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Note

Method of measurement of urinary homovanillic acid and vanillylmandelic acid by gas chromatography–mass spectrometry suitable for neuroblastoma screening

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Although the outlook for clinically evident neuroblastoma is generally poor, presymptomatic detection of this, one of the commoner solid malignant tumours of early childhood, is associated with a favourable response to therapy. In over 92% of cases the presence of the tumour, which is derived from the primitive neural crest, is marked by an excessive urinary excretion of one or both of the catecholamine metabolites homovanillic (HVA, 3-methoxy-4-hydroxyphenylacetic acid) and vanillylmandelic acid (VMA, 3-methoxy-4-hydroxymandelic acid) [1]. In Japan, Sawada and co-workers [2–4] introduced population screening of six-month-old infants using a simple spot test for the qualitative detection of VMA only. They were able to identify, and successfully treat, a number of apparently healthy babies although their relatively crude method produced high false positive and false negative rates. Now that modern technology provides more effective analytical methods the screening of infant population is gaining general support [5].

A number of procedures for the quantitative estimation of HVA and VMA have been published. These have generally employed either gas chromatography with flame ionisation detection (GC–FID) [1,6] or high-performance liquid chromatography (HPLC) [7,8] both of which lack the specificity associated with gas

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chromatography–mass spectrometry (GC–MS), probably the most comprehensive and versatile instrumental method of analysis currently available [9]. Using MS detection the interfering peaks encountered with GC–FID [7] are eliminated, the need for additional confirmary methods obviated and the rate of sample analysis increased above that of either GC–FID or HPLC.

In this note we describe a GC–MS method capable of screening up to 144 urine samples per day for both HVA and VMA, employing a simplified organic extraction followed by rapid derivatization at room temperature.

EXPERIMENTAL

Materials

Highly absorbent filter paper, Schleicher & Schuell No. 903, was obtained from Anderman (Kingston upon Thames, U.K.), α-phenylhydracrylic acid (APHA), VMA and HVA from Sigma (Poole, U.K.) and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) from Pierce and Warriner (U.K.) (Chester, U.K.). Analytical-grade ethyl acetate, concentrated hydrochloric acid and sodium chloride were bought from BDH (Poole, U.K.).

Sample collection

Urine specimens were obtained from six-month-old infants by health visitors either at home or on a clinic visit. The urine sample was collected from freshly urine-soaked napkins (not faecally soiled) by placing a 9×11 cm piece of the absorbent filter paper inside the folded napkin and pressing down until saturated. The wet filter papers were then dispatched in self-sealing plastic bags to the laboratory where they were air-dried and stored at room temperature prior to analysis.

Sample preparation

A 27-cm^2 section cut from the dried filter paper was fanfolded to fit into a 16-ml polypropylene tube and the internal standard solution containing 10 μg of APHA was added. Internal standard was also added to 5 ml of the aqueous standard solution containing 1 μg/ml each of HVA and VMA. Each sample was eluted with 5 ml distilled water by shaking for 15 min on a mechanical shaker. The creatinine content of a 200-μl aliquot was determined by standard Technicon RA500 methodology.

Aqueous samples and standards were acified with 100 μl concentrated hydrochloric acid, saturated with 3 g sodium chloride and the acidic metabolites were extracted with 6 ml ethyl acetate by shaking for 15 min. After transfer to a second polypropylene tube, the organic phase was evaporated to dryness under a stream of nitrogen at 60 °C. The trimethylsilyl (TMS) derivatives were formed by the addition to the dried residue of 100 μl MSTFA at room temperature for at least 1 h.

GC–MS analysis

The GC system used was a Hewlett Packard 5890 fitted with a 7673A (100 place) autosampler, Hewlett Packard Ultra 2 capillary column (12.5 m $\times$ 0.2 mm I.D., 0.33 μm film thickness) and HP5970 mass-selective detector. Helium carrier

gas flow-rate was 1.0 ml/min (head pressure, 70 kPa). Split injections (ratio 70:1) were made using a 1-μl sample. A two-step temperature programme was run from 150 to 160°C at 5°C/min followed by 160 to 212°C at 20°C/min. A ballistic ramp to 270°C held for 4 min was used to clean the column prior to the next injection. The mass spectrometer, connected directly to the capillary column outlet, was operated at 70 eV. SIM was carried out for *m/z* 280 (APHA), *m/z* 209 and 326 (HVA) and *m/z* 297 (VMA) with dwell times of 10 ms to give a cycle time of 70 ms. Quantitation was on the basis of peak area for the ratios HVA/APHA and VMA/APHA.

The urinary HVA and VMA excretion were expressed as μg/mg of creatinine.

RESULTS AND DISCUSSION

Fig. 1 illustrates a GC–MS profile (SIM mode) of a typical urine specimen showing smooth, symmetrical peaks corresponding to APHA, HVA and VMA with no interferences due to extraneous substances. The retention times for these peaks were reproducible, with coefficients of variation of less than 0.06% for all peaks during the course of analysis of 75 samples.

The mass spectrum of the TMS derivative of HVA has three major fragments, *m/z* 326, 209 and 267, of which *m/z* 209 and 326 were selected for quantitation; VMA has a single fragment at *m/z* 297 and APHA a single fragment at *m/z* 280. The *m/z* 73 and 147 fragments, present in all TMS compounds, were ignored. The intra-assay imprecision for HVA improved from a coefficient of variation of 9.2% if one ion was used to 1.8% when the abundance of two ions were combined. Single ions were monitored for VMA (*m/z* 297) and APHA (*m/z* 280).

Peak purity was verified by repeated analysis of the sample in the full-scan mode using a run time of 5 min. In this mode the ratios of the ion intensities for HVA could be checked by monitoring *m/z* 267; this was found to have approximately 65% of the abundance of *m/z* 209 and 43% of the species with *m/z* 326. Similarly for VMA the relative abundances of *m/z* 73 could be compared with the

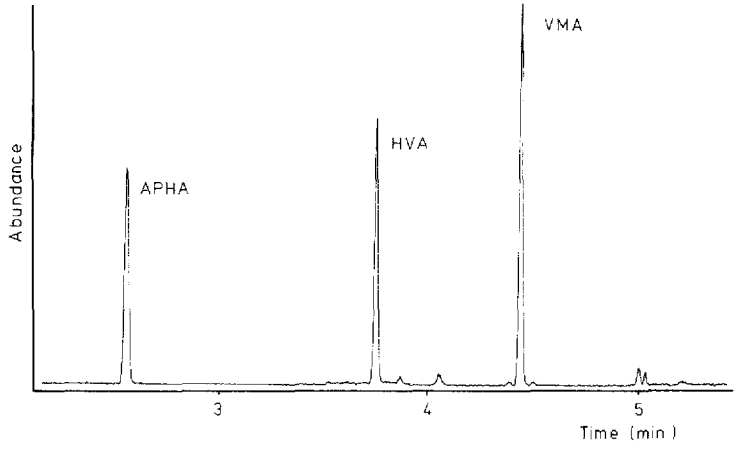


Fig. 1. GC–MS–SIM profile of typical urine sample. Note the absence of interfering peaks.

ion m/z 297 and the resulting ratios checked against the authentic standard. In addition, the spectra can be checked for the presence of additional fragments which might indicate interference from underlying peaks by comparison with the computer's in-built library of spectra.

The working range of the method was found to be linear for both HVA and VMA over the concentration range 10–4000 μg/l. Recoveries, determined by adding 500 μg each of HVA and VMA to four urine samples (concentration 578–1069 μg/l), were 100% for HVA and 101% for VMA. The imprecision of the method is shown in Table I.

Although dihydroxybenzoic acid (DBHA) has been used as an internal standard in the assay of HVA and VMA by GC methods, we have encountered a number of the urine samples that produced measurable DHBA interference. In some cases this was due to the excretion of DHBA itself; in other samples hippuric acid, or some other unidentified compound co-chromatographing with DHBA, produced minor ions in the mass-selective detector at m/z 193, 355 or 370 which could be misinterpreted by SIM mode analysis. We have not come across similar problems with APHA and therefore prefer the latter as the internal standard.

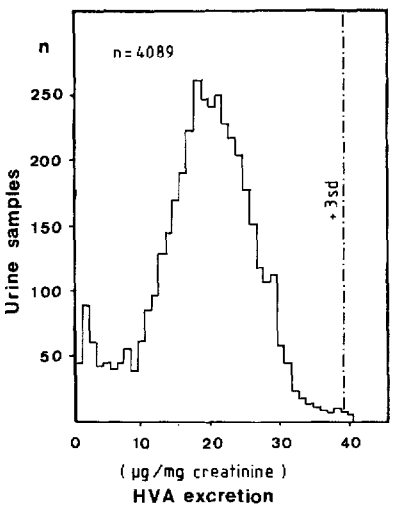
The use of capillary GC with split injection permits the short chromatographic separations essential for the routine analysis of the large number of samples encountered in population screening programmes. The metabolites under investigation are eluted from the column within 5 min (APHA 2.536 min, HVA 3.732 min and VMA 4.423 min). A complete analysis can then be carried out within 9 min, offering a real analysis rate of six specimens per hour. In terms of running costs, this increased analysis rate gives GC–MS an economic advantage over GC–FID.

The method is currently being used to screen infants born in four health districts of the Northern Region of England; the detailed results of these studies are to be published elsewhere. The measurement of the HVA and VMA excretion has so far been made in 4089 six-month-old infants; the values obtained for HVA and VMA have a normal distribution (Figs. 2 and 3), with a mean (± S.D.) excretion

TABLE I

INTER-ASSAY AND INTRA-ASSAY IMPRECISION FOR HVA AND VMA

Compound	Mean concentration (μg/mg of creatinine)	Coefficient of variation (%)	n
Intra-assay			
HVA	2.17	5.1	12
	9.17	0.9	9
VMA	2.18	4.1	12
	6.3	2.2	9
Inter-assay			
HVA	15.5	5.9	14
	6.5	8.7	13
VMA	17.3	10.0	14
	5.0	9.5	13



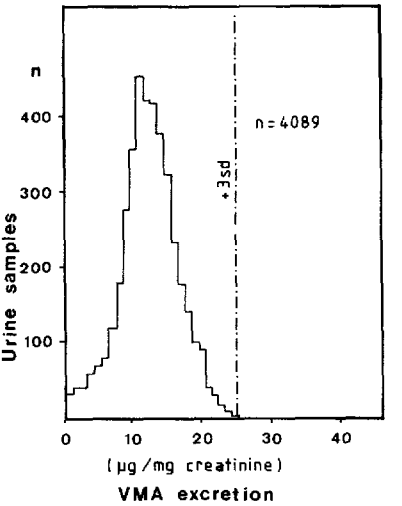


Fig. 2. Distribution of HVA excretion in 4089 infants aged six months. Mean value, 17.0 μg/mg of creatinine; S.D., 7.3 μg/mg of creatinine; the 3S.D. cut-off limit at 39.0 μg/mg of creatinine is indicated.

Fig. 3. Distribution of VMA excretion in six-month-old infants (mean, 11.4 μg/mg of creatinine; S.D., 4.5 μg/mg of creatinine). The 3S.D. cut-off limit at 25.0 μg/mg of creatinine is indicated.

for HVA of 17.0 $\pm$ 7.3 μg/mg of creatinine and for VMA of 11.4 $\pm$ 4.5 μg/mg of creatinine. Upper cut-off limits, equivalent to 3S.D. above the mean value, correspond to 39.0 μg/mg of creatinine for HVA and 25.0 μg/mg of creatinine for VMA.

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