Gas-Chromatographic Profiling of Urinary Acidic and Alcoholic Catecholamine Metabolites

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Urinary acidic and alcoholic catecholamine metabolites were isolated separately from 24-h urines by simple extraction procedures. After derivatization, extracts were analyzed by means of simultaneous gas chromatography on two different stationary phases. The quality of the assay was checked by running "normal" and enriched commercial (Hyland) control urines in each series. Normal values are given for vanilmandelic acid, homovanillic acid, and 3-methoxy-4-hydroxyphenylethylene glycol, expressed as a function of age. We present excretion patterns showing these metabolites and also 3,4-dihydroxyphenylacetic acid, vanillactic acid, 3,4-dihydroxyphenylethylene glycol, and vanilmandelol for 12 patients with neurogenic tumors. Values obtained for vanilmandelic acid by this method correlate well with those obtained by the electrophoretic/colorimetric method of Hermann [Am. J. Clin. Pathol. 41, 373 (1964)].

Additional Keyphrases: diagnostic aids · neural crest (neurogenic) tumors · cancer · electrophoresis · mass spectrometry · normal values · age-related values · pediatric chemistry · newborns

Measurement of urinary acidic and alcoholic catecholamine metabolites is invaluable in the diagnosis, followup, and prognosis of patients with tumors derived from the neural crest (1–5). There are reports of abnormally high urinary excretion of VMA3 1–6 and MHPG (1, 2, 5, 6), both of which are metabolites of norepinephrine and epinephrine, and HVA (1, 2, 4–6), a dopamine metabolite, associated with neuroblastoma, ganglioneuroblastoma, or ganglioneuroma. Increased urinary excretion of VMA (5, 6) and MHPG (5, 6) in patients with pheochromocytoma has been noted.

Many (7–18) methods have been described for purifying and quantitating these compounds, but few publications deal with the quantitative determination of more than two metabolites by one analytical method. Consequently, many different, complicated, and time-consuming techniques are necessary when one must measure more than two metabolites.

This paper describes the determination of several acidic and alcoholic catecholamine metabolites in an extract of acidified urine containing VMA, HVA, DOPAC, and VLA, and in a urinary extract obtained after enzymatic hydrolysis at pH 6.2, containing MHPG, DHPG, and VE. The extracts were analyzed by simultaneous gas chromatography on two different stationary phases.

Materials and Methods

Standards and Reagents

VMA, HVA, MHPG, DOPAC, VLA, DHPG, DOMA, and pOHPAA were purchased from Sigma Chemical Co., St. Louis, Mo. 63178; VE from Calbiochem, La Jolla, Calif. 92037; propylgallate from Koch Light, Colnbrook, Bucks, England; resorcinol from BDH Chemicals Ltd., Poole, England; Helix pomatia juice from l'Industrie Biologique Française, Gennevilliers, France; and ethyl acetate (gC-spectroscopic quality) from Baker Chemicals, Deventer, Holland. All other chemicals were "suprapur" or AR grade reagents from Merck, Darmstadt, Germany. Glass-distilled water was used throughout.

The concentration of standard solutions of the knowns was 0.1 g/liter of methanol, except for propylgallate (1 g/liter of methanol) and resorcinol (2 g/liter of water). All were stored at 4 °C and prepared every four months.

Samples

Twenty-four-hour urine specimens were acidified to pH 1 with concentrated hydrochloric acid as a preservative. Urines were either processed immediately or stored at −20 °C until analysis. Creatinine was measured according to Chasson et al. (19).

Aliquots of urine were processed separately, one for acidic, the other for alcoholic metabolites. We analyzed the processed samples by gas chromatography, injecting

Received Nov. 29, 1976; accepted Jan. 28, 1977.
each sample onto two different stationary phases simultaneously.

Procedures

*Extraction and derivatization of urinary acidic catecholamine metabolites.* To a 5-ml aliquot of centrifuged urine, add 0.1 ml of propylgallate solution and 0.5 ml of resorcinol solution. Adjust the pH to 1 with concentrated hydrochloric acid and saturate the urine with sodium chloride. Extract twice with 5 ml of ethyl acetate and separate the aqueous layer from the organic layer by centrifugation. Dry the combined ethyl acetate layers over anhydrous sodium sulfate and evaporate at 40 °C under a stream of nitrogen. Perform the whole procedure in 15-ml glass-stoppered tubes, extracting by vigorous mixing on a vortex-type mixer for 1 min. In each series include a blank, containing 5 ml of distilled water, a control urine (we used lot No. 521L002AA; Hyland Labs., Costa Mesa, Calif. 92626) containing 4.5 ± 0.9 mg of VMA per liter, as determined by the Hyland Laboratories by the method of Pisano et al. (20), and the same control urine enriched with 8.0 mg of each of the known compounds per liter. Silylate the dried extracts by adding 0.1 ml of bis(trimethylsilyl)trifluoroacetamide and heating for 10 min at 80 °C in a heating block.

*Hydrolysis, extraction, and derivatization of alcoholic catecholamine metabolites.* Enzymatically hydrolyze the sample by adjusting 3 ml of centrifuged urine to pH 6.2 with 4 mol/liter sodium hydroxide solution, adding 1 ml of sodium acetate buffer (2.5 mol/liter, pH 6.2), 0.5 ml of resorcinol solution, and two drops of chloroform, and incubating overnight (about 18 h) with 0.1 ml of the *Helix pomatia* preparation. In each series, treat 3 ml of distilled water (blank) and two 3-ml portions of the control urine as described above, except add the knowns to one aliquot of the control urine after hydrolysis. For the extraction and derivatization of alcoholic metabolites, follow the same procedure as described for acidic metabolites, except maintain the pH of the urine at 6.2.

*Gas chromatography, quantification, and mass spectrometry.* Gas-chromatographic analysis was performed with a Becker Model 420 instrument equipped with two 2-m coiled-glass columns (i.d., 2 mm) packed with 3% OV-1 and 3% OV-225, respectively, both on Supelcoport 80-100 mesh (Supelco Inc., Bellefonte, Pa. 16823), and dual flame-ionization detectors. The oven temperature was programmed from 110–220 °C at 2.5 °C/min, detector and injection temperature were set at 220 °C, and the nitrogen flow rate was 20 ml/min. Aliquots of 1–3 μl were injected simultaneously on both columns.

The different compounds in the chromatogram were quantitated by comparing their peak heights with the corresponding peak heights in the chromatogram of a standard, with propylgallate as an internal standard. Correction factors for differences in extraction recovery between propylgallate and the compounds measured were calculated by comparing the difference in calculated amounts of these compounds in the enriched control urine and the non-modified control urine with the actual amounts added.

Peak identity was checked by combined gas chromatography/mass spectrometry on a Varian MAT 112 gas chromatograph/mass spectrometer unit equipped with 2-m columns containing 3% OV-1 or 3% OV-225 (i.d., 1.2 mm). Ionization energy was 70 eV, slit separator temperature 260 °C, source temperature 250 °C, and helium flow rate 6 ml/min.

*Electrophoretic/colorimetric determination of VMA.* One hundred urine aliquots from normal individuals and patients with neural crest tumors were also taken for the determination of VMA according to the method described by Hermann (9).

Results and Discussion

Specificity of the Assay

Injecting the same sample on two different stationary phases generally reduces the risk of falsely high results for a metabolite, caused by interference of impurities.

Extracts of acidified urine especially give rise to complicated gas chromatograms (12, 21) that can be difficult to interpret quantitatively. However, the calculation of VMA and HVA peaks in the OV-225 gas chromatogram gave satisfactory results. The homogeneity of these peaks was confirmed by gas chromatography/mass spectrometry. VMA and HVA peaks in the OV-1 chromatogram showed interference by impurities.

DOPAC generally was measured with highest accuracy on OV-1 except for extracts containing large amounts of hippuric acid, in which case the DOPAC peak was overlapped completely.

VLA, detectable in some urines of patients with neurogenic tumors, was calculated on the basis of the OV-225 gas chromatogram, because this compound partly overlaps propylgallate in the OV-1 gas chromatogram under the conditions described above.

Unlike De Quattro et al. (22) and Messiha et al. (7), we were unable to detect DOMA excretion in the urine of healthy control persons or in the urine of patients with neurogenic tumors.

Determinations of pOHPAA, a diet-dependent tyrosine metabolite, gave comparable results on OV-1 and OV-225 chromatograms.

The calculations of the alcoholic catecholamine metabolites MHPG, DHPG, and VE gave similar results on OV-1 and OV-225 gas chromatograms. Mass spectra of the corresponding peaks showed their homogeneity. DHPG and VE were detectable only in the urine of patients with neurogenic tumors.

Analytical Recovery Studies

The extraction recovery of the knowns relative to the internal standard, as measured by enriching control urines with 8.0 mg/liter (see Methods), are shown in Table 1. As a consequence of the low extraction recovery
of DHPG, the necessary correction was made for the amounts measured in urine.

Correlation between VMA as Measured by Gas Chromatography and Electrophoresis

As a screening technique for detecting neural crest tumors, the electrophoretic/colorimetric procedure as described by Hermann (9) for urinary VMA was performed. VMA values obtained by the gas-chromatographic method for 100 persons were compared with those obtained by this technique (Figure 1). Results were divided into group I (0–25 μg/mg of creatinine) and group II (>25 μg/mg of creatinine). The correlation coefficients were 0.947 and 0.985; the equations of the regression lines were calculated as y = 0.89x − 0.56 and y = 1.03x + 0.45, respectively, y being the present technique in each case. The somewhat higher amounts found for the electrophoretic/colorimetric method in group I, where peak-background ratio is relatively low, could be due to interference from extraneous material as already suggested by Wilk et al. (23).

Normal Values

Normal values of the main urinary catecholamine metabolites VMA, HVA, and MHPG were determined for healthy control persons ranging in age from infants to 71 years, and expressed in μg/mg of creatinine. Because of the increase in excretion of creatinine during the first years of life, the results of these measurements are expressed as a function of age as can be seen in Figure 2. For each metabolite a typically normal excretion as a function of age is obtained as outlined by Applegarth et al. (24). The curves reach a constant level after the thirteenth year of life. Newborns and infants in the first months of postnatal life showed a tendency to excrete more MHPG rather than VMA, the quantitatively most important metabolite of norepinephrine and epinephrine at later age. We saw no sex related difference in amounts excreted. Excretion values for adults were in accord with those reported by Wilk et al. (23) for VMA, Roginsky et al. (14) and Stott et al. (11) for HVA, and Fellows et al. (15) and Kahane et al. (10) for MHPG.

Neuroblastoma, Ganglioneuroblastoma, Ganglioneuroma, and Pheochromocytoma

Gas chromatograms of urinary acidic and alcoholic catecholamine metabolites of a patient with neuro-

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Table 1. Extraction Recoveries Relative to the Internal Standard (Mean ± 1 SD)

<table>
<thead>
<tr>
<th>Acidic metabolites</th>
<th>Alcoholic metabolites</th>
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<tbody>
<tr>
<td>VMA 85.6 ± 8.5%</td>
<td>MHPG 80.8 ± 13.6%</td>
</tr>
<tr>
<td>HVA 101.9 ± 9.7%</td>
<td>DHPG 37.8 ± 6.9%</td>
</tr>
<tr>
<td>DOPAC 103.8 ± 6.4%</td>
<td>VE 117.6 ± 10.4%</td>
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<tr>
<td>VLA 94.5 ± 7.6%</td>
<td></td>
</tr>
<tr>
<td>DOMA 23.9 ± 2.7%</td>
<td></td>
</tr>
<tr>
<td>pOHPPAA 94.8 ± 9.7%</td>
<td></td>
</tr>
</tbody>
</table>

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Fig. 1. Correlation between vanillmandelic acid (VMA) values obtained by the gas-chromatographic (GLC) method described above and those obtained by the electrophoretic/colorimetric (ELECTR.) method of Hermann (9)

n, no. measurements; cc, correlation coefficient

Fig. 2. Normal urinary excretion values of vanillmandelic acid (VMA), homovanillic acid (HVA), and 3-methoxy-4-hydroxyphenylethylene glycol (MHPG), expressed in μg/mg of creatinine (CREAT) as a function of age

Fig. 3. Gas chromatograms showing urinary acidic (A), and alcoholic (B) catecholamine metabolites excreted by a patient with neuroblastoma

Flame-ionization detector (FID); VMA, vanillmandelic acid; HVA, homovanillic acid; MHPG, 3-methoxy-4-hydroxyphenylethylene glycol; DOMA, 3,4-dihydroxyphenylethylene glycol; VE, vanillyl alcohol; pOHPPAA, p-hydroxyphenylacetic acid; PG, propylgallate; R, resorcinol
Table 2. Urinary Excretion Patterns for 12 Patients with Neurogenic Tumors

<table>
<thead>
<tr>
<th>No.</th>
<th>Diagnosis</th>
<th>Age</th>
<th>Sex</th>
<th>VMA</th>
<th>HVA</th>
<th>DOPAC</th>
<th>VLA</th>
<th>pOHPPA</th>
<th>NMHP</th>
<th>DHHP</th>
<th>VE</th>
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<tr>
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<td>F</td>
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<td>nm</td>
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<td>19 y</td>
<td>M</td>
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<td>6.7</td>
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<td>nm</td>
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<td>0.2</td>
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<tr>
<td>11</td>
<td>PH*</td>
<td>46 y</td>
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</table>

* Patients of the Department of Internal Medicine, University Hospital, Groningen.

NB, neuroblastoma; GNB, ganglioneuroblastoma; GN, ganglioneuroma; PH, pheochromocytoma; y, years; m, months; F, female; M, male; nm, not measurable; nd, not determined.

We thank the Central Laboratory, Division of Internal Medicine (Head Drs. E. W. Kwarta) for the electrophotographic/colorimetric determinations of VMA, Mr. J. van der Meulen for the mass spectral analysis, Ir. H. J. G. M. Derks for his advice, and Dr. A. Groen for his encouragement.

References


