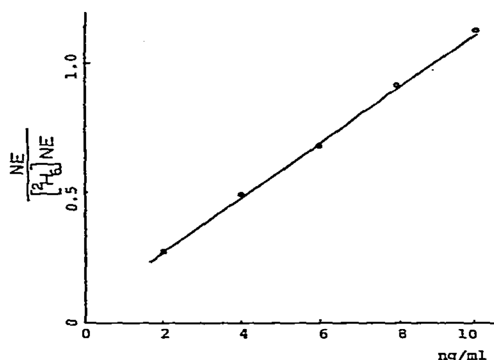

Fig. 4. Calibration curve for NE from artificial CSF. [$^2H_6$]NE (7 ng) was used as an internal standard.

Simultaneous quantitation of norepinephrine and dopamine-β-hydroxylase activity by gas chromatography–chemical ionization mass fragmentography

An attempt was made to determine the DBH activity in human serum by mass fragmentography. The principle of this method is based on the determination of the [$^2H_2$]NE formed by incubation of [$^2H_2$]DA as a substrate. Table III shows the *m/e* values for the determination of DBH activity by chemical ionization mass fragmentography, when isobutane or ammonia is employed as a reagent gas. In the

TABLE III

SUITABLE *m/e* VALUES FOR THE SIMULTANEOUS DETERMINATION OF THE ASSAY FOR DBH ACTIVITY AND ENDOGENOUS NOREPINEPHRINE BY MASS FRAGMENTOGRAPHY

Substrate	*Product*	*m/e values for MF*	
		*Isobutane**	*Ammonia**
(HO)$_2C_6H_3$–CH_2–$C(^2H)_2$–NH_2	(HO)$_2C_6H_3$–CH(OH)–$C(^2H)_2$–NH_2	592	773
	(HO)$_2C_6H_3$–CH(OH)–CH_2NH_2 (endogenous)	590	771
	(HO)$_2C_6^2H_3$–C(OH)(^2H)–$C(^2H)_2$–NH_2 (internal standard)	596	777

* Reagent gas.

case of isobutane chemical ionization mass fragmentography, the peaks at *m/e* 592 and 596 were selected for monitoring, corresponding to [MH — PFPOH]$^+$ of [$^2H_2$]-NE and [$^2H_6$]NE as an internal standard. The ions at *m/e* 773 and 777 corresponding to [M + NH_4]$^+$ of [$^2H_2$]NE and [$^2H_6$]NE were used when ammonia was employed as a reagent gas.

It is known that the rate of reaction of a deuterated substrate in an enzymatic reaction is less than that of undeuterated species because of a biological isotope effect. Hence, in this experiment, the ion-cluster technique was used to investigate if this isotope effect occurred in the assay for the DBH activity. An equimolar mixture of unlabelled and deuterated DA was employed and, prior to mass fragmentographic analysis, the peak heights of the ions at *m/e* 590 and 592 were equated using a gain control and backing circuit in an MID instrument. If there is no difference in the rates of NE formation from the protium- or deuterium-labelled substrates, the peak heights of NE and [$^2H_2$]NE should be identical. As shown in Fig. 5, a slight biological isotope effect was observed for the deuterium species[13].

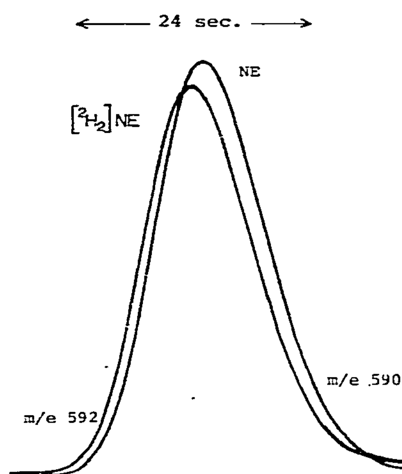

Fig. 5. Biological isotope effect on dopamine-β-hydroxylase activity in human serum. Substrate, 1:1 mixture of DA and [$^2H_2$]DA.

Fig. 6 shows typical mass fragmentograms obtained when measuring the DBH activities in 2 and 4 μl of human serum. The peak height of [$^2H_2$]NE produced by 4 μl of human serum was exactly twice that in the case of 2 μl. The DBH activity in this serum was calculated to be 25.2 nmole per 10 μl of serum per hour, which is in good agreement with that obtained by the spectrophotometric method[8]. This indicates that the biological isotope effect of the deuterated species on the enzymatic reaction may be negligible in the determination of DBH activity. Fig. 6C shows the inhibition of the DBH activity by the addition of 10^{-5} *M* 5-(4′-chlorobutyl)-picolinic acid, which is a specific inhibitor of DBH[14], to the reaction mixture.

An attempt was made to simultaneously determine the endogenous NE and DBH activity in biological materials by gas chromatography–chemical ionization mass fragmentography. Table III summarizes the *m/e* values of the ions used for this analysis. Fig. 7 shows a typical mass fragmentogram obtained by the simul-

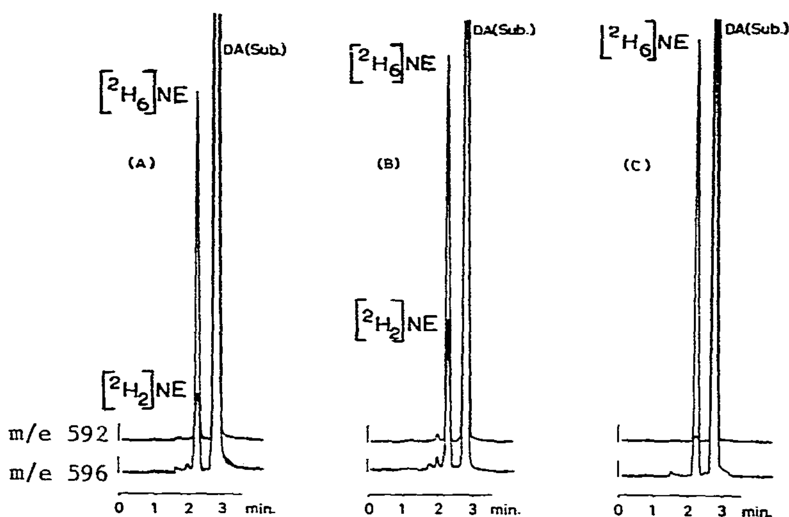

Fig. 6. Assay for the DBH activity in human serum by chemical ionization (isobutane) mass fragmentography. Sample: A, 2 μl of human serum; B, 4 μl of human serum; C, 4 μl of human serum + 10^{-5} *M* 5-(4′-chlorobutyl)picolinic acid.

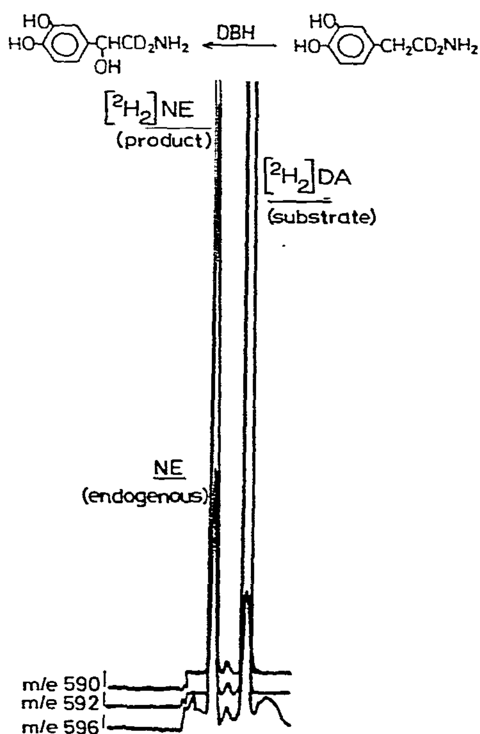

Fig. 7. Simultaneous determination of endogenous norepinephrine and DBH activity in rat adrenal by gas chromatography–chemical ionization mass fragmentography. Reagent gas, isobutane. Peaks: *m/e* 590, endogenous norepinephrine; *m/e* 592, enzymatic reaction product; *m/e* 596, internal standard.

taneous analysis of endogenous NE and DBH activity in a piece of rat adrenal. The amounts of endogenous NE and the DBH activity in this tissue were found to be 152 ng/mg of rat adrenal and 2.7 nmole/mg of rat adrenal per hour, respectively. The detection limit of this DBH activity by this method was estimated to be 0.1 pmole/mg of rat adrenal per hour.

In conclusion, we have developed a new procedure for the extraction and clean-up procedure of biogenic amines and their acidic metabolites from biological materials. This procedure has been applied to the simultaneous determination of the endogenous norepinephrine and DBH activity in rat adrenal by use of [$^{2}H_2$]dopamine as a substrate. It may be possible to extend this technique to the simultaneous analysis of biochemically important substances and to the activities of their related enzymes in biological materials.

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