"Total" Acidic Metabolites of Catecholamines in Urine as Determined by Hydrolysis with Hydriodic Acid and Liquid Chromatography: Application to Patients with Neuroblastoma and Melanoma

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We describe a method for determining the "total" excretion of acidic metabolites of catecholamines by measuring 3,4-dihydroxyphenylacetic acid (DOPAC) formed by hydriodic acid hydrolysis of 4-hydroxy-3-methoxyphenylacetic acid (HVA), 4-hydroxy-3-methoxymandelic acid (VMA), and their conjugates. The DOPAC in the diluted hydrolysate is measured directly by liquid chromatography with electrochemical detection. Normal values, expressed in relation to excretion of creatinine, vary as a function of age. For healthy subjects, the mean DOPAC value after hydrolysis was 1.15 times that for unconjugated HVA, VMA, and DOPAC combined. Preliminary results for patients with neuroblastoma and melanoma indicate the potential usefulness of the method for diagnosis and prognosis of patients with neural crest tumors that produce dopa or catecholamines.

Additional Keyphrases: vanillylmandelic acid ∙ homovanillic acid ∙ electrochemical detection ∙ neural crest tumors ∙ age-related effects ∙ reference interval ∙ screening

Catecholamines are metabolized and excreted as phenylethanol acetic acids and their conjugates (1). 4-Hydroxy-3-methoxyphenylacetic acid (HVA) and 4-hydroxy-3-methoxymandelic acid (VMA) are the major acidic metabolites in urine, formed from dopamine and from norepinephrine + epinephrine, respectively (Figure 1). Determination of HVA and VMA in urine aids diagnosis of malignant tumors arising from cells of the neural crest such as neuroblastoma (2−4), pheochromocytoma (2, 4, 5), and melanoma (6, 7), which possess a common metabolic pathway for conversion of tyrosine to dopa. However, determination of HVA and VMA requires lengthy chromatographic analysis (8) or extraction of the analytes (9).

In addition to HVA and VMA, minor metabolites of catecholamines are excreted in urine: 3,4-dihydroxyphenylacetic acid (DOPAC) (1), 3,4-dihydroxymandelic acid (DOMA) (1, 2), and the 4-O-methylated metabolites, iso-HVA and iso-VMA (1, 10). These acidic metabolites are also present as sulfate and glucuronate conjugates (11). Thus, the urinary excretion of HVA and VMA may be affected by the activities of catecholamine-metabolizing enzymes. The "total" excretion of acidic metabolites of catecholamines might thus reflect well the overall turnover of catecholamines and be of clinical value in screening for diseases affecting catecholamine production.

The ether bond of methoxy group is cleaved by heating with concentrated hydriodic acid (HI); this also reduces the alcoholic hydrazyl group to hydrogen. By combining these two reactions, the acidic metabolites of catecholamines (HVA, VMA, and DOMA) can be converted to a single compound, DOPAC (Figure 1). We describe here a simple, rapid method for determining the "total" excretion of acidic metabolites of catecholamines by use of hydrolysis with HI and liquid chromatography. We used the method to obtain reference values for adults and children of various age groups. We also present preliminary results on neuroblastoma and melanoma patients, obtained to assess the feasibility of the method.

Materials and Methods

Apparatus. We used a Model L-2000 chromatograph (Yanaco, Kyoto, Japan) equipped with a 250 mm × 4.6 mm (i.d.) C18 reversed-phase column (Yanapak ODS-A; average particle size, 7 μm). The detector was a Yanaco VMD-101 electrochemical detector.

Reagents. DOPAC, HVA, VMA, dopa, and dopamine were purchased from Sigma Chemical Co., St. Louis, MO, and concentrated HI (870 g/kg) was from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Other chemicals were of analytical grade. 5-S-Cysteinyl-dopa was prepared according to the method of Ito et al. (12). "Ultrapure" water prepared by use of the "Milli-Q" system (Millipore, Bedford, MA) was used throughout this study.

We prepared a stock standard solution of DOPAC (100 μg/mL) in 0.1 mol/L HCl and stored it at −30 °C. A working 10 μg/mL standard solution was prepared just before use by diluting 0.5 mL of the stock solution to 5.0 mL with 0.4 mol/L HCl.

Urine specimens. From normal adults and melanoma patients we collected 24-h urine specimens in bottles containing, as preservative, 50 mL of acetic acid and 1 g of sodium metabisulfite (13). Untimed urine specimens from healthy children and patients with neuroblastoma were adjusted to pH 1 with 6 mol/L HCl. All specimens were stored at −30 °C until analysis.

Fig. 1. Metabolism of catecholamines and chemical conversion of HVA, VMA, and DOMA to DOPAC

Thin arrows represent the metabolic pathway of catecholamines; thick arrows represent the chemical conversion of HVA, VMA, and DOMA to a single compound, DOPAC, by reaction with HI.

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3 Nonstandard abbreviations: HVA, 4-hydroxy-3-methoxyphenylacetic acid; VMA, 4-hydroxy-3-methoxymandelic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; DOMA, 3,4-dihydroxymandelic acid.

Received March 4, 1985; accepted April 25, 1985.
HI hydrolysis and chromatographic analysis. To a screw-capped tube, add 500 µL of urine, 20 µL of concentrated (500 g/kg) H₃PO₄, and 500 µL of concentrated HI. Heat in an oil bath at 140 °C for 45 min, cool, then add 4.0 mL of 0.4 mol/L HClO₄. Centrifuge, and inject 10 µL of the diluted hydrolysate directly into the chromatograph.

The chromatographic conditions are as follows: mobile phase, 950 mL of potassium phosphate buffer (0.2 mol/L, pH 2.3, containing 0.1 mmol of Na₂EDTA per liter) plus 50 mL of acetonitrile; column temperature, 45 °C; flow rate, 1.0 mL/min; detector potential, +600 mV. Calculate results by comparing the height of the DOPAC peak with that for the working standard solution. We injected an aliquot of the standard solution after every fifth sample.

Comparison procedures. Urinary HVA and VMA were determined by the method of Fujita et al. (8) at an oxidation potential of +650 mV vs an Ag/AgCl reference electrode. Free DOPAC in urine was determined under the following chromatographic conditions: mobile phase, same as above except at pH 3.0; column temperature, 50 °C; flow rate, 0.7 mL/min; detector potential, +600 mV. We determined urinary dopa, dopamine, and 5-S-cysteinyldopa by the method of Ito et al. (13).

Results

Hydrolysis with HI

As Figure 2 shows, HI hydrolysis of a 100 µg/mL standard solution at 140 °C for 45 min converted 100% of the HVA and 98% of the VMA to DOPAC. After 45 min, no trace of HVA remained in either reaction mixture. In the HI hydrolysis at 140 °C of urine samples from five normal subjects and a patient with neuroblastoma, the DOPAC values were greatest at 30 to 45 min, and had decreased slightly by 60 min (Figure 3). We therefore hydrolyzed for 45 min.

Chromatography

We used an oxidation potential of +600 mV for greater selectivity and to reduce the interference from HI, which appears as a broad peak at the elution front. Figure 4 shows chromatograms of hydrolyzed urine samples from normal subjects and from patients with neuroblastoma or metastatic melanoma. The DOPAC peak appears at about 12 min, and no large peaks appear thereafter in the chromatograms for normal subjects. Thus, samples can be injected every 15 min.

Fig. 3. Time course of HI hydrolysis of urine
A, DOPAC after hydrolysis with HI at 140 °C of urine samples from normal adults and infants; A, HVA or VMA after HI hydrolysis at 140 °C in urine samples from patients with neuroblastoma; read the latter results from the right-hand y-axis (0 to 2000 µmol/L)

Fig. 4. Chromatograms of urine samples from (A) a normal infant, (B) a normal adult, and patients with (C) neuroblastoma or (D) metastatic melanoma
DOPAC values after HI hydrolysis: (A), 19 µg/mL (28 nmol/mol of creatinine); (B), 10 µg/mL (6.0 nmol/mol of creatinine); (C), 275 µg/mL (217 nmol/mol of creatinine); (D), 39 µg/mL (48 nmol/mol of creatinine)

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Analytical Variables

Linearity. Peak height was linearly related to DOPAC concentration from 5 to 1000 µg/mL, a range that covers the DOPAC concentrations in all the urine samples we examined.

Precision. Within-run precision was evaluated by analyzing 10 aliquots of a urine sample, day-to-day precision by analyzing aliquots of another urine sample stored at −30°C during the study. For a mean DOPAC concentration of 54.6 (SD 1.1) µmol/L, the within-run CV was 2.0% (n = 10). The day-to-day CV, for a mean concentration of 34.4 (SD 2.4) µmol/L, was 6.4% (n = 10).

Analytical Recovery. The analytical recoveries after HI hydrolysis of DOPAC, HVA, and VMA added to a urine sample to give a concentration of 100 µg per milliliter of urine were 100.7 (SD 0.5), 96.8 (SD 0.4), and 95.2 (SD 2.6%), respectively (n = 5 each).

Stability. We evaluated the stability of DOPAC in the diluted hydrolysate by analyzing three hydrolysates that had been stored at room temperature for three days. The value obtained was 99.2 (SD 2.0)% of the original concentration.

Clinical Evaluation

Reference Values. Table 1 shows the age-related urinary excretion of DOPAC after HI hydrolysis (HI-DOPAC), in comparison with those of unconjugated HVA, VMA, and DOPAC. When expressed as millimole per mole of creatinine, the HI-DOPAC excretion decreased with age until adulthood, as has been reported for HVA and VMA excretion (14). The age-related HI-DOPAC values were 1.08 to 1.22 (mean 1.15) times greater than those of the sum of HVA, VMA, and DOPAC ("total"). Among 72 normal subjects we examined, the highest ratio for HI-DOPAC/total was 1.57; in only four did the ratio exceed 1.50; the lowest ratio was 0.89, with eight ratios being <1.00.

Neuroblastoma. Table 2 shows data on the urinary excretion of acidic metabolites of catecholamines by four patients with neuroblastoma. All had increased excretion of HI-DOPAC as well as increased HVA and VMA, as compared with the reference values in Table 1. One patient (no. 4) had an abnormally high ratio for HI-DOPAC/total.

Melanoma. Table 3 shows data on the urinary excretion of acidic metabolites of catecholamines and dopamine-related metabolites by melanoma patients with and without metastatic lesions in stage I and IV. In all the patients without metastatic lesions, the urinary excretion of HI-DOPAC was normal, as was 5-S-cysteinyl-dopa. For all four patients with metastatic lesions, however, values for 5-S-cysteinyl-dopa exceeded the upper limit of normal (0.11 mmol/mol creatinine, n = 19; unpublished data). Of these four patients, three excreted increased amounts of both HI-DOPAC and HVA. One patient (no. 7) had an abnormally high ratio for HI-DOPAC/total.

Discussion

When heated with HI, HVA is converted quantitatively to DOPAC, and VMA is also quantitatively converted to DOPAC via HVA. Taking advantage of this reaction, we developed a method to determine the "total" amount of urinary acidic metabolites of catecholamines as DOPAC. The method is simple and rapid: as many as 25 samples can be analyzed within a working day without the use of an autosampler. The precision and accuracy of the method also sufficed for clinical use. The preliminary results on neuroblastoma and melanoma patients exemplify the potential usefulness of the present method.

The value for HI-DOPAC was 1.15 times that for unconjugated HVA, VMA, and DOPAC combined. Most of the difference may be ascribed to the conjugation of these metabolites. Musket and Green (15) reported that 12% of HVA and 33% of DOPAC are conjugated, whereas VMA is excreted only as the free compound. Moreover, besides HVA and VMA, several minor metabolites may give rise to DOPAC by HI hydrolysis, e.g., D OMA, iso-HVA, and iso-VMA. According to Musket et al. (17), HI-DOPAC, and iso-HVA account for 90% of the overall turnover of dopamine, and VMA, DOMA, and iso-VMA account for 48% of the overall turnover of norepinephrine + epinephrine. Therefore, the HI-DOPAC value should account for 70% or more of the total catecholamine produced.

LaBrosse et al. (2) and Soldin and Hill (4) find HVA and VMA assays to be almost equally effective in detecting neuroblastoma; thus, determination of DOPAC after hydrolysis with HI appears to be as useful as the separate determination of HVA and VMA. A potential disadvantage of our method, however, may be that it cannot be used to estimate the HVA/VMA ratio in particular or the ratio of dopamine metabolites to norepinephrine + epinephrine metabolites in general, data that may have a prognostic value in neuroblastoma (2). Nevertheless, the simplicity and rapidity of the present method warrant further studies as a screening method for neuroblastoma.

In melanocytes tyrosine is oxidized by tyrosinase to dopa and then to dopaquinone. In a complex series of nonenzymic reactions, dopaquinone is converted to the black pigment, eumelanin (16). In the presence of cysteine or glutathione, dopaquinone reacts rapidly to form cysteinyl-dopas, among which 5-S-cysteinyl-dopa is the major isomer. Further oxidation of cysteinyl-dopas give rise to a reddish pigment, pheomelanin (16). Some of these melanin precursors, dopa
and 5-S-cysteinyl-dopa, are secreted into the blood and metabolized. At present, urinary 5-S-cysteinyl-dopa, because of its resistance to decarboxylation and conjugation (17), may be one of the best biochemical markers for the diagnosis and prognosis of melanoma in advanced stages (7).

On the other hand, dopa secreted from melanoma cells may be rapidly decarboxylated to dopamine, which in turn is metabolized to DOPAC, HVA, and their conjugates. Goodall and Alton (18) showed that after infusion of [3-14C]L-dopa in humans, 32% of the radioactivity was recovered as HVA (18%), DOPAC (8.6%), VMA (0.7%), and their conjugates. We found an increased excretion of HI-DOPAC as well as of HVA in three of four patients with metastatic lesions. Trapeznikov et al. (19) and Morgan et al. (20) demonstrated higher concentrations of HVA in urine of melanoma patients with advanced metastasis.

Although our data confirm the usefulness of urinary 5-S-cysteinyl-dopa as a marker of metastatic melanoma, in one case (no. 6 in Table 3) 5-S-cysteinyl-dopa excretion was marginally high, but HI-DOPAC excretion was eightfold that of the normal subjects. This may further support the usefulness of the present method with melanoma patients who excrete predominantly dopa metabolites rather than 5-S-cysteinyl-dopa.

Finally, the abnormally high HI-DOPAC/total ratios in two patients (no. 4 in Table 2 and no. 7 in Table 3) suggest abnormally high ratios of conjugation. In such cases, the HI-DOPAC value should better reflect the catecholamine turnover than would the value for unconjugated HVA or VMA.

### Table 3. Urinary Excretion of Catecholamine Acidic Metabolites and Dopa-Related Metabolites by Patients with Melanoma

<table>
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<tr>
<th>Subjects</th>
<th>HI-DOPAC</th>
<th>Total*</th>
<th>HVA</th>
<th>VMA</th>
<th>DOPAC</th>
<th>Dopa</th>
<th>Dopamine</th>
<th>S-S-CD*</th>
<th>HI-DOPAC/total</th>
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<td><strong>Without metastatic lesions</strong></td>
<td></td>
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<td></td>
<td></td>
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<td>0.15</td>
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<td>1.8</td>
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<td>5.8</td>
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<td>ND*</td>
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*The sum of unconjugated HVA, VMA, and DOPAC. 5-S-Cysteinyl-dopa. *All at stage I. †All at stage IV. ‡Not determined.

References