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SIMULTANEOUS DETERMINATION OF THE MAJOR ACIDIC METABOLITES OF CATECHOLAMINES AND SEROTONIN IN URINE BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION AFTER A ONE-STEP SAMPLE CLEAN-UP ON SEPHADEX G-10; INFLUENCE OF VANILLA AND BANANA INGESTION

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SUMMARY

A simple method is described for the simultaneous determination of vanilmandelic acid (VMA), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindole-3-acetic acid (5-HIAA) and homovanillic acid (HVA) in urine. The compounds are isolated by a one-step sample clean-up on Sephadex G-10, separated by ion-pair reversed-phase liquid chromatography and detected electrochemically. A single analysis is completed within 65 min. Sample clean-up did not cause losses of the compounds of interest. The detection limits in urine were 0.4, 0.8, 1.0 and 1.6 μmol/l for VMA, DOPAC, 5-HIAA and HVA, respectively. 3,4-Dihydroxymandelic acid and vanillic acid (VA) were also detectable, but, under the chromatographic conditions used, they were not resolved from interfering components. VA and 5-HIAA could be analysed separately in the Sephadex G-10 eluate if more restrictive sampling conditions were used. Ingestion of bananas caused an increase of VMA, DOPAC, 5-HIAA and HVA in 24-h urine. After ingestion of vanilla an increased excretion of VA was observed, while the excretion of VMA, DOPAC and HVA was unaffected.

INTRODUCTION

Excretion of acidic metabolites of catecholamines and serotonin (5-HT) in urine is clinically of interest in the diagnosis and follow-up of treatment of tumours secreting these compounds [1–3]. During the past few years, many methods using high-performance liquid chromatography (HPLC) and electrochemical (ED) or fluorimetric detection have been described for the analysis of the acidic metabolites. In general, the compounds of interest are extracted from urine with organic solvents or by chromatography, both resulting in losses. For the simul-

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taneous analysis of vanilmandelic acid (VMA) and homovanillic acid (HVA), buffer changes or gradient elution are used [4–6].

Here we report a simple method for the simultaneous determination of VMA, 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindole-3-acetic acid (5-HIAA) and HVA in urine by isocratic HPLC and ED after a sample clean-up on Sephadex G-10, yielding quantitative recoveries of the compounds. The method has been used to investigate the influence of vanilla and banana eating on the occurrence of interferences in the HPLC elution profile and on the amounts of the compounds of interest excreted in 24-h urine.

EXPERIMENTAL

Apparatus

HPLC analyses were performed using a system incorporating a Perkin-Elmer Series 10 pump, a Perkin-Elmer ISS 100 autoinjector and a Bioanalytical Systems amperometric detector, consisting of an LC-4B control unit, a TL-5A amperometric flow-cell with glassy carbon, stainless-steel and Ag/AgCl as working, auxiliary and reference electrode, respectively (Perkin-Elmer, Gouda, The Netherlands). The injector and detector were connected on-line to a Perkin-Elmer 3230 Labdata system equipped with LIMS/CLAS software.

Stainless-steel Hyperchrome HPLC columns, 125×4.6 mm I.D. (Salm & Kipp, Breukelen, The Netherlands), were packed with ODS-Hypersil 5 μm (Shandon Southern Products, Astmoor, U.K.) at 63 MPa by the balanced-density slurry technique, on a column-packing installation designed at our institute, using a Haskel pump Type DSTV-150 (Ammann Technik, Stuttgart, F.R.G.). The slurry and packing solvents were isopropanol and methanol, respectively (Merck, Darmstadt, F.R.G.).

Chemicals

Iso-HVA was a gift of Hoffmann-La Roche (Mijdrecht, The Netherlands). 3-Hydroxy-4-methoxyphenylglycol (iso-MHPG) was synthesized by reduction of iso-VMA (Fluka, Buchs, Switzerland) with lithium aluminium hydride [7]. The other reference compounds were obtained from Sigma (St. Louis, MO, U.S.A.). Pic A (tetrabutylammonium phosphate) was from Waters Assoc. (Etten-Leur, The Netherlands) and Sephadex G-10 from Pharmacia (Woerden, The Netherlands).

Sample clean-up of urine, HPLC separation and detection

For the simultaneous analysis of VMA, DOPAC, 5-HIAA and HVA, 0.25 ml of urine (pH 2.8, adjusted with 3 *M* hydrochloric acid) was poured into a column containing Sephadex G-10 (70×5 mm I.D.), equilibrated with 0.1 *M* acetic acid. The resin was washed with 2.5 ml of 0.1 *M* acetic acid. The compounds were eluted with 10 ml of sodium acetate (0.1 *M*, pH 5.5). The pH of the eluate was adjusted to 4.1 by the addition of 0.1 ml of glacial acetic acid. For the analysis of vanillic acid (VA) in urine, the same procedure was used, except that the first 2 ml and the last 5 ml of the sodium acetate eluate were discarded. The Sephadex

G-10 columns were regenerated by rinsing with 5 ml of 0.1 *M* ammonium hydroxide followed by 5 ml of 0.1 *M* acetic acid.

Analyses were carried out by injecting 100-μl aliquots (solutions containing a mixture of reference compounds or sodium acetate eluates) onto the ODS-Hypersil 5-μm column. The column was eluted isocratically at a flow-rate of 1.5 ml/min with a solution containing sodium acetate (25 m*M*) and tetrabutylammonium phosphate (5 m*M*), adjusted to pH 4.2 with glacial acetic acid. Detection was done electrochemically at +750 mV vs. Ag/AgCl.

Analysis of vanillin and analogues in vanilla sugar

The content of vanillin (3-methoxy-4-hydroxybenzaldehyde), isovanillin and ethylvanillin (3-ethoxy-4-hydroxybenzaldehyde) in vanilla sugar was determined with capillary gas chromatography on a fused-silica column. For this purpose a sample was dissolved in 80% (v/v) ethanol and injected without derivatization.

Diet and 24-h urine collection

Six male and six female apparently healthy subjects (25–42 years) participated at the same time in a study on the influence of banana and vanilla ingestion on the excretion of metabolites of catecholamines and serotonin. During a period of six days, three different diets were prescribed, each for two consecutive days.

(1) A control diet, being their habitual food pattern without bananas, pineapple, tomatoes, chocolate, nuts and products containing vanilla.

(2) A vanilla diet, being the control diet supplemented with 7 g of vanilla sugar, containing 110 mg vanillin per g and no detectable amounts of ethylvanillin and isovanillin (<1 mg/g).

(3) A banana diet, being the control diet supplemented with four bananas per day.

Each subject received each diet in a randomized order according to four Latin squares, balanced for sex and carry-over effects from the previous treatment, with subjects and sub-periods of two days as rows and columns, respectively [8].

On the second day of each diet 24-h urine was collected. Collection started after voiding morning urine. Urine was collected in polyethylene containers. Volumes were measured and aliquots were stored at $-20\,^{\circ}$C until analysis.

Effects of diet and sex were investigated with split-plot analysis of variance (ANOVA) with factors sex, diet, their interaction and day of collection.

RESULTS

Applicability of the assay

Typical HPLC elution profiles of a solution containing the acidic reference compounds coeluting from the Sephadex G-10 column (see below), a pooled urine sample and a pooled urine sample after addition of known amounts of these reference compounds are shown in Fig. 1. The HPLC procedure provided a good separation of the reference compounds. In urine samples VMA, DOPAC, 5-HIAA and HVA were separated from other compounds yielding an electrochemical sig-

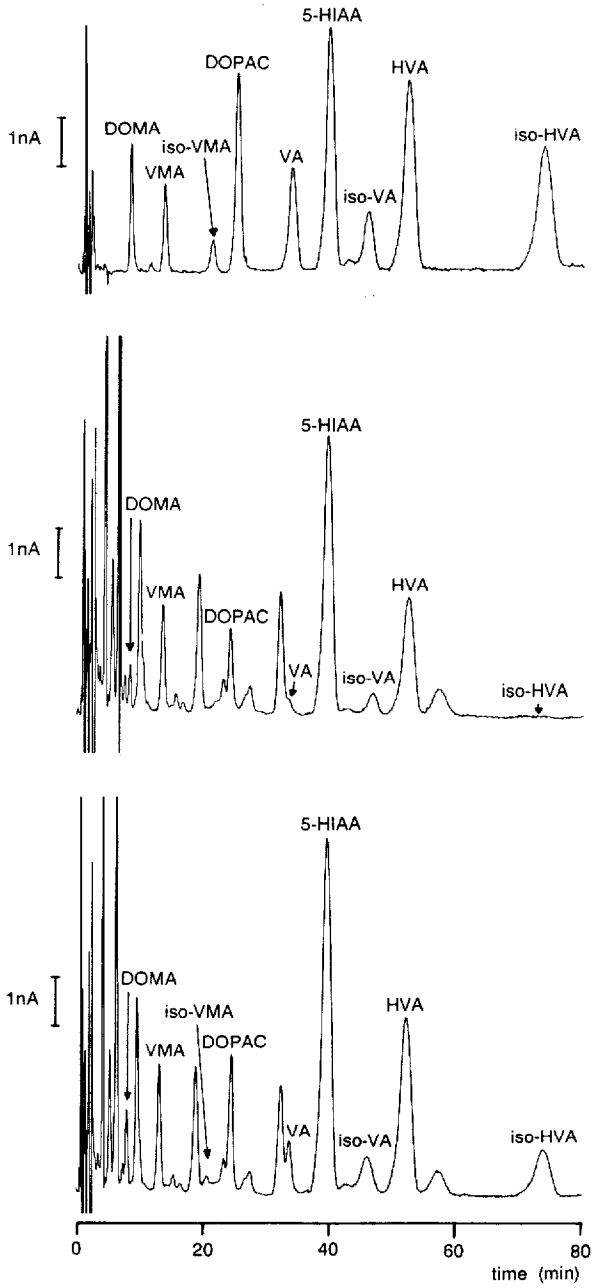

Fig. 1. HPLC elution profiles of: (top) 100 μl of a mixture of reference compounds containing 25 pmol DOMA, 50 p mol (iso-)VMA, DOPAC and 5-HIAA, and 100 pmol (iso-)VA and (iso-)HVA; (middle) 100 μl of a Sephadex G-10 eluate of a pooled urine sample; and (bottom) 100 μl of a Sephadex G-10 eluate of the same urine sample after increasing the endogenous concentrations by 3.75 μmol/l for DOMA, 7.5 μmol/l for (iso-)VMA, DOPAC and 5-HIAA, and 15 μmol/l for (iso-)VA and (iso-)HVA.

TABLE I

RECOVERIES OF THE COMPOUNDS ADDED TO URINE SAMPLES

On ten days, a urine sample was randomly chosen and its endogenous concentrations were increased by 15.5 μmol/l for VMA and HVA and by 9.5 μmol/l for DOPAC and 5-HIAA, respectively. Single determinations were done on the samples before and after the addition.

Compound	Recovery (mean ± S.D.) (%)
VMA	100.9 ± 8.5
DOPAC	100.5 ± 8.5
5-HIAA	97.5 ± 9.2
HVA	101.5 ± 10.4

nal. For optimal separation the pH of the mobile phase had to be between 4.15 and 4.25. In general, 3,4-dihydroxymandelic acid (DOMA) and VA could not be reliably measured due to interferences. Under the HPLC conditions used iso-VA had almost the same retention time as an unknown component in the elution profile. Small changes in pH of the mobile phase resulted in a more pronounced difference between both compounds. Incidentally an interference of 5-HIAA was observed. In those cases 5-HIAA could be analysed separately by using more restrictive sampling conditions during Sephadex G-10 chromatography (see below). The same holds true for VA.

Assuming a signal-to-noise ratio of at least 3, the detection limits of the assay described in this paper were 1.0, 2.0, 2.5 and 4.0 pmol for VMA, DOPAC, 5-HIAA and HVA, respectively. This corresponds to 0.4, 0.8, 1.0 and 1.6 μmol/l for VMA, DOPAC, 5-HIAA and HVA, respectively, in urine. In practice, these limits suffice for determinations in 24-h urine.

Recovery and precision

Recoveries were determined by the addition of known amounts of the compounds to urine samples of healthy individuals. The results are summarized in Table I. The analysis of the compounds showed quantitative recoveries.

To determine the precision of the assay, a pooled urine sample was analysed over a period of six months under routine conditions. The results are presented in Table II.

Sephadex G-10 elution profile

The elution profile on Sephadex G-10 was studied to optimize the analytical recovery of the compounds of interest and to investigate whether structurally related compounds and methyluric acids (metabolites of caffeine) interfered with or could be included in the assay, and whether analogues could be used (if desired) as internal standards.

The results are shown in Fig. 2. By discarding the first 2.75 ml of the eluate after pouring the sample onto the Sephadex column and collecting the next 10 ml, the acidic compounds (iso-)VMA, (iso-)HVA, DOPAC, (iso-)VA and 5-

TABLE II

PRECISION OF THE ASSAY

The precision was calculated with analysis of variance from the data of a pooled urine sample analysed in duplicate on 53 different days over a period of six months under routine conditions.

Compound	Concentration (μmol/l)			
	Mean	$S.D._{between}$	$S.D._{within}$	$S.D._{overall}$*
VMA	20.0	1.58	1.15	1.95
DOPAC	7.2	1.53	0.97	1.81
5-HIAA	25.9	1.84	1.59	2.43
HVA	26.1	2.10	1.59	2.64

*Precision of a single assay: $(S.D._{overall})^2 = (S.D._{between})^2 + (S.D._{within})^2$

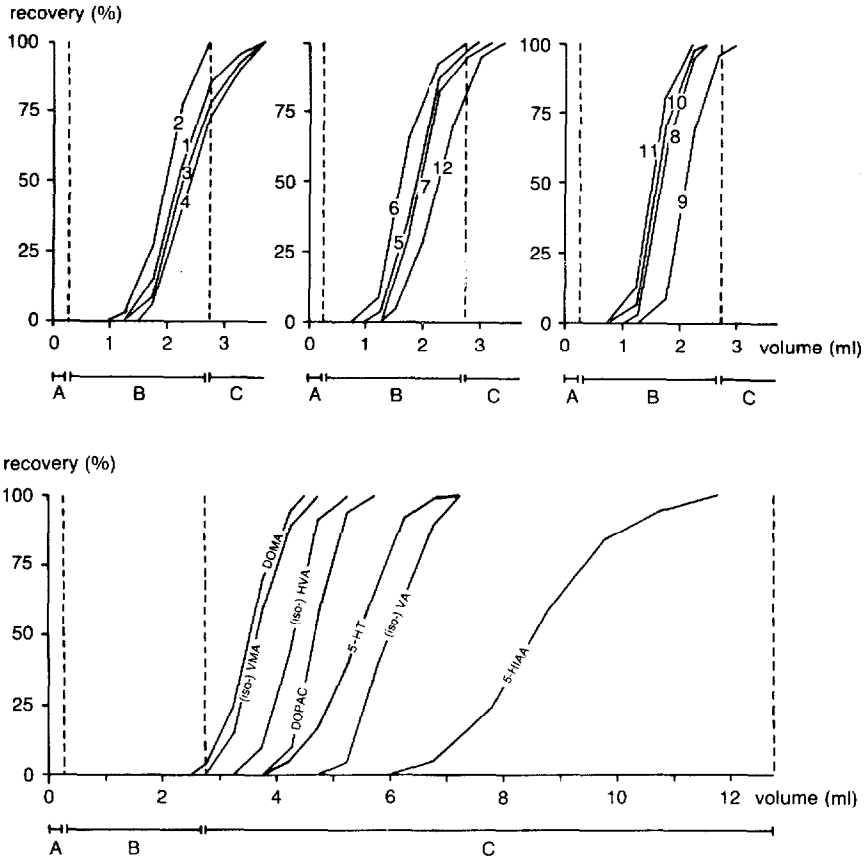

Fig. 2. Sephadex G-10 elution profiles. After pouring a sample of 250 μl (A) on the column (7 cm × 5 mm I.D.) the resin was subsequently washed with 2.5 ml (B) of acetic acid (0.1 *M*) and 10 ml (C) of sodium acetate (0.1 *M*, pH 5.5). Curves: 1 = epinephrine; 2 = norepinephrine; 3 = dopamine; 4 = salsolinol; 5 = normetanephrine; 6 = metanephrine; 7 = 3-methoxytyramine; 8 = 1,3-dimethyluric acid; 9 = 1-methyluric acid; 10 = 3,7-dimethyluric acid; 11 = 1,3,7-trimethyluric acid; 12 = (iso-)MHPG.

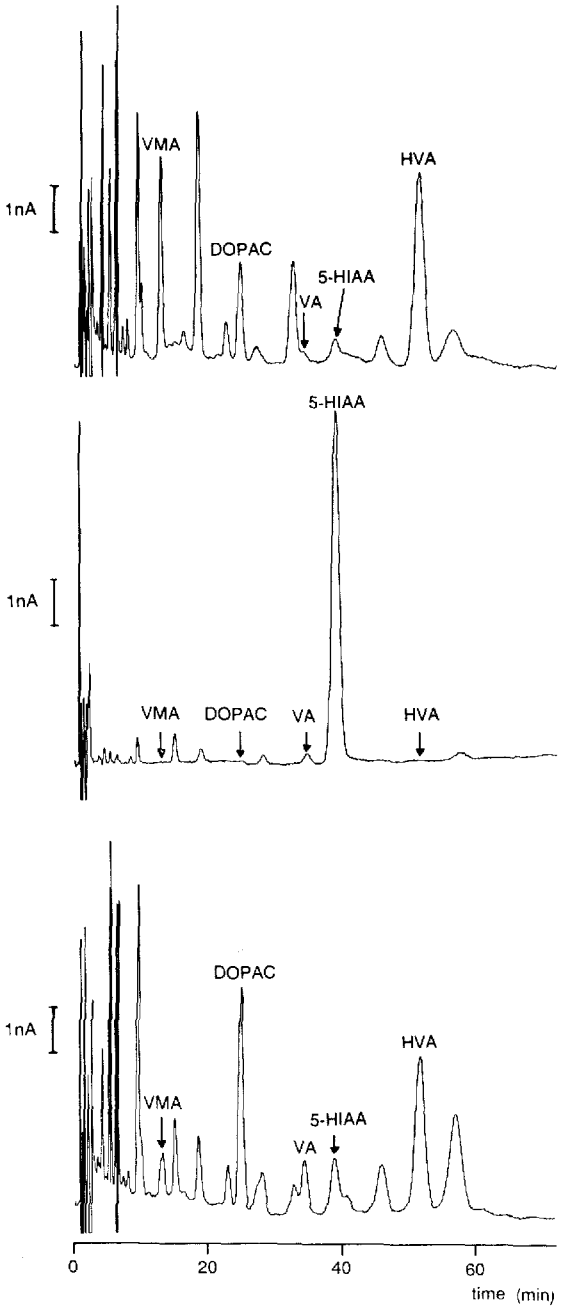

Fig. 3. HPLC elution profiles of: (top) 50 μl of the fraction (3 ml) of the Sephadex G-10 eluate containing VMA, DOPAC and HVA; (middle) 100 μl of the fraction (7 ml) containing 5-HIAA; and (bottom) 100 μl of the fraction (3 ml) containing VA.

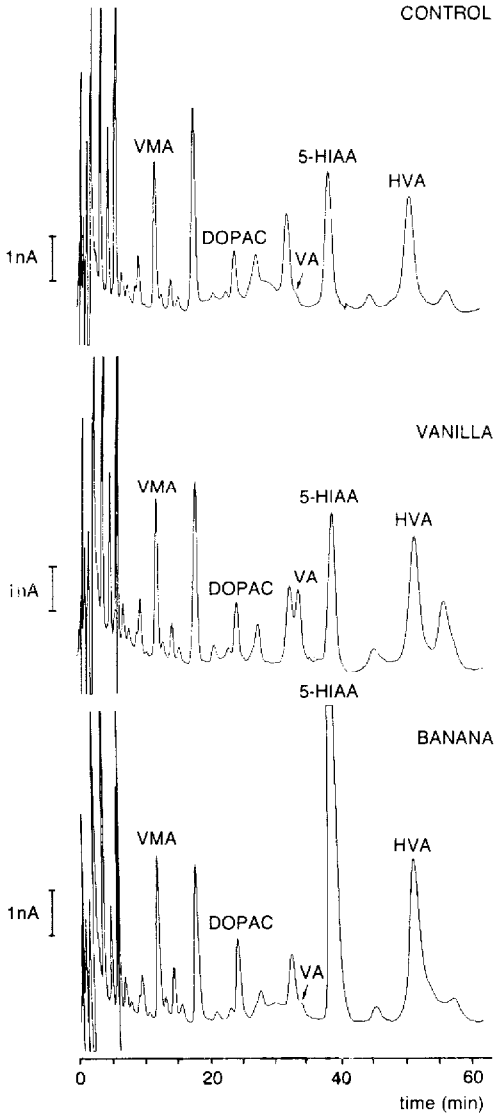

Fig. 4. HPLC elution profiles of 100 μl of Sephadex G-10 eluates of pooled control (top), vanilla (middle) and banana (bottom) urine samples. The samples were constituted by mixing aliquots (1:100 of the 24-h volume) of the corresponding urine of each subject.

HIAA were quantitatively recovered, while a slight loss was observed for DOMA. As mentioned above, the latter compound was not well separated in the HPLC elution profile. 5-HT coeluted with the acidic compounds, but under the HPLC conditions used, it eluted immediately after the void volume .

The caffeine metabolites, norepinephrine and metanephrine were quantitatively removed by the sample clean-up. More than 70% each of epinephrine, dopamine, salsolinol, normetanephrine, 3-methoxytyramine and (iso-)3-methoxy-4-hydroxyphenylethyleneglycol [(iso-)MHPG] were discarded. They were eluted within 6 min from the HPLC column and thus did not interfere in the assay.

TABLE III

INFLUENCE OF BANANA AND VANILLA INGESTION ON THE EXCRETION IN 24-H URINE

Compound	Sex	Diet			S.E.D.* between sexes	S.E.D.* within sexes	*P*-value		
		Control	Vanilla	Banana			Sex	Diet	Sex×diet
VMA	Male	23.6	22.3	25.4	3.43	1.25	0.037	0.049	N.S.
(μmol)	Female	15.5	15.3	16.9					
DOPAC	Male	7.2	8.7	10.5	2.06	1.40	N.S.	0.002	N.S.
(μmol)	Female	7.8	7.2	12.4					
5-HIAA	Male	23.1	24.2	74.8	5.66	4.18	N.S.	<0.001	N.S.
(μmol)	Female	22.7	21.9	79.4					
HVA	Male	28.5	29.4	39.0	3.74	3.05	0.007	<0.001	N.S.
(μmol)	Female	20.5	20.5	27.8					
VA	Male	4.4	41.6	8.1	9.28	8.68	N.S.	<0.001	N.S.
(μmol)	Female	6.8	42.0	6.3					
Creatinine	Male	14.9	14.8	15.9	0.99	0.60	<0.001	N.S.	N.S.
(mmol)	Female	11.3	10.7	10.9					
Volume	Male	1540	1860	1720	375	286	N.S.	N.S.	N.S.
(ml)	Female	1790	1230	1530					

*Standard error of the difference (S.E.D.) between two means; *t*-values (18 degrees of freedom) can be calculated by dividing the difference between two means by the corresponding S.E.D. N.S. = not significant.

Separate analysis of 5-HIAA, VA and 5-HT

As shown in Fig. 2, collection of the Sephadex G-10 eluate as two fractions (3 and 7 ml, respectively) offers the possibility to analyse 5-HIAA separately, while VMA, HVA and DOPAC can still be analysed simultaneously. Typical HPLC elution profiles are presented in Fig. 3. It is apparent from the same figures that analysis of VA without visible interferences can be achieved by discarding the first 2 ml and the last 5 ml of the sodium acetate eluate.

The Sephadex G-10 elution profiles also indicate that selective sample collection might also be used for the analysis of 5-HT. However, as indicated above, the mobile phase used for the separation of the acidic metabolites was not suitable for this purpose.

Influence of vanilla and banana ingestion

The influence of vanilla and banana ingestion on the HPLC elution profiles is shown in Fig. 4. The data about their effect on the excretion of VMA, DOPAC, 5-HIAA, HVA and VA are summarized in Table III. No visible interferences of the compounds to be analysed were observed. Neither creatinine excretion nor the volume of 24-h urine was significantly influenced, indicating proper urine collection. Men showed higher values than women for the excretion of VMA, HVA and creatinine.

Vanilla ingestion caused an increased excretion of VA and of an unidentified compound eluting after HVA. The excretion of VMA, DOPAC, 5-HIAA and HVA was not affected. Banana consumption caused a considerable increase of 5-HIAA

excretion, a moderate increase of DOPAC and HVA and a small but significant increase of VMA. The excretion of VA was not affected. No significant differences were observed for the effects of diet between men and women.

DISCUSSION

The adsorption properties of Sephadex G-10 have been used for the extraction of catecholamines, 5-HT, metabolites and analogues from supernatants of acid precipitates of nervous tissue, plasma and serum [9–14]. Furthermore, Sephadex G-10 chromatography has been successfully applied as second step in the sample clean-up of urine in the analysis of catecholamines [15] and metanephrines [16]. For the analysis of HVA and 5-HIAA in urine one-step sample clean-up procedures using Sephadex G-10 have been reported [15,17,18]. In the present paper, we showed that simultaneous analysis of VMA, DOPAC, 5-HIAA and HVA can be achieved using merely Sephadex G-10 chromatography as sample clean-up.

Sephadex G-10 chromatography in sample clean-up has several advantages compared with ion-exchange chromatography or extraction with organic solvents. Owing to the weak interactions of the compounds with Sephadex G-10 quantitative recoveries easily can be obtained. This implies that analyses can be performed without using internal standards. In practice, Sephadex G-10 columns are easy to handle and can be used many times without appreciable loss of the chromatographic properties [9,10,12,16,18].

The data presented confirm the general assumption that in studies on the metabolism of biogenic amines a low-amine diet should be followed. Ingestion of bananas, which are known to contain considerable amounts of amines [19], caused a substantial increase of 5-HIAA excretion. This is in accordance with findings of other investigators [3,19]. The effect of ingestion of amine-containing products on the excretion of VMA, DOPAC and HVA has hardly been described. Our results clearly showed an increased excretion of these compounds as well, although the effect on VMA excretion was relatively small.

Ingestion of vanillin-containing food may interfere in assays in which catecholamine metabolites are oxidized to vanillin prior to analysis. In our assay, an effect of vanillin was neither expected nor found, except for the excretion of VA to which ingested vanillin is apparently metabolized.

We conclude that Sephadex G-10 is a reliable, versatile and inexpensive tool for the sample clean-up of urine in the determination of the major acidic metabolites of catecholamines and serotonin. In studies on the metabolism of these compounds it should be realised that intake of amine-containing food can influence the results obtained.

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