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DETERMINATION OF NINE CATECHOLAMINE METABOLITES AND 5-HYDROXYINDOLACETIC ACID IN URINE BY CAPILLARY GAS CHROMATOGRAPHY

E. B. M. DE JONG⁺, B. P. M. HORSTEN and H. M. J. GOLDSCHMIDT*

Department of Clinical Chemistry and Haematology, St. Elisabeth Hospital, 5000 LC Tilburg (The Netherlands)

SUMMARY

A method is described for the simultaneous determination of nine urinary acidic and alcoholic catecholamine metabolites and urinary 5-hydroxyindolacetic acid. Incubation of a urine sample in the presence of ascorbic acid, glucuronidase and acylase and subsequent extraction with ethyl acetate precedes derivatization to trimethylsilyl compounds, capillary gas chromatographic separation and flame-ionization detection.

The automated dual injection procedure and the analytical characteristics of the proposed method are reported in detail. Special attention is paid to problems that occur in analysis on a routine basis.

INTRODUCTION

Adrenaline, as a hormone, and noradrenaline together with dopamine, as neurotransmitters, are the main physiologically active compounds of the catecholamines. Their determination in plasma is difficult because of the low concentrations (0.5–1.5 pmol/ml), and the interpretation is troublesome as rapid variations frequently occur. Therefore, the measurement of catecholamine metabolites and related compounds in urine, instead of plasma, is still of invaluable importance in many diseases, *e.g.*, neurogenic tumours. It is important not only in diagnosis or for monitoring therapy, but also in the prognosis of diseases.

The significance of vanilmandelic acid (VMA), 3-methoxy-4-hydroxyphenylethylene glycol (MHPG) and homovanillic acid (HVA) in urine was illustrated by Wadman *et al.*[1] and Muskiet *et al.*[2]. Other acidic metabolites of interest are 3,4-dihydroxyphenylacetic acid (DOPAC), vanillactic acid (VLA), 3,4-dihydroxymandelic acid (DOMA) and *p*-hydroxyphenylacetic acid (*p*-OHPAA). In addition to MHPG, we have focused our attention on 3,4-dihydroxyphenylethylene glycol (DHPG) and vanilethanol (VE), as alcoholic metabolites. 5-Hydroxyindolacetic acid (HIAA), a serotonine metabolite, was also of interest as a more abundant urinary acid.

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* Deceased August 4th, 1982.

Although the importance of high-performance liquid chromatography in the separation of the above compounds is increasing capillary gas chromatography is still the method of choice, especially in routine clinical applications, because of the high resolution and reproducibilities achieved.

EXPERIMENTAL

Apparatus

A Model 5880A gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) with two terminals, containing a print–plot mechanism and a functional keyboard (level IV) and two alphanumeric keyboards, equipped with a 7672A automatic sampler, two capillary injectors (splitless mode), two flame-ionization detectors and a hydrogen generator from General Electric (Chrompack, Middelburg, The Netherlands). Two fused-silica columns (wall-coated open-tubular) coated with CP Sil 5 (25 m × 0.25 mm I.D.) were obtained from Chrompack. A Model A141 universal pH/mV meter from Ankersmit (Breda, The Netherlands) was used.

Reagents and materials

DHPG, VLA, MHPG, pOHPAA, HVA, DOMA, VE, VMA, DOPAC, HIAA and docosanoic (behenic) acid were purchased from Sigma (St. Louis, MO, U.S.A.), tetracosane from Fluka (Buchs, Switzerland), acylase (15 U/mg) from Serva (Heidelberg, F.R.G.), β-glucuronidase (*Escherichia coli*) from Boehringer (Mannheim, F.R.G.), Sep-Pak C_{18} columns (1 cm × 1 cm I.D.) from Waters (Etten-Leur, The Netherlands), N,O-bistrimethylsilyltrifluoroacetamide (BSTFA) from Applied Science (State College, PA, U.S.A.) and dichloromethane and ethyl acetate (GC–spectrophotometric quality) from Baker (Deventer, The Netherlands). All other chemicals were obtained from E. Merck (Darmstadt, F.R.G.) and were of the highest available quality. Dichloromethane was dried on molecular sieve 4Å (E. Merck).

The Sep-Pak C_{18} columns were prepared for analysis by washing them successively with 10 ml of water, 10 ml of methanol, and 10 ml of water at a rate of 2 ml/min. The water was demineralized by ion exchange. Acylase (10 mg) was dissolved in 7.5 ml of phosphate buffer (0.07 M KH_2PO_4–Na_2HPO_4, pH 6.4; Merck). This solution was transferred into a prepared Sep-Pak C_{18} column and drawn through it at a rate of 30 ml/min. Glucuronidase was purified analogously.

Urine specimens from a patient were collected for 24 h and stored at $-20°C$ until analysed. The creatinine content was determined according to a modified Jaffe[3] method on a continuous flow system.

Urine specimen preparation

A four-fold dilution of 1 ml of centrifuged urine with the described phosphate buffer, containing 4.0 mg of acylase per 3 ml, was followed by the addition of 400 μl of glucuronidase and 100 μl of a 0.57 M ascorbic acid solution. Hydrolysis was performed for 24 h at 37°C. Subsequent extraction of the unconjugated catecholamine metabolites and HIAA from 1 ml of diluted urine was performed at pH 2.5 and 1.0 with ethyl acetate. Tetracosane and docosanoic acid (0.24 μmol) were added as internal standard before the first extraction. The separation by means of dehydration at $-80°C$ and the evaporation of the latter phase under nitrogen at 37°C made the metabolites ready for derivatization (see Fig. 1).
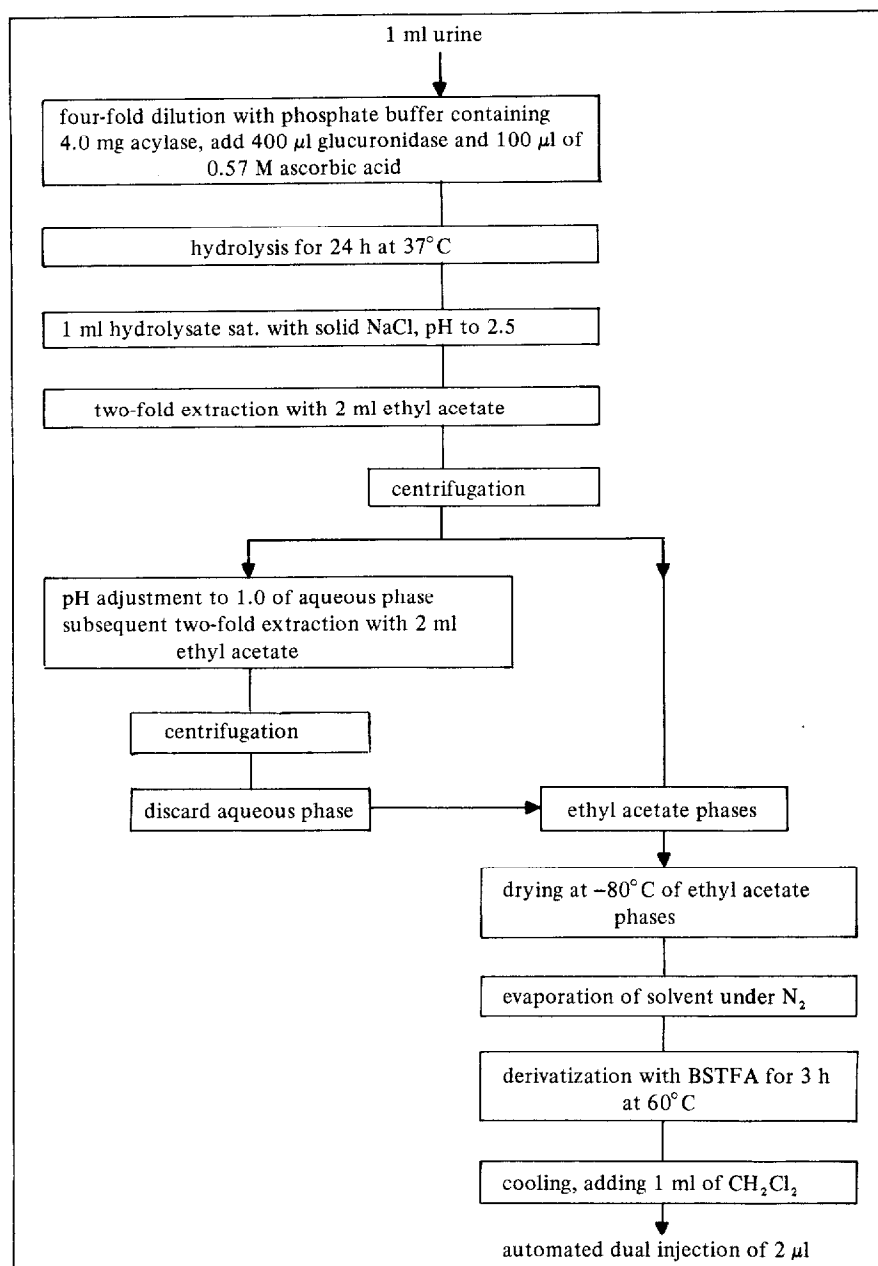
Fig. 1. Outline of the analysis.

Derivatization

Derivatization into trimethylsilyl compounds was performed at 60°C for 3 h in a heating block with 100 μl of BSTFA. After cooling to room temperature and adding 1 ml of dried dichloromethane the sample was ready for injection.
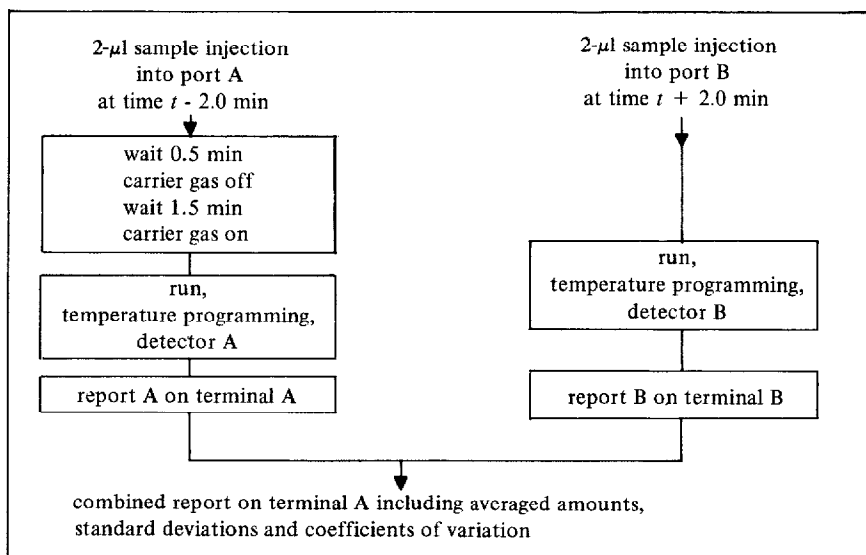
Fig. 2. Schematic diagram of the dual automated injection and analysis. Time t is an arbitrary starting time for the procedure.

Automated gas chromatographic procedure

The splitless injection was automated by means of a sampler, holding a series of samples up to a maximum of 99 and the procedure outlined in Fig. 2. Parallel injections were performed in such a way that the samples were analysed by the same temperature programme and processed simultaneously. This was accomplished by means of a valve that turned the carrier gas off, but only after the sample had reached the beginning of the column, where it remained because of the low temperature (50°C). After injection on the parallel channel, the two samples were subjected to the same analysis and the results, after checking the retention times, were averaged.

Injection into channel A at time $t - 2.0$ min was followed by a 2-min clean-up procedure to remove the excess of solvent and 0.5 min for the sample to reach and settle at the beginning of column A. After 2 min, the analysis started in column B with the clean-up procedure. The combined report included standard deviations and coefficients of variation of the means, calculated according to Sokal and Rohlf[4].

Helium was used as the carrier gas at a flow-rate of 1 ml/min. The oven temperature was kept at 50°C for 1 min then increased at 25°C/min to 120°C, kept at 120°C for 4 min, increased at 2°C/min to 180°C, and increased again at 6°C/min to 240°C, where it was kept for 30 min (see Fig. 3). The temperature of the splitless injectors was 250°C and that of the detectors 300°C.

Analytical duplicates were used for the parallel determinations described. The coefficients of variation of the averaged amounts were used as a measure of quality assurance. We also processed every urine specimen spiked with the components of interest.

RESULTS AND DISCUSSION

Optimization of the analysis

The optimal choice of, e.g., silylation reagents and conditions, oven temperature programming, clean-up and extraction, resulted in a gas chromatogram as shown in Fig. 3.
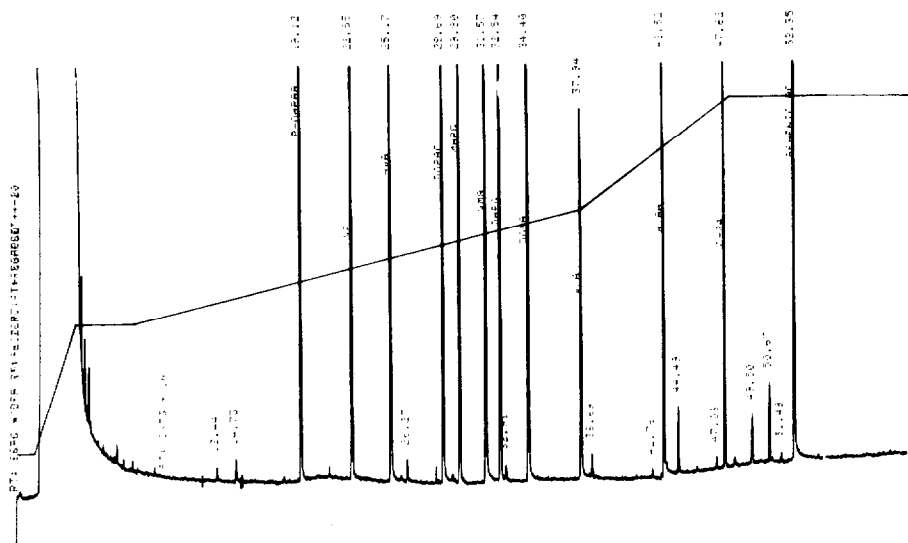
Fig. 3. Gas chromatogram of a standard mixture containing 0.06 μmol/ml of dichloromethane, with retention time in minutes on abscissa and flame-ionization detector response on ordinate with $8 \cdot 10^{-12}$ A f.s. The line represents the oven temperature. For explanation of abbreviations, see text.

Ascorbic acid

The need to add ascorbic acid, which probably acts as an antioxidant, to the hydrolysis medium depended on the specimen. Omitting it resulted in very low recoveries of MHPG, DOMA, DHPG and DOPAC in certain samples. These urine samples were indistinguishable from others by any other criterion. We added ascorbic acid to the urine in such an amount (57 μmol/ml) that we could see its peak in the chromatogram (at a retention time of approximately 35 min, between DOMA and VLA). The excess of ascorbic acid did not interfere with any of the compounds of interest.

In spiked urine samples and with tetracosane as internal standard the recoveries of MHPG, DOMA, DHPG and DOPAC were 73.2 $\pm$ 14.6, 77.2 $\pm$ 11.6, 35.8 $\pm$ 9.5 and 114.6 $\pm$ 5.4%, respectively (mean value, $\bar{x} \pm$ S.D., $n = 10$). Muskiet and co-workers[2,5] reported very low recoveries of some of these components. For VMA, HVA, VLA, p-OHPAA and VE we found 126.2 $\pm$ 7.1, 130.0 $\pm$ 4.8. 123.0 $\pm$ 3.4, 134.6 $\pm$ 9.9 and 99.6 $\pm$ 7.0%, respectively. Behenic acid as internal standard yielded the following recoveries: MHPG 95.3 $\pm$ 12.0, DOMA 79.6 $\pm$ 8.3, DHPG 54.5 $\pm$ 23.7, DOPAC 95.6 $\pm$ 11.8, VMA 108.8 $\pm$ 6.8, HVA 101.1 $\pm$ 9.6, VLA 101.6 $\pm$ 5.8, p-OHPAA 98.7 $\pm$ 12.1, VE 98.2 $\pm$ 8.8 and HIAA 87.8 $\pm$ 13.4% ($\bar{x} \pm$ S.D., $n = 7$). There is a distinct difference between these recoveries and those of standards estimated in 0.15 M sodium chloride solution: the latter gave better recoveries.

Urinary hippuric acid interference

Hippuric acid, formed in the liver as a conjugate of glycine and benzoic acid and excreted in the urine, interferes strongly with DOPAC. The added acylase solves this problem by converting hippuric acid into glycine and benzoic acid, which does not interfere in the analysis.

Effect of pH of the aqueous phase

Extraction of the unconjugated catecholamine metabolites and HIAA at two pH values (1.0 and 2.5) yielded better recoveries specially of MHPG, DHPG (also according to Biondi et al.[7]) and DOMA. Four-fold extraction from 0.15 M sodium chloride solution at pH 1.0 yielded recoveries of 77.1, 35.5 and 68.5%, respectively. Sequential two-fold extraction at pH 2.5 and 1.0 resulted in 99.4, 68.0 and 107.8%, respectively.

Calibration

We tested various potential internal standards, *e.g.*, β-resorcylic acid, 3,4,5-trihydroxybenzoic acid, propyl gallate, palmitic acid and stearic acid. However, they interfered with the analysis of DOPAC, ascorbic acid, VLA, an unknown urinary component and an unknown urinary component, respectively. The results presented here were obtained with tetracosane as internal standard, although we have found docosanoic acid to be more satisfactory (Figs. 3 and 8). The calibration mixture was assayed to obtain the correct response factors for each specimen.

Analytical characteristics of the procedure

Detection limits. The detection limits for VLA were $3.0 \cdot 10^{-11}$ g/sec on channel A and $2.5 \cdot 10^{-11}$ g/sec on channel B, and for DOMA were obtained $2.0 \cdot 10^{-11}$ and $1.9 \cdot 10^{-11}$ g/sec, respectively. The detection limits were about $3.0 \cdot 10^{-10}$ g. The noise was $5 \cdot 10^{-14}$ A at $8 \cdot 10^{-12}$ A f.s., as shown in Figs. 3–5 and 8, for both channels. Calculations were made according to Deelder[8].

Linearity. We tested the linearity of the response; the results are shown in Table I. A linear equation giving the relationship between the peak-area ratio (y) and the concentration of a particular component (x) is reported, as well as the correlation coefficient, r.

A graphical representation of the linear response for HVA is shown in Fig. 6 as an example.

Reproducibility of retention times. Within 1 day there were no detectable differences in the retention times of a component. The average of 10 analyses over a period of 3 months gave the results shown in Fig. 7. All of the coefficients of variation were less than 1%. However, there is a significant difference between channels A and

TABLE I

LINEARITY CHECK OF THE PEAK-AREA RATIO WITH REGARD TO THE CONCENTRATION OF THE DERIVATIVES

No.	Compound	Dynamic range ($\mu mol/ml$)	Regression line	R	No. of standards
1	*p*-OHPAA	0.003 –0.3	$y = 8.14x - 0.03$	0.99991	7
2	Ve	0.003 –0.3	$y = 9.23x - 0.01$	0.99998	7
3	HVA	0.003 –0.3	$y = 8.08x - 0.04$	0.9997	7
4	DOPAC	0.003 –0.3	$y = 9.21x - 0.04$	0.9998	7
5	MHPG	0.0015–0.15	$y = 19.74x - 0.01$	0.99998	7
6	VMA	0.003 –0.3	$y = 9.10x - 0.06$	0.9995	7
7	DHPG	0.003 –0.3	$y = 11.32x - 0.01$	0.99992	7
8	DOMA	0.003 –0.3	$y = 10.24x - 0.07$	0.9996	7
9	VLA	0.003 –0.3	$y = 8.92x - 0.06$	0.9993	7
10	HIAA	0.003 –0.3	$y = 9.55x - 0.04$	0.99988	7

Fig. 4. Gas chromatogram of hippuric acid containing 2.8 μmol/ml dichloromethane, obtained by the entire analytical procedure but without acylase. Detector settings as in Fig. 1. The abbreviations indicate the retention times at which particular components are expected; thus, the interference with DOPAC is obvious.

Fig. 5. Gas chromatogram of hippuric acid; as in Fig. 4 but with the inclusion of acylase in the hydrolysis medium.

B. The cryogenic focusing procedure caused a four-fold worsening of the retention time reproducibility. It was still acceptable, in our opinion, for a period of 3 months. In addition, Fig. 7 shows that components with high boiling points are evidently less influenced by this cryogenic focusing procedure. Therefore, the carrier gas flow-rate in channel A recovered sufficiently fast after the holdup.

Reproducibility of concentrations in standard mixtures. No difference could be observed in reproducibility between channels A and B. The average reproducibility

TABLE II

URINARY CATECHOLAMINE METABOLITES AND HIAA LEVELS OF SEVEN PATIENTS IN MILLIMMOLES PER MOLE OF CREATININE

No.	Patient	Age (yrs)	p-OHPAA	VE	HVA	DOPAC	MHPG	VMA	DHPG	DOMA	VLA	HIAA
1	VA	20	7.7	2.5	1.7	1.3	0.3	1.1	4.1	—	—	3.5
2	KE	29	12.2	0.2	2.7	2.2	0.9	4.8	—	0.1	0.7	1.9
3	SP	30	10.9	1.5	2.5	3.2	0.6	3.1	—	1.8	0.9	3.4
4	SM	16	32.4	11.2	2.9	—	0.6	2.9	—	—	—	4.1
5	VO	64	7.4	—	2.3	0.5	3.3	3.1	—	0.7	1.1	4.9
6	VD	47	12.3	2.2	3.8	6.7	1.1	3.5	—	—	—	3.5
7	SA	51	8.1	1.7	1.8	2.1	0.8	2.9	—	—	0.4	1.6
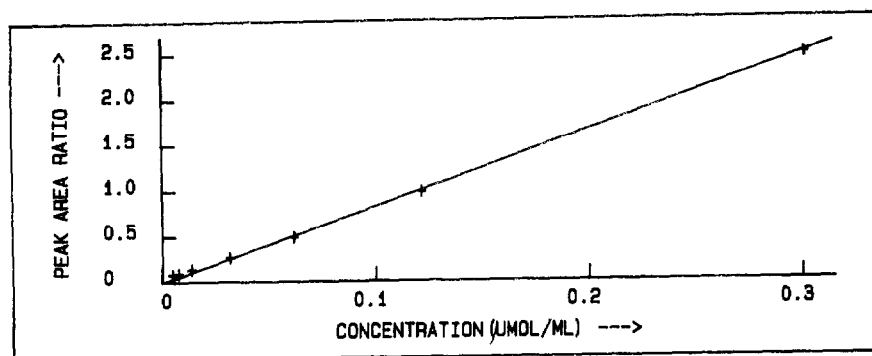
Fig. 6. The peak-area ratio with tetracosane as internal standard for different concentrations of HVA per millilitre of urine.
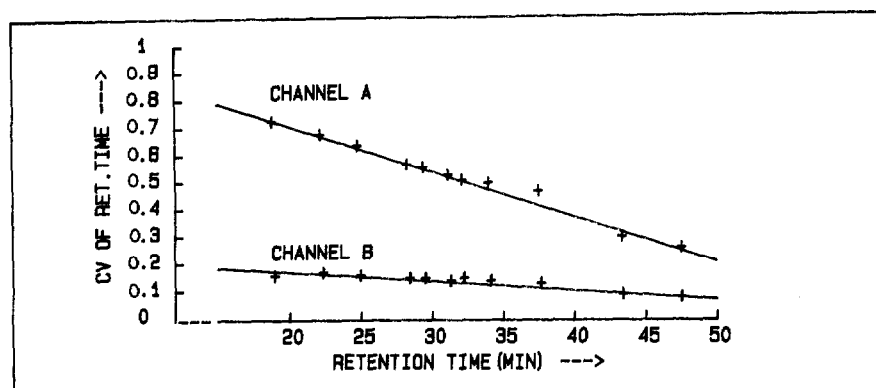

Fig. 7. Relationship between the retention time and the coefficient of variation (CV) of the retention time for both channels.

of the overall analysis, at a concentration of 0.06 μmol/ml, was 4.9% ($n = 20$). The relative standard deviation of duplicate measurements at the same level was 3.1% ($n = 20$).

Reproducibility of concentrations in urine specimens. The overall relative standard deviation was 30.4% ($n = 6$) at an average concentration of 0.0433 μmol/ml, with tetracosane as internal standard, while behenic acid gave 19.9% ($n = 10$) at a concentration of 0.056 μmol/ml level.

Urine specimens

The proposed procedure is not entirely selective for the compounds under study, and therefore one has to be aware of interferences all the time, especially with samples from patients under medication, subject to diets and various function tests. If confirmation of identification by mass spectrometry is not possible, it is necessary to spike the specimens with standards to ensure that the peak identification is correct. For instance, VE and DHPG were found to interfere frequently with unknown urinary components.

It is clear that there is no interference by vanillin, as mentioned in the spectrophotometric method for VMA by Addanki *et al.*[6] and requiring extremely strict
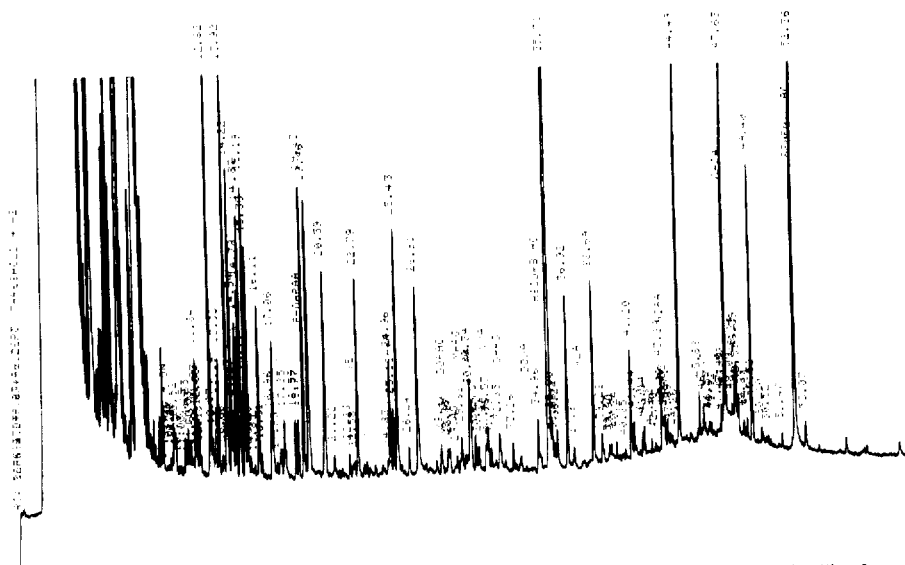
Fig. 8. Gas chromatogram of a normal urine; axes and conditions similar to those in Fig. 3.

diets. However, although there is no methodological interference, vanillin-containing foods can cause falsely elevated catecholamine metabolites or HIAA levels. This applies also to many drugs, which may cause either elevated or decreased catechol-amine levels. Fig. 8 shows a gas chromatogram of a normal urine, and Table II gives some creatinine-standardized results.

CONCLUSIONS

We have been able to separate nine catecholamine metabolites and HIAA by means of their trimethylsilyl derivatives by a quantitative procedure taking 1–2 days. The method yielded very clean chromatograms and did not suffer from interference from hippuric acid. We have been using this method routinely for 6 months, during which period the dual injection system proved to be extremely useful.

REFERENCES

1 S. K. Wadman, D. Ketting and P. A. Voûte, *Clin. Chim. Acta*, 72 (1976) 49–68.
2 F. A. J. Muskiet, D. C. Fremouw-Ottevangers, B. G. Wolthers and J. A. de Vries, *Clin. Chem.*, 23 (1977) 863–867.
3 M. Z. Jaffé, *Z. Physiol. Chem.*, 10 (1856) 391–400.
4 R. R. Sokal and F. J. Rohlf, *Biometry*, Freeman, San Francisco, CA, 1981, pp. 59.
5 F. A. J. Muskiet, M. C. Stratingh, G. J. Stob and B. G. Wolthers, *Clin. Chem.*, 27 (1981) 223–227.
6 S. Addanki, E. R. Hinnenkamp and J. F. Sotos, *Clin. Chem.*, 22 (1976) 310–314.
7 P. A. Biondi, G. Fedele, A. Motta and C. Secchi, *Clin. Chim. Acta*, 94 (1979) 155–161.
8 R. S. Deelder, *Instrumental Analysis, Part 2: Chromatography*, Technical University Press, Eindhoven, 1972, pp. 71–78 (in Dutch).