Journal of Chromatography, 145 (1978) 1—9
Biomedical Applications

CHROMBIO. 085

MASS FRAGMENTOGRAPHIC DETERMINATION OF VANILMANDELIC ACID, HOMOVANILLIC ACID AND ISOHOMOVANILLIC ACID IN HUMAN BODY FLUIDS

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(Received April 12th, 1977)

SUMMARY

Vanilmandelic, homovanillic and isohomovanillic acids in body fluids were efficiently isolated by liquid chromatography on an Amberlite XAD-4 column and by organic extraction in a special apparatus. The purified metabolites were converted into their trifluoroacetylhexafluoroisopropanol esters and analyzed by mass fragmentography. The working curves of the metabolites were linear from 0.5 to 5 ng injected. The minimum detectable concentrations of all the metabolites were 2 ng/ml for plasma and cerebrospinal fluid, and 120 ng/ml for urine. The metabolite concentrations in plasma, cerebrospinal fluid and urine of normal persons and patients were determined.

INTRODUCTION

Vanilmandelic acid (VMA), homovanillic acid (HVA) and isohomovanillic acid (iso-HVA) are the main metabolic end-products of catecholamines. Determination of these metabolites in human body fluids is essential to the diagnosis of, for example, neuroblastoma, phaeochromocytoma and Parkinson's disease.

The quantitative analyses of the metabolites in body fluids are usually carried out by fluorimetry [1], thin-layer chromatography [2, 3], gas chromatography (GC) [4—9] and high-speed liquid chromatography (HSLC) [10—12]. However, they are susceptible to interference of many other constituents in human body fluids, and a highly selective and sensitive analysis is desired. Recently, Sjöquist et al. reported a determination of VMA [13] or HVA [14] in human body fluids by mass fragmentography (MF). Karoum et al. [15] also determined VMA and HVA in spinal fluids and brain tissues by MF.

In previous work, we reported briefly a determination method of the metab-

olites by GC [16]. In this paper, we describe the development of a simultaneous determination method for VMA, HVA and iso-HVA in body fluids by MF.

EXPERIMENTAL

Materials

VMA, HVA, trifluoroacetic anhydride (TFAA) and hexafluoroisopropanol (HFIP) were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Iso-HVA was kindly supplied by Dr. I. Kuruma of Nippon Roche (Tokyo, Japan). 3-Methoxy-4-hydroxyphenylethanol (HMPE), obtained from Aldrich (Milwaukee, Wis., U.S.A.), was dissolved in dioxane and stored at —20° until used as an internal standard. *n*-Hexane for absorption spectrometry was obtained from Wako Junyaku Kogyo (Tokyo, Japan). Bovine albumin fraction V and rabbit γ-globulins fraction II were obtained from Sigma (St. Louis, Mo., U.S.A.) and Miles Labs., (Kankakee, Ill., U.S.A.), respectively. Ethyl acetate and 6 *M* hydrochloric acid were used after distillation. All the other chemicals used were of reagent grade purity.

Samples

Venous blood was added with heparin (Novo, Copenhagen, Denmark) and centrifuged to obtain plasma. Three ml of plasma or cerebrospinal fluid (CSF), which was acidified with 180 μl of 6 *M* hydrochloric acid and stored at —20°, was added with 1.6 μg of HMPE.

Urine was collected in a polyethylene bottle containing about 30 ml of 6 *M* hydrochloric acid during 24 h and stored at —20°. Seventy-eight μg of HMPE, 2 ml of water and 1.5 g of sodium chloride were added to 3 ml of the urine to make a sample solution.

Preparation of Amberlite XAD-4 column

Amberlite XAD-4 (Rohm and Haas, Philadelphia, Pa., U.S.A.) was ground by a rotary grinder in water and the particles of 60—150 mesh were collected and further fractionated by sieves in water. The particles were washed with water, 1 *M* sodium hydroxide, water, 1 *M* hydrochloric acid, water, methanol acetone, *n*-hexane, acetone, methanol and water, successively. The adsorbent was stored in water together with sodium azide until used. The particles were transferred with water into a glass tube of 0.45 cm (I.D.) to make columns of various heights.

Clean-up and derivatization of the metabolites in samples

Three ml of the sample solution from plasma or CSF were poured onto Amberlite XAD-4 columns of 6 or 10 cm height and different meshes. Each column was washed with 15 ml of 0.1 *M* formic acid and eluted with 5 ml of 50% methanol and 5 ml of 100% methanol. The two eluates were mixed and concentrated to dryness in vacuo. The residue was dissolved in 5 ml of water, and 50 μl of 6 *M* hydrochloric acid and 1.5 g of sodium chloride were added.

The solution thus obtained or the sample solution from urine was extracted

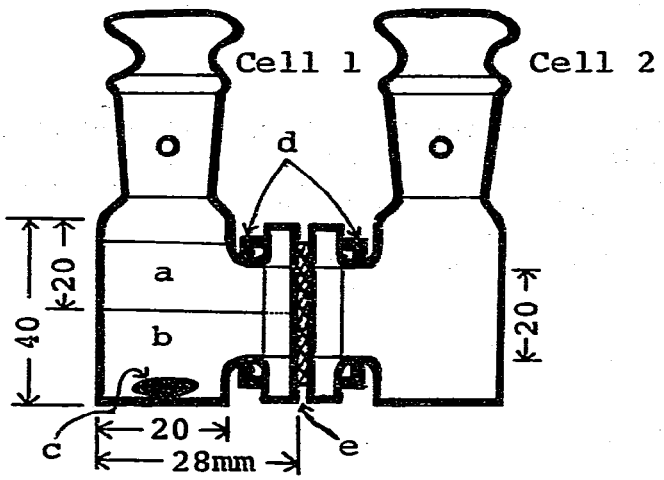

Fig.1. Solvent extraction apparatus. A PTFE membrane filter (extra coarse, 30—60 μm, Chemplast, Wayne, N.J., U.S.A.) was clamped between the twin glass cells. Cell 2 was stoppered, and 5 ml of the aqueous solution and 3 ml of ethyl acetate were poured into cell 1. Then cell 1 was stoppered and stirred for 5 min. By turning off the stoppers, the organic phase penetrated through the filter into cell 2. a = Ethyl acetate phase; b = aqueous phase; c = magnetic stirring rod; d = clamp; e = PTFE filter.

twice with 3 ml of ethyl acetate with an apparatus* shown in Fig. 1. All the volume for plasma and CSF or, for urine, 100 μl of the organic phase was collected into a 10-ml test tube (13 × 1.3 cm I.D.) with a glass stopper and evaporated in vacuo. Next, the residue was dried over phosphorus pentoxide in vacuo for 15 min.

To the dried residue 300 μl of TFAA and 150 μl of HFIP were added. The tube was stoppered and heated in an oil-bath at 75° for 1 h. The tube was kept in ice and the excess of the reagents were removed under a stream of dried nitrogen with a moisture-protecting attachment. Two hundred μl of *n*-hexane were added immediately to the residue, and 1 or 2 μl of the solution was injected into a gas chromatograph [16] or a gas chromatograph—mass spectrometer.

Mass fragmentography

A gas chromatograph—mass spectrometer Hitachi Model-52 fitted with a multiple-ion detection system was used at the Central Research Laboratory, Mitsubishi Chemical Industry Co. (Kanagawa-ken, Japan) under the following conditions. A 1 m × 3 mm (I.D.) glass tube was packed with 2% OV-1 on Chromosorb W (AW-DMCS). The column inlet pressure of the carrier gas (helium) was 1.0 kg/cm^2 (40 ml/min). The temperatures of column, column injector and ion source were 120°, 200° and 200°, respectively. The ionizing potential was 20eV.

RESULTS

As shown in Table I, a column of Amberlite XAD-4, 10 cm high and 80—

*The apparatus was a modification of that of T. Igarashi and S. Tamura [17].

TABLE I

THE EFFECTS OF THE COLUMN HEIGHT AND THE PARTICLE SIZE OF AMBERLITE XAD-4 ON THE RECOVERY PERCENTAGE OF THE METABOLITES BY GC

Metabolite	60—80 mesh		80—115 mesh		115—150 mesh
	6 cm	10 cm	6 cm	10 cm	10 cm
VMA in water	70	100	99	102	—
HVA in water	98	96	100	98	—
iso-HVA in water	97	103	105	100	—
VMA in plasma	44	80	70	103	96
HVA in plasma	107	106	96	105	103
iso-HVA in plasma	103	105	100	98	105

150 mesh, was found to be suitable for purification of protein-containing body fluids such as plasma. By the use of the column, even VMA, the weakest adsorbing metabolite, was quantitatively adsorbed. More than 95% of proteins were removed by washing the column with 0.1 *M* formic acid as shown in Fig. 2; otherwise the proteins would disturb the next extraction step with an organic solvent by emulsification. As shown in Fig. 2 and Table I, the elution of the metabolistes and the internal standard from the column was completed with 50 and 100% methanol. On the other hand, the ordinary deproteinization with perchloric acid or acetone gave less recovery of the metabolites. For urine, which contained the metabolites at higher concentrations and no protein, the chromatographic step was unnecessary (Fig. 2 and Table I).

The next step, extraction with ethyl acetate of the acidified and salted sample solution [6], was effective in removing from the samples traces of proteins, electrolytes, uric acid and urea, which would disturb the GC of the metabolites. As an example, 5 ml solution of 1.5 g sodium chloride in 0.06 *M*

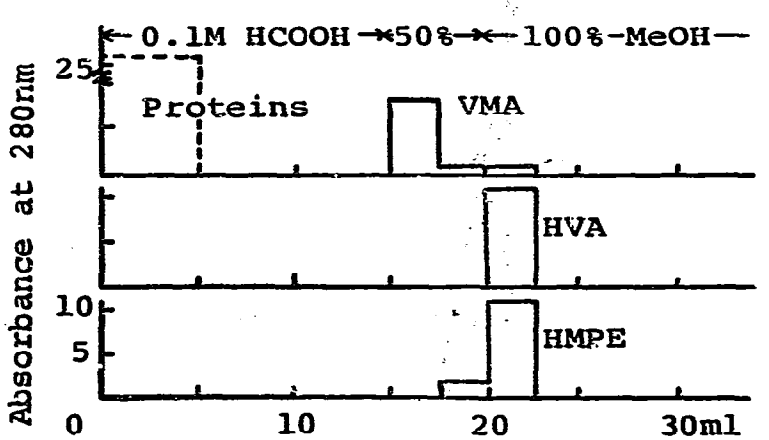

Fig.2. Elution profile of VMA, HVA, HMPE and proteins from Amberlite XAD-4. Three ml of 3.5 m*M* aqueous solutions of the metabolites or 3 ml solution of 180 mg of albumin and 30 mg of γ-globulin in 0.01 *M* phosphate—0.15 *M* sodium chloride (pH 7.4) were applied to the XAD-4 column (10 × 0.45 cm I.D.).

hydrochloric acid (45.8 mS/cm) was extracted with 3 ml of ethyl acetate twice, the organic phase was evaporated to dryness and the residue was dissolved in 5 ml of water. The conductivity was much reduced to 0.54 μS/cm. In the case of a urine of 6.3 mS/cm, treated similarly, the conductivity of the final solution became 50 μS/cm. The absorbance at 280 nm of the residue dissolved in 5 ml of water was 0.012 when 5 ml of a solution saturated with uric acid of 0.531 absorbance was extracted. Further, 0.5 *M* urea in water at the normal urinary concentration was also removed by the extraction, as judged from the disappearance of the GC peak of the derivative, which would overlap with the VMA peak.

On the other hand, *p*-hydroxyphenylacetic acid and hippuric acid in urine were not removed, and they interfered with the following derivatization. The interference was neglected when 100 μl of the organic phase were analyzed. The recoveries of 8 μg each of VMA, HVA and iso-HVA added to the sample solution of normal urine were within 95—105% by MF.

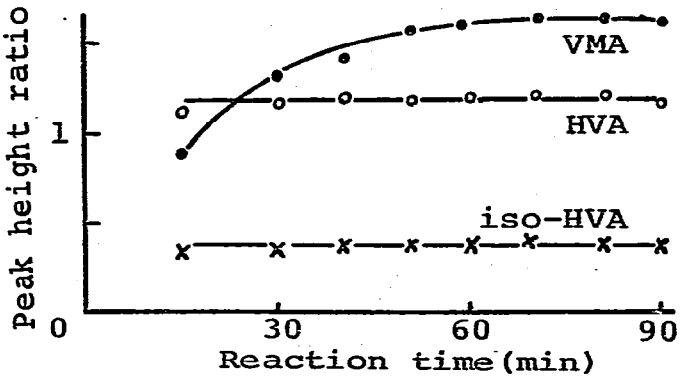

Fig.3. Rate of formation of the trifluoroacetyl-hexafluoroisopropanol ester derivatives of the metabolites. A mixture of 10 μg of VMA, 7.5 μg of HVA, 17 μg of iso-HVA and 78 μg of HMPE, dried over phosphorus pentoxide, was reacted with the reagents under the conditions described in the text then analyzed by GC [16].

An hour was enough for the derivatization, as shown in Fig. 3. The derivatized metabolites were identified by their mass spectra as shown in Fig. 4. The major ions of 345, 428, 428 and 360 *m/e*, respectively from the spectra of VMA, HVA, iso-HVA and HMPE derivatives were used for the MF.

The MF patterns of the metabolites from the authentic mixture and normal plasma are shown in Fig. 5. The working curves for the metabolites obtained by the mass fragmentographs were linear from 0.5 to 5 ng injected as shown in Fig. 6. The minimum detectable concentrations of all the metabolites were 2 ng/ml for plasma and CSF, and 120 ng/ml for urine. In ordinary body fluids, the content of endogenous HMPE was negligible compared with the amount of the internal standard, so that the correction was unnecessary. The contents of the metabolites in body fluids were measured and are summarized in Table II.

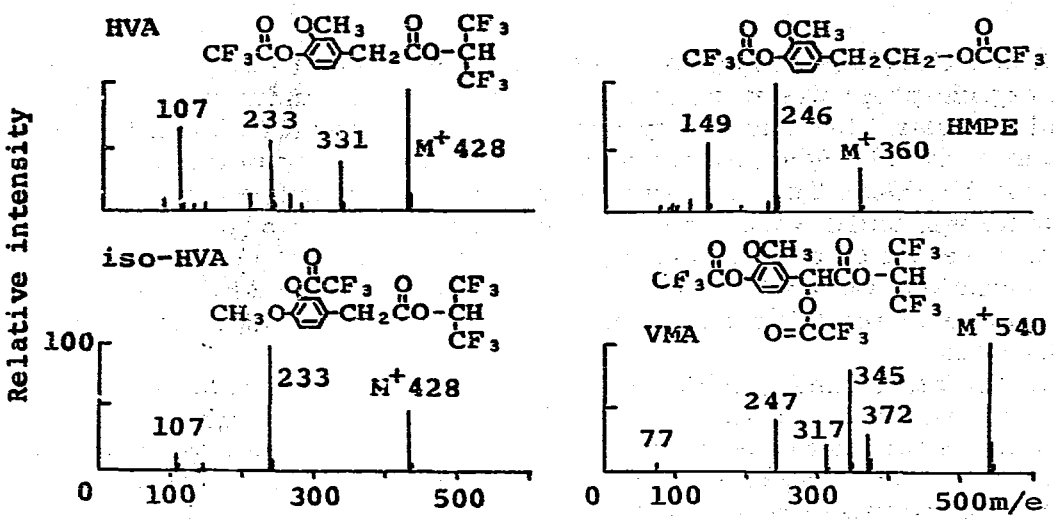

Fig.4. Mass spectra of the derivatives.

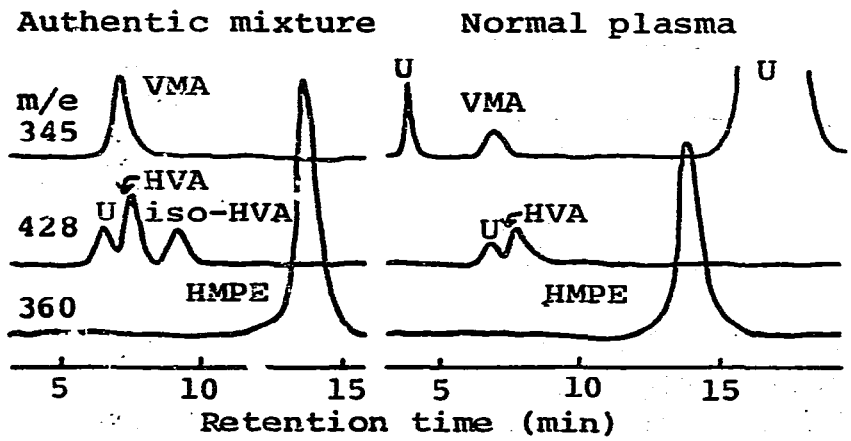

Fig.5. Mass fragmentographic patterns of the metabolites from the authentic mixture and normal plasma. U, unknown peaks.

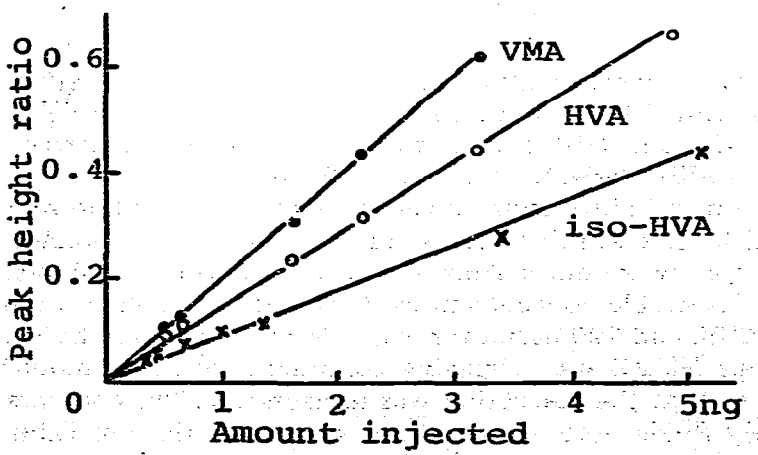

Fig.6. Working curves of the metabolites. Fifteen ng of HMPE were injected.

TABLE II

CONCENTRATION OF THE METABOLITES IN HUMAN BODY FLUIDS BY MF

Subject	Sex	Age (years)	Concentration						
			Plasma* (ng/ml)		CSF* (ng/ml)		Urine (mg/day)		
			VMA	HVA	VMA	HVA	VMA	HVA	iso-HVA
Normal	M	27	20	9	—	—	1.6	4.2	0.1
	M	35	15	11	—	—	1.3	3.9	0.1
	M	25	17	19	—	—	—	—	—
	M	30	10	8	—	—	—	—	—
	F	52	14	4	12	40	—	—	—
Parkinson's**	M	16	18	16	6	<2	1.5	4.5	0.1
Neuroblastoma	M	3	—	—	—	—	2.0	15	0.4

*Iso-HVA was not detected in plasma and CSF.

**Administration of 3,4-dihydroxyphenyl-L-alanine (L-DOPA) as a medicine was stopped fo several days before sampling. Lack of influence of L-DOPA was confirmed by the observatio that the excretion of VMA and HVA in urine analyzed by HSLC [10, 11] was in the norma range.

DISCUSSION

We have developed a reliable method for the simultaneous determination of VMA, HVA and iso-HVA in body fluids, using convenient and efficient clean-up techniques and highly selective and sensitive MF. MF determinations of these metabolites in body fluids have been reported, but a simultaneous determination of the metabolites from a normal person has not been reported. The comparable data are summarized in Table III. Sjöquist et al. [13, 14] purified the metabolites from plasma by the use of an Amberlite XAD-2 column, although, according to our experiments, the recovery of VMA from the column was not so good. On the contrary, quantitative adsorption and sharp elution of the metabolites on the Amberlite XAD-4 used in this method were carried out as shown in Fig. 2 and Table I. As shown in Fig. 1, with the solvent extraction apparatus using a PTFE membrane we have been able to separate completely the organic and water phases without standing or nervous watching. The derivatization of the metabolites by past methods consisted of two steps of methylation and acylation, whereas the present method offers one step of derivatization.

ACKNOWLEDGEMENTS

This work was supported by grants from the Ministry of Health and Welfare and the Ministry of Education, Science and Culture.

TABLE III

COMPARATIVE DATA FOR METABOLITE CONCENTRATIONS OBTAINED BY MF

Author	N	Concentration					
		Plasma (pmole/ml)		CSF (pmole/ml)		Urine (nmole/ml)	
		VMA	HVA	VMA	HVA	VMA	HVA
Present authors	*	51—100	22—100	60	220	6.6—8.1	21—29
Sjöquist [13]	10	31—71	—	14—25**	—	11.3±0.21	—
Sjöquist et al. [14]	1	—	48	—	420	—	20
Gordon et al. [18, 19]	6	25—130	55—110	—	—	—	—
Karoum et al. [15]	5	—	—	2.3±0.9***	280±44***	—	—
Jimerson et al. [20]	34	—	—	5.2±0.9***	150±18***	—	—
Narasimhachari et al. [21]	17	—	27—450§	—	0.02—0.38§	—	—

*Plasma (N=5), CSF (N=1) and urine (N=2).
**Neurological patients.
***Psychiatric patients.
§Free plus conjugates.

We are deeply grateful to Drs. T. Nakanishi, of the University of Tsukuba School of Medicine, and Y. Shimada, of the University of Tokyo School of Medicine, for supplying samples.

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