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SIMULTANEOUS ANALYSIS OF TWELVE BIOGENIC AMINE METABOLITES IN PLASMA, CEREBROSPINAL FLUID AND URINE BY CAPILLARY COLUMN GAS CHROMATOGRAPHY—HIGH-RESOLUTION MASS SPECTROMETRY WITH SELECTED-ION MONITORING

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SUMMARY

A procedure is described for the simultaneous determination of twelve acidic and alcoholic metabolites of trace and catecholic biogenic amines in plasma, cerebrospinal fluid and urine by capillary column gas chromatography—high-resolution mass spectrometry. Protein precipitation with sulphosalicylic acid, derivatization with two different reagent systems, final sample clean-up with a buffer wash and a program for automatically changing the reference mass of the mass spectrometer to suit each group of compounds as they are eluted from the column, are the main novel features of the procedure. A brief description of the synthesis of the deuterium-labelled internal standards is provided. The procedure is applied to biological samples and a comparison to reported values is given.

INTRODUCTION

The trace amines (phenylethylamine, *m*- and *p*-tyramine, *m*- and *p*-octopamine, tryptamine and phenylethanolamine), the catecholamines (dopamine, adrenaline and noradrenaline) and 5-hydroxytryptamine have been implicated in a number of psychiatric and neurological disorders, such as schizophrenia [1—4], depression [2, 4—6], agoraphobia [7], aggression [8—10], hyperkinesis [11, 12] and Parkinsonism [13], as well as in stress [14] and dietary migraine [15]. The levels of the major acid metabolites of these amines, phenylacetic (PAA), *m*- and *p*-hydroxyphenylacetic (*m*-HPA, *p*-HPA), mandelic (MA), *m*- and *p*-hydroxymandelic (*m*-HMA and *p*-HMA), indoleacetic (IAA), 5-hydroxy-indoleacetic (5-HIAA), 3,4-dihydroxyphenylacetic (DOPAC), homovanillic

(HVA) and vanilmandelic (VMA) acids and 3-methoxy-4-hydroxyphenyl-ethylene glycol (MHPG), may serve as a measure of the turnover of the amines and thus of their relative importance in the aetiology of these diseases. Furthermore, since the precursor amines may not function independently from one another, it would be useful to have measures of all their major metabolites in order to ascertain the possible inter-relationship between them in the various psychiatric disorders.

Hitherto only a few of these acids have been measured simultaneously in any one sample. For example, PAA, *m*-HPA and *p*-HPA were measured in the plasma of normals [16], schizophrenics [1], agoraphobics [7] and aggressive psychopaths [8, 9], and *m*-HPA and *p*-HPA in rat brain [17] by gas chromatography—mass spectrometry (GC—MS) as either their trifluoroethylpentafluoro-propionyl or methylheptafluorobutyryl derivatives.

The catecholamine and serotonin metabolites, DOPAC, HVA, VMA, MHPG and 5-HIAA have been quantitated in cerebrospinal fluid (CSF) and other fluids by a number of GC—MS procedures, usually only one or two at a time. Notable methods are those using the pentafluoropropionylpentafluoro-*n*-propyl ester derivative [18—24], and the methyl esters with pentafluoropropionates [25] or heptafluorobutyrates [26].

HVA, VMA, DOPAC, MHPG and 5-HIAA in urine have been determined by GC of their *tert.*-butyldimethylsilyl derivatives [27]. Seven acids were determined simultaneously in plasma [28] by GC—MS as their methylpentafluoropropionyl derivatives and nine in urine by capillary GC [29] as their trimethylsilyl derivatives. Because of the volatility of PAA, MA and IAA and their fluorinated derivatives, most of the above procedures are unsuitable. Furthermore, *p*-HMA, VMA and MHPG undergo side-reactions during derivatization with fluorinated alcohols [30], thus markedly reducing sensitivity. In this paper we report an analytical procedure considerably modified from the earlier reported procedures, and apply it to the determination of the twelve acidic metabolites in plasma, CSF and urine.

EXPERIMENTAL

Materials

Aqueous solutions were prepared from deionized, double-distilled water. Glassware was soaked and washed in Contrad 70 (Canlab), rinsed with distilled water, soaked in chromic acid and finally rinsed several times with distilled water. Organic solvents were glass-distilled HPLC grade (Caledon, Georgetown, Canada). Derivatizing reagents were purchased as follows: pentafluoropropionic anhydride (PFPA) from Chromatographic Specialities (Brockville, Canada), 2,2,2-trifluoroethanol (TFEOH) from Aldrich (Milwaukee, WI, U.S.A.) and acetyl chloride from BDH (Toronto, Canada). The authentic proteo acids, *m*-HPA, *p*-HPA, DOPAC, HVA, MHPG piperazine salt, phenylpropionic, *p*-hydroxyphenylpropionic and 5-methoxyindoleacetic acids, were purchased from Aldrich; *m*-HMA, *p*-HMA, 5-HIAA, MA, PAA from Sigma (St. Louis, MO, U.S.A.); and IAA from Fisher (New York, NY, U.S.A.). Deuterium-labelled reagents (toluene-d_8, phenol-d_6, iodomethane-d_3, lithium aluminum deuteride and ethanol-d_1) were purchased from Aldrich and deuterium-labelled

TABLE I

DEUTERIUM INCORPORATION OF THE SYNTHESIZED LABELLED METABOLITES

Acid	d_0	d_1	d_2	d_3	d_4	d_5
PAA*	0	0	0	0	3.5	96.5 §
MA**	0	0	0	0	5.3	94.7 §
IAA**	0.0	3.9	96.1 §	0.0	0	0
m-HPA*	0.0	0.0	0.8	6.5	30.5 §	62.2
p-HPA*	0.0	0.0	2.2	6.1	86.1 §	5.6
m-HMA**	0	0	38.1	61.9 §	0	0
p-HMA**	0	0	0	3.2	96.7 §	0
VMA**	0	0	0.8	99.2 §	0	0
HVA*	0.2	0.3	4.0	95.5 §	0	0
DOPAC**	0	0	1.7	9.6	34.9	53.8 §
5-HIAA**	0	0.9	99.1 §	0	0	0
MHPG***	0	0	1.8	98.2 §	0	0

*Determined by GC—MS of the molecular ion of the trifluoroethylpentafluoropropionyl derivative.
**Determined by GC—MS of the molecular ion of the methylpentafluoropropionyl derivative.
***Determined by GC—MS of the molecular ion of the tripentafluoropropionyl derivative.
§Isotopomer used for internal standard (the ion and mass used in the analysis are given in Table II).

standards (MHPG-d_3 piperazine salt and 5-HIAA-α,α-d_2) from Merck Sharp and Dohme (Montreal, Canada). Proteo VMA and the other deutero acids were synthesized as described briefly below. The deuterium incorporation of all the synthesized and purchased deuterium-labelled metabolites is presented in Table I.

Syntheses

DOPAC-d_5, HVA-d_3, m-HPA-d_4, p-HPA-d_4 and m-HMA-d_3. The labelled acids were prepared from the unlabelled acids by exchange with 9% ^{2}HCl in ^2H_2O. The acid (200 mg) was dissolved (or suspended) in 3 ml of 9% ^{2}HCl in ^2H_2O and heated at 80°C for 24 h (HVA), 90°C for 24 h (DOPAC) or 110°C for 72 h (m-HPA, p-HPA, m-HMA) in a sealed acylation tube. The solvent was then lyophilized. Three such exchanges were carried out. Deuterium incorporation was determined by GC—MS of the trifluoroethylpentafluoro-propionyl derivative (for HVA, DOPAC, m-HPA, p-HPA) or methylpenta-fluoropropionyl derivative (for m-HMA) (Table I). The HVA-d_3 was found to contain 8% DOPAC-d_5 arising from demethylation of the HVA.

Indole-3-acetic acid-α,α-d_2. An adaptation of the method of Beck and Sedvall [31] for the synthesis of 5-hydroxyindole-3-acetic acid-α,α-d_2 was used. A solution of gramine and sodium cyanide in [^{2}H]ethanol and deuterium oxide was refluxed for four days, cooled, poured into ice-cold water and acidified with concentrated hydrochloric acid to give a slightly off-white precipitate. The product, which contained about 2% of unlabelled indole-3-acetic acid, was dissolved in 10% NaO^{2}H in ^2H_2O and heated at reflux for 24 h, cooled and acidified with hydrochloric acid to give the desired product.

Mandelic acid-d_5. Benzoic acid-d_5, prepared by permanganate oxidation of

toluene-d_8, was converted to benzoyl chloride-d_5 which was then refluxed with a suspension of cuprous cyanide in acetonitrile according to the procedure of Normant and Piechucki [32] yielding benzoyl cyanide-d_5. The cyanide was hydrolyzed in concentrated hydrochloric acid, the clear solution was diluted with water, extracted with diethyl ether, the extracts were dried and evaporated to leave benzoylformic acid as a yellow oil which was not purified further, but reduced with sodium borohydride using the procedure of Reid and Siegel [33] to give mandelic acid-d_5 (m.p. 116—117°C, literature 118—119°C).

Phenylacetic acid-d_5. Benzyl chloride-d_5 (prepared from benzoic acid-d_5 via the alcohol) was converted to the Grignard reagent which was poured onto crushed dry ice. After all the dry ice had evaporated, the product was isolated by the addition of concentrated hydrochloric acid and extraction with diethyl ether, m.p. 75—76°C.

Vanilmandelic acid and vanilmandelic acid-d_3. VMA and VMA-d_3 were prepared according to the procedure of Markey et al. [34].

p-Hydroxymandelic acid-d_4. Phenol-d_6 was treated with glyoxylic acid in 10% sodium hydroxide in a manner similar to that described for VMA. The brownish oil obtained from the ethyl acetate extract of the acidified reaction solution was crystallized from diethyl ether—light petroleum (b.p. 60—75°C) to give a white solid, m.p. 110—112°C. The product contained 11% of *o*-hydroxymandelic acid-d_4, as determined from the retention times and mass spectra of the two peaks observed in the gas chromatogram of the methylpenta-fluoropropionyl derivative.

Calibration curves were prepared for all the metabolites using a fixed amount of deuterium-labelled internal standard and varying amounts of the corresponding proteo acid.

Instrumentation

GC—MS analysis was performed with a VG 70-70 F double-focussing mass spectrometer which was equipped with an HP 5700 gas chromatograph. The column, J & W Scientific bonded phase silica, DB-1, 60 m × 0.32 mm I.D., was inserted directly into the ion source and operated with a helium flow-rate of about 30 cm/s. To detect the compounds, the oven was programmed iso-thermally at 140°C for 8 min, then 10°C/min to 240°C and held for 8 min. For selected-ion monitoring (SIM) analysis, the mass spectrometer was operated at 5000 resolution. The accelerating voltage was controlled by a VG DIGMID multiple-ion detection (MID) controller and the magnet (under field control) by a separate precision four-channel voltage source. This magnetic MID con-troller was connected to the DIGMID so that the choice of DIGMID program (see Table II) selected the magnetic field. With this system one could select a reference mass in the spectrum of heptacosafluorotri-*n*-butylamine or per-fluorokerosene using the magnetic multiple-ion detector in conjunction with the magnetic feedback circuitry, and the ions of the compounds to be detected using the accelerating voltage ratio controlled by the DIGMID. This maximised sensitivity over conventional high-resolution SIM at constant magnetic field as the use of different reference mass ions allowed the acceleration voltage to be kept close to the maximum (4 kV) and yet still permitted high-resolution

TABLE II

GC—MS PARAMETERS FOR THE ANALYSIS OF ACID AND ALCOHOL METABOLITES

Program	Reference mass	Compound	Mass		Ion measured (elemental composition)	Retention time (min:sec)
I	213.9903	PAA-TFE	H	218.0555	M^+	6:00
			d_5	223.0868	$(C_{10}H_9F_3O_2)$	
II	213.9903	MA-Me-PFP	H	253.0288	$M^+ - COOCH_3$	8:26
			d_5	258.0602	$(C_{10}H_6F_5O_2)$	
III	375.9807	m-HPA-TFE-PFP	H	380.0297	M^+	9:15
			d_4	384.0564	$(C_{13}H_8F_8O_4)$	
		p-HPA-TFE-PFP	H	380.0297	M^+	9:42
			d_4	384.0546	$(C_{13}H_8F_8O_4)$	
		m-HMA-Me-diPFP	H	415.0028	$M^+ - COOCH_3$	10:34
			d_3	418.0216	$(C_{13}H_5F_{10}O_4)$	
IV	375.9807	p-HMA-Me-diPFP	H	415.0028	$M^+ - COOCH_3$	11:40
			d_4	419.0279	$(C_{13}H_5F_{10}O_4)$	
		DOPAC-Me-diPFP	H	415.0028	$M^+ - COOCH_3$	12:08
			d_5	420.0342	$(C_{13}H_5F_{10}O_4)$	
		MHPG-tri-PFP	H	445.0134	$M^+ - CH_2OCOC_2F_5$	12:54
			d_1	446.0197	$(C_{14}H_7F_{10}O_5)$	
		HVA-TFE-PFP	H	410.0400	M^+	13:02
			d_3	413.0589	$(C_{14}H_{10}F_8O_5)$	
V	375.9807	VMA-ME-diPFP	H	445.0134	$M^+ - COOCH_3$	14:10
			d_3	448.0322	$(C_{14}H_7F_{10}O_5)$	
VI	325.9839	IAA-Me-PFP	H	335.0581	M^+	17:24
			d_2	337.0707	$(C_{14}H_{10}F_5NO_3)$	
VII	375.9807	5-HIAA-Me-diPFP	H	438.0188	$M^+ - COOCH_3$	18:39
			d_2	440.0314	$(C_{15}H_6F_{10}NO_3)$	

analysis. In operation, the magnet was set to the first reference mass and the GC cycle initiated. After each group of compounds had eluted from the gas chromatograph, the DIGMID program was increased and the next reference mass automatically came into focus. The mass reference ions and ions detected are indicated in Table II.

Biological samples

Urine was frozen at −18°C within 2 h of collection after addition of 10% EDTA and 4% thioglycolic acid. CSF was collected from the lumbar sac of fasting patients undergoing diagnostic pneumoencephalography and stored at −70°C until the analyses were performed. Blood samples were collected into vacuumized tubes containing sodium citrate. Platelets and plasma were separated by differential centrifugation and the plasma stored at −70°C until the first analysis and at −18°C thereafter.

Procedure

A stock solution in ^2H_2O of 25 ml of a mixture of all the deuterium-labelled acids was prepared such that, when diluted, 100 µl would contain two to five times the quantity of unlabelled acids expected to be present in the biological sample (see Table III for the exact quantities). The 25 ml of stock solution was divided into twelve 2-ml portions and frozen in individual vials at −18°C until needed for an analysis at which time a vial was thawed and 1 ml diluted. Unused standard stock and diluted solutions were discarded after one experiment and were not saved for another day. An aqueous mixture of three acids (phenylpropionic, *p*-hydroxyphenylpropionic and 5-methoxyindole-3-acetic

TABLE III

AMOUNT (ng) OF INTERNAL STANDARD ADDED TO BIOLOGICAL SAMPLES

Internal standard	Plasma	CSF	Urine
PAA-d$_2$	258.2	42.0	1269.6
MA-d$_3$	27.7	N.A.*	423.7
m-HPA-d$_4$	26.7	43.7	2077.1
p-HPA-d$_4$	99.8	38.6	4748.5
m-HMA-d$_3$	14.6	N.A.	102.0
p-HMA-d$_4$	37.8	N.A.	1643.2
VMA-d$_3$	27.3	N.A.	1003.4
HVA-d$_3$	58.5	37.7	1651.2
DOPAC-d$_5$	19.3	40.0	582.3
MHPG-d$_5$	13.2	N.A.	203.2
IAA-d$_2$	170.5	46.3	1702.2
5-HIAA-d$_2$	80.9	33.5	1894.9

*N.A. = Not added.

acid) at a concentration of about 10 μg each per 100 μl was used for the purpose of acting as carrier (i.e. to improve the extraction of the desired acids). A mixture of ascorbic acid and EDTA in water (100 μl, 1 mg/ml each) was prepared fresh for each analysis and added to each blank, standard and sample as anti-oxidant.

To 0.50 ml plasma, 0.50 ml CSF or 0.20 ml urine were added 100 μl of diluted internal standard, 100 μl of carrier solution and 100 μl of anti-oxidant solution. Plasma and CSF were deproteinized by the addition of 0.5 ml of 10.0% sulphosalicylic acid (SSA). Urine samples were treated with 0.20 ml of 0.2 M barium chloride to precipitate sulphate and phosphate ions and acidified with 0.40 ml of 10% SSA. All samples were centrifuged at 6000 g for 20 min at 20°C after precipitation of protein and sulphate.

The supernatant solutions (pH 1–2) were decanted from the pellet, and saturated with sodium chloride (about 0.5 g). This solution was then extracted (vortex-mixing) with 2 ml of glass-distilled ethyl acetate three times. The extract containing the acidic (and glycol) metabolites was divided into two approximately equal portions. Each was separately concentrated to about 200 μl and transferred to a 1-ml Reacti-Vial (Pierce, Rockford, IL, U.S.A.), 5 μl triethylamine were added and the solution was evaporated to dryness in a stream of nitrogen at 60°C. Removal of traces of water was achieved by adding 200 μl benzene and again evaporating to dryness in a stream of nitrogen.

The residue from one portion of the extract was derivatized with 200 μl methanolic hydrogen chloride (prepared by the cautious addition of 5 ml acetyl chloride to 40 ml of stirred, ice-cold methanol) at room temperature for 30 min. The methanolic hydrogen chloride was evaporated in a stream of nitrogen just barely to dryness. Extreme caution must be exercised here because the methyl esters of PAA, MA and IAA are very volatile. The residue was treated with 50 μl pentafluoropropionic anhydride and heated at 80°C for 60 min.

The residue of the second portion was derivatized with TFEOH and PFPA. PFPA (50 μl) and TFEOH (50 μl) were added to the residue and heated at

80°C for 60 min. The vial was cooled to about 30°C and the solution was concentrated to about 40—50 μl (the very volatile TFEOH would be completely evaporated). Finally, another 50 μl PFPA was added and the solution heated again at 80°C for 60 min.

The final PFPA solutions of the two derivative mixtures were combined and concentrated to 25 μl in a stream of nitrogen. Hexane (200 μl) was added and the solution was washed (vortex for 30 s each washing) with 100 μl of 1 M phosphate buffer (pH 6.0) twice. The hexane layer was transferred to a clean Reacti-Vial, concentrated to about 5 μl, and 0.5 μl was injected onto the GC—MS capillary column. The GC retention times and exact masses of the ions measured are given in Table II. With each batch of biological samples, two blanks (each 0.5 ml distilled water) and two check solutions of a mixture of known amounts of all the acids were analyzed in exactly the same way.

RESULTS AND DISCUSSION

The main feature of this procedure distinguishing it from most earlier ones is the capability of analysing a large number of biogenic amine metabolites at high sensitivity in one GC—MS run. This requires derivatization with two different reagent systems and precipitation of protein prior to extraction.

Derivatization

The necessity of employing two different derivatizing reagents arises from the special requirements of the particular acids we wished to analyze. Other workers and ourselves have been able to analyse PAA as a fluorinated ester, either as trifluoroethyl (TFE) [1] or the pentafluoro-*n*-propyl (PFnP) [35] which can be easily prepared in a one-step reaction with a mixture of the appropriate fluorinated alcohol (TFEOH or pentafluoro-*n*-propanol, PFPOH) and a fluorinated anhydride such as PFPA [20]. This reaction may be readily extended to the phenolic acids *m*-HPA, *p*-HPA and DOPAC since the PFPA in the reaction mixture acetylates the phenolic groups. Although the reaction mixture will also derivatize β-hydroxy acids, the products are more complex. We observed, confirming the results of Godse et al. [30], that fluorinated alcohols attack the benzylic carbon of *p*-hydroxy-substituted mandelic acids and MHPG and thus at least two products are obtained for each, an ether and an ester, hence the extra peaks for *p*-HMA observed in Fig. 1. In fact, as many as five products are sometimes observed in these derivatizations; the number and proportions of the products vary according to as-yet-undetermined factors. Four of these have been identified by GC—MS for VMA (Fig. 2). This problem can be eliminated by using the methyl ester instead of the trifluoroethyl ester, resulting in an increase in signal size by a factor of ten. Unfortunately, the methyl ester is completely unsatisfactory for PAA. The molecular ion of methyl-PAA has a mass of only 150 (compared to 218 for TFE-PAA) and does not contain any mass-deficient atoms. As a result, fragment and molecular ions from the numerous other compounds present in biological samples make unpredictable and frequently large contributions to the PAA signal. *m*-HPA, *p*-HPA and HVA are derivatized somewhat more efficiently by TFEOH—PFPA, whereas the methyl-PFP derivatives of the other acids are to be preferred.
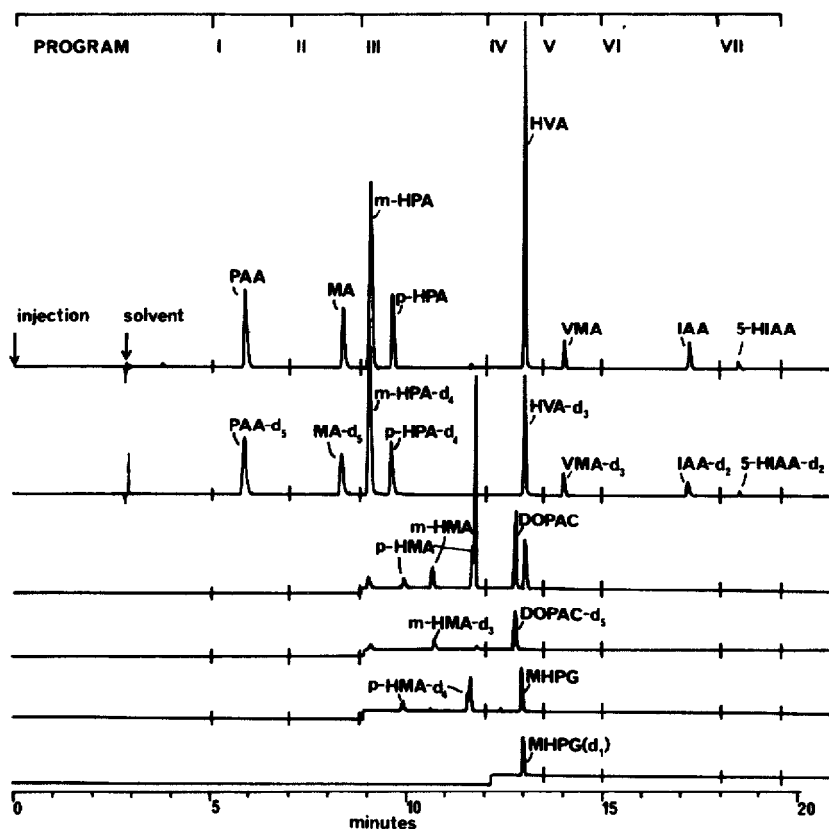
Fig. 1. Mass chromatogram of a standard mixture of twelve biogenic amine metabolites and their deuterium-labelled internal standards.
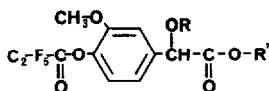

Fig. 2. Proposed structures of the four products of the derivatization of VMA with PFPA and TFEOH. (1) R = COC_2F_5; R' = CH_2CF_3. (2) R = COC_2F_5; R' = C_2F_5; (3) R = CH_2CF_3; R' = CH_2CF_3; (4) R = H; R' = CH_2CF_3.

Therefore, by dividing the ethyl acetate extract in half and derivatizing each half with the two different reagents and then combining the derivatives, we take advantage of the favourable characteristics of each derivative, maximizing sensitivity and selectivity, yet at the same time retain most of the efficiency of a single derivatization since we require only one extraction and one injection into the GC—MS system. A mass chromatogram of a standard mixture of all twelve metabolites is shown in Fig. 1.

An attempt was made to reduce formation of the ether in the derivatization of p-HMA, VMA and MHPG by reaction of the acid mixture initially with PFPA to form the acyl derivatives of the alcoholic and phenolic groups and then with TFEOH—PFPA to make the carboxylic ester. However, we observed that if an internal standard is labelled with deuterium in the alkyl chain,

as was the case with *m*-HPA, *p*-HPA, IAA and 5-HIAA, some of the deuterium label is lost due to keto—enol isomerization if traces of water are present. A small amount of the d_2 label is also lost when using the usual TFEOH—PFPA reaction mixture, but it appears to be relatively negligible, i.e. 2—3% of the d_2 is converted to d_1. We also observed that all the mandelic acids react much more poorly with TFEOH—PFPA than do the corresponding phenylacetic acids, even allowing for the formation of the fluorinated ether by-products.

Protein precipitation

Sulphosalicylic acid is the preferred protein precipitating agent because sample volumes are low, final samples are clean and recoveries are relatively high. Perchloric acid and hydrochloric acid proved to be unsuitable for protein precipitation because carry-over of traces of the mineral acid in the ethyl acetate extract inhibited the derivatization. Triethylamine added to the ethyl acetate extract forms salts of the acid metabolites, allowing the solvent to be evaporated to dryness and the residue to be azeotropically dried by the evaporation of benzene without loss of the volatile acids (PAA, MA, IAA). In the final step of the procedure, excess reagent is hydrolyzed and removed in a buffer wash rather than simply blowing to dryness in a stream of nitrogen as is customary [23, 24] because the derivatives of PAA, MA, IAA and the phenolic acids are too volatile and would be lost. Providing a buffer of pH 6.0 is used for the wash, hydrolysis of the derivatives is kept to a minimum.

Quantitative analysis

The slopes of the calibration curves are presented in Table IV. Values less than 1.0 are due chiefly to the fact that the internal standards are not isotopically pure, although chemical impurities would also contribute to a lowering of the slope.

The precision and accuracy of the procedure were determined for a standard aqueous mixture of the twelve metabolites. The coefficients of variation for the analyses of plasma, CSF and urine samples (Table V) were less than 10% for PAA, PHPA, HVA and IAA which are present in relatively large quantities, but

TABLE IV

SLOPES OF CALIBRATION CURVES FOR TWELVE METABOLITES

Acid	Slope ± S.E.	Number of data points
PAA	0.89 ± 0.06	44
p-HPA	0.73 ± 0.08	42
m-HPA	0.31 ± 0.03	43
MA	0.76 ± 0.08	42
VMA	0.95 ± 0.10	42
p-HMA	0.71 ± 0.06	44
m-HMA	0.61 ± 0.03	19
MHPG	0.98 ± 0.05	5
DOPAC	0.81 ± 0.06	7
IAA	0.71 ± 0.07	47
5-HIAA	1.04 ± 0.11	24
HVA	0.36 ± 0.05	44

TABLE V

COEFFICIENTS OF VARIATION (%) FOR THE ANALYSIS OF METABOLITES IN BIOLOGICAL FLUIDS

Each value (a percentage) is an average of fourteen samples each analyzed three times. N.M. = Not measured; N.D. = not detected.

Metabolite	Plasma	CSF	Urine
PAA	6.1	11.4	8.8
MA	13.1	N.M.	4.8
m-HPA	15.3	64.3	5.2
p-HPA	3.2	12.6	4.1
m-HMA	N.D.	N.M.	8.6
p-HMA	20.1	N.M.	4.6
VMA	12.0	N.M.	4.0
HVA	4.1	11.9	8.8
DOPAC	25.9	N.D.	9.9
MHPG	15.1	N.M.	11.4
IAA	6.4	17.3	9.0
5-HIAA	9.3	7.0	8.3

generally 10—20% for the other acids which are present in amounts of less than 10 ng/ml (or 5 ng in the 0.5-ml samples analyzed). For metabolites present in exceedingly small amounts, such as m-HPA in CSF and DOPAC in plasma, the coefficients of variation are even higher. The reproducibility of the GC—MS part of the analytical procedure was determined by injecting samples three consecutive times and comparing the calculated values; the coefficients of variation are less than 10%, which is similar to the coefficients of variation for the entire procedure, suggesting that a part of the variation in duplicate sample results is due to GC—MS factors. These may arise from errors in peak-height

TABLE VI

COMPARISON OF MEAN VALUES FOR UNCONJUGATED ACIDS IN HUMAN PLASMA, LUMBAR CEREBROSPINAL FLUID AND URINE REDUNDANT WITH A RANGE OF VALUES REPORTED IN THE LITERATURE

Acid	Plasma (ng/ml)			CSF (ng/ml)			Urine (mg per 24 h)		
	This paper	Literature	Ref.	This paper	Literature	Ref.	This paper	Literature	Ref.
PAA	71.4	107—622	16, 40	10.8	11—32	40, 41	1 7	0.7	6, 46
m-HPA	5.3	0—21	16, 28	0.5	1—4	41	5.8	3—15	6, 46
p-HPA	68.5	7—54	16, 28	6.0	6—9	41	25.1	15—54	6, 46
MA	13.6	10—80	35	N.M.*	—	—	0.22	—-	
m-HMA	N.D.**	—	—	N.M.	—	—	0.10	0.02—0.13	45
p-HMA	8.4	5—36	28	N.M.	—	—	1.91	1 7	6
IAA	292.3	260	39	5.1	17	42	10.1	7.0***	3
5-HIAA	34.0	7—13	38	18 6	30	43	4.78	5.5—8 0	29, 36
HVA	7.9	5—29	28, 38	20.1	7—101	24	5.37	4.1—6.0	29, 36
DOPAC	0.7	2—20	28, 38	N.D.	0.4	43	0.91	1.5—4.0	29, 36
VMA	5.4	8—25	28	N.M.	0.4—0.6	44	4.02	3.5—5.4	29, 36
MHPG	3.2	4—10	28	N.M.	8	43	0.16	0.13— 1.8	36, 37

*N.M. = Not measured; literature values not quoted for these
**N.D. = Not detected.
***Units are μg/mg of creatinine.

measurements (significant for small peaks) or from somewhat variable GC—MS parameters which may differentially alter the ionization or fragmentation of the proteo and deutero isomers. Frequent cleaning of the injection port liner is essential for good reproducibility of values for repeated injections, particularly for 5-HIAA.

The means of the determinations of ten samples each of plasma, CSF and urine are compared with a range of reported values in Table VI and are close to or within the reported ranges. In plasma we were unable to detect m-HMA, which was also in very low concentration in urine and our value agrees with literature values for the latter. Similarly DOPAC concentration in CSF is extremely low and was found to be below our level of detectability. Recoveries of DOPAC were very low, reflecting its strong hydrophilic character.

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