Normal Reference Intervals for Free Catecholamines and Their Acid
Metabolites in 24-h Urines from Children, as Determined by Liquid
Chromatography with Amperometric Detection

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We used two methods of reversed-phase liquid chromatography
with amperometric detection, one for assay of unconjuga-
tated norepinephrine, epinephrine, and dopamine in urine, the
other for determination of unconjugated vanillylmandelic
acid and homovanillic acid. The catecholamines were ex-
tracted from urine by passage through a weak-cation ex-
change resin, then through alumina. Vanillylmandelic acid
and homovanillic acid were isolated from urine by a two-step
solvent-extraction procedure. The between-day CV was 5% for
norepinephrine, 3% for epinephrine, 3% for dopamine, 4.5% for
vanillylmandelic acid, and 6.4% for homovanillic acid. Reference ranges for these analytes in 76 children,
ages 3–16 years, are reported.

Additional Keyphrases: reversed-phase chromatography · nor-
epinephrine · epinephrine · dopamine · vanillylmandelic
acid · homovanillic acid · neural crest tumors · pediatric
clinical chemistry

Measurement of urinary catecholamine metabolites—
vanillylmandelic acid (VMA), homovanillic acid (HVA), or
metanephrines—has first been used for diagnosis of neural
crest tumors, because the metabolites are excreted in larger
quantity than are the catecholamines.1 However, determina-
tion of only one of these metabolites lacks diagnostic speci-
ficity. It appears now that determination not only of the
catecholamines but also of one or more of their metabolites
is necessary for diagnosing these tumors. Liquid chromato-
graphy (LC) with amperometric detection has proved to be
a useful tool in this regard (1–3). Reference data for the
urinary excretion of the catecholamines and their meta-
bolites by adults have been widely published, but only a few
authors (4–9) have reported the normal values for children.
Moreover, these data are not directly usable because of
differences in the sample collection (untimed urine specimen
or 24-h urine collection) and in the expression of results (per
gram of creatinine or per 24 h).

Here we describe an LC procedure with amperometric
detection for urinary free catecholamines, VMA, and HVA.
We have used these methods to determine normal values for
children.

Materials and Methods

Apparatus. The LC-system included a Model 870 pump and
a column compartment (Du Pont Instruments, Wil-
mington, DE 19898), a wisp 710 B autosampler (Waters
Associates, Milford, MA 01757), a Vista 402 chromatogra-
phy data system (Varian, Walnut Creek, CA 94596), and a
Model LC4B/17 dual-amperometric detector (Bioanalytical
Systems, West Lafayette, IN 47906).

The analytical column, a 4.6 × 250 mm reversed-phase
column, packed with Spherisorb 5 octadecylsilane (Phase
Sep, Queensferry, Great Britain) was protected by a small
4.6 × 50 mm precolumn packed with 30- to 40-μm particles
of Permporphase C18 (Du Pont Instruments).

Reagents. Norepinephrine (NE), epinephrine (E), dopa-
mine (DA), VMA, and HVA were obtained from Sigma
Chemical Co., St. Louis, MO 63178. 3,4-Dihydroxybenz-
ylamine (DHBA) was purchased from Aldrich Chemical Co.,
Milwaukee, WI 53233. Cation-exchange resin (Amberlite
CG-50, type I, 100–200 mesh) was from Prolabo, Paris
75011, France. Heptane sulfonate was from Eastman
Organic Chemicals, Rochester, NY 14650. Alumina (neutral
activity, grade I; Woelm, Eschwege, F.R.G.) was prepared
as recommended by Anton and Sayre (10) and stored in a
desiccator. Acetonitrile, "HPLC" grade, was purchased from
Carlo-Erba, Milano, Italy. All other chemicals were of
analytical grade and were used without further pretreat-
ment.

Catecholamine stock solutions were prepared separately
at a concentration of 100 mg/L in 10 mmol/L HCl. A
working standard mixture containing, per liter, 2.5 mg of
NE, 0.5 mg of E, and 25 mg of DA was made in 10 mmol/L
HCl. DHBA, used as internal standard for catecholamine
analysis, was prepared at a concentration of 2.5 mg/L in
10 mmol/L HCl. VMA and HVA standard solutions were
prepared separately at a concentration of 200 mg/L in 10
mmol/L HCl. All were stable at 4 °C for at least one
month.

Urine samples. Twenty-four-hour urine specimens were
collected in polyethylene bottles with 5 mL (for children
younger than five years) or 10 mL (for children older than
five years) of concentrated hydrochloric acid as preservative.
Aliquots were stored at 4 °C for as long as two weeks.

Assay of urinary catecholamines. To 1 mL of urine ad-
20 μL of 10 g/L sodium metabisulfite solution, 100 μL of 50 g/L
EDTA solution, 50 μL of 2.5 mg/L DHBA solution, and 500
μL of 0.1 mol/L phosphate buffer, pH 6.5. Using a pH meter,
adjust the pH to 6.5 ± 0.2 with 1 mol/L NaOH. Transfer
the mixture to a 2.2-mL propylene conical tube containing
500 μL of the Amberlite resin. Cap and agitate for 2 min in
a reciprocal shaker. Allow the resin to settle, then aspirate
the liquid. Wash the resin with 1.4 mL of distilled water.
Remove the wash by aspiration. Wash the resin once again.
To elute the catecholamines, add 1 mL of mol/L HCl, shake
for 2 min, centrifuge, and transfer the supernatant liquid in
a 1.5-mL polypropylene conical tube containing 20 μL of 10
g/L sodium metabisulfite solution, 100 μL of 50 g/L EDTA
solution, and 250 μL of 3 mol/L Tris buffer, pH 8.4. Titrate
the mixture to pH 8.4 ± 0.2. Add 50 mg of alumina and
shake the tube for 5 min. Aspirate and discard the super-
natant fluid. Wash the alumina twice with 1.4-mL portions
of water. Aspirate the wash and add 250 μL of 1 mol/L acetic

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1 Nonstandard abbreviations: VMA, vanillylmandelic acid; HVA,
homovanillic acid; LC, liquid chromatography; NE, norepinephrine;
E, epinephrine; DA, dopamine; and DHBA, 3,4-dihydroxybenzyl-
amine.
Acid. Agitate the tube for 5 min, then centrifuge. Transfer the supernatant liquid to an autosampler vial. Inject 40 μL into the LC system. If the analysis cannot be performed promptly, store samples at 4 °C, at which temperature they are stable for at least 24 h.

The catecholamines were quantified by the peak-height ratio method, with DHBA as internal standard. The mobile phase (pH 2.5) contained, per liter, 0.1 mol of potassium dihydrogen phosphate, 0.05 mol of phosphoric acid, 0.3 mmol of EDTA, and 1 mmol of heptane sulfonate. The flow rate was 1.1 mL/min and the column temperature was 35 °C. The dual-amperometric detector was operated in series mode at 0.75 and 0.075 V vs a Ag/AgCl reference electrode.

Assay of urinary VMA and HVA. In a 1.5-mL polypropylene conical tube, mix 50 μL of urine, 50 μL of 2 mol/L HCl, and 400 μL of a mixture of ethylacetate/dichloromethane (8/2, by vol). Cap and agitate for 5 min, then briefly centrifuge. Transfer 200 μL of the organic phase to a new tube. Add 500 μL of 0.1 mol/L potassium phosphate solution containing 0.4 g of EDTA per liter. Shake for 10 min, then briefly centrifuge. Remove the organic phase by aspiration. Evaporate the remaining organic solvent by holding the open tube for 5 min at 30 °C under nitrogen. Centrifuge once again and transfer to an autosampler vial. Inject 20 μL into the LC system. VMA and HVA were quantified by comparing peak heights with those of calibration samples according to the external standard method.

The mobile phase was the same as that used for the catecholamines assay plus 30 mL of acetonitrile per liter. The flow rate was 1.8 mL/min. The column temperature was 40 °C and the dual-amperometric detector was operated in series mode at 0.8 and 0.6 V.

Results and Discussion

LC profiles of urinary catecholamines and their acid metabolites from a healthy child are presented in Figures 1 and 2, respectively. The overall absolute analytical recovery was 43% for NE, 41% for E, 41% for DA, and 43% for DHBA. The within-run CV (n = 12) was 1.1% for NE, 1.9% for E, and 0.8% for DA. The between-day CV (n = 6) was 5% for NE, 3% for E, and 3% for DA. The detection limit was 1 μg/L for E, and linearity was good up to 500 μg/L. The overall absolute analytical recovery was observed to be 73% for VMA and 55% for HVA. The within-run CV (n = 12) was 7.5% for VMA and 3.6% for HVA. The between-day CV (n = 6) was 4.5% for VMA and 6.4% for HVA. The detection limit was 0.25 mg/L for VMA and 0.5 mg/L for HVA. The linearity was good up to 20 mg/L.

Urinary excretion of NE, E, DA, VMA, and HVA was determined for 24-h urine specimens from 76 children, ages 3–16 years. The children were healthy and under normal conditions of their daily life. Measurement of creatinine excretion was used to verify that 24-h urine collection was complete.

Fig. 1. Chromatograms of urinary catecholamines obtained for a normal child (NE = 49 μg/L, E = 8 μg/L, DA = 348 μg/L, UA = undetectable). IS = internal standard (3,4-dihydroxybenzylamine)

Fig. 2. Chromatograms of an acid extract of a urine sample from a normal child (VMA = 1.7 mg/L, HVA = 1.8 mg/L, 5 HIAA = 5-hydroxyindole-3-acetic acid)
Table 1. Reference Intervals for Free Catecholamines and Their Acid Metabolites in Urine of Normal Children

<table>
<thead>
<tr>
<th>Age range, yr</th>
<th>3–6</th>
<th>9–10</th>
<th>10–16</th>
</tr>
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<tbody>
<tr>
<td>N*</td>
<td>35</td>
<td>17</td>
<td>24</td>
</tr>
<tr>
<td>NA, µg/24 h</td>
<td>12.7 ± 4.7</td>
<td>20.5 ± 5.7</td>
<td>32.6 ± 6.9</td>
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<tr>
<td></td>
<td>(5.3 – 26.0) b</td>
<td>(11.1 – 32.6)</td>
<td>(15.2 – 46.0)</td>
</tr>
<tr>
<td>µg/g creat.</td>
<td>49.1 ± 16.4</td>
<td>46.0 ± 12.9</td>
<td>41.1 ± 9.3</td>
</tr>
<tr>
<td></td>
<td>(20.7 – 84.4)</td>
<td>(26.7 – 69.3)</td>
<td>(29.0 – 60.5)</td>
</tr>
<tr>
<td>A, µg/24 h</td>
<td>2.4 ± 0.9</td>
<td>4.1 ± 2.2</td>
<td>4.1 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>(0.9 – 5.0)</td>
<td>(2.0 – 9.8)</td>
<td>(1.8 – 9.4)</td>
</tr>
<tr>
<td>µg/g creat.</td>
<td>3.2 ± 3.5</td>
<td>8.0 ± 3.5</td>
<td>6.5 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>(4.1 – 18.3)</td>
<td>(4.1 – 16.6)</td>
<td>(1.8 – 12.2)</td>
</tr>
<tr>
<td>DA, µg/24 h</td>
<td>163 ± 56</td>
<td>200 ± 74</td>
<td>292 ± 56</td>
</tr>
<tr>
<td></td>
<td>(38 – 309)</td>
<td>(43 – 340)</td>
<td>(216 – 401)</td>
</tr>
<tr>
<td>µg/g creat.</td>
<td>603 ± 174</td>
<td>479 ± 188</td>
<td>396 ± 86</td>
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<tr>
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<td>(239 – 995)</td>
<td>(86 – 806)</td>
<td>(234 – 864)</td>
</tr>
<tr>
<td>VMA, mg/24 h</td>
<td>1.7 ± 0.4</td>
<td>2.5 ± 0.4</td>
<td>3.5 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>(1.0 – 2.6)</td>
<td>(2.0 – 3.2)</td>
<td>(2.3 – 5.2)</td>
</tr>
<tr>
<td>mg/g creat.</td>
<td>6.8 ± 1.7</td>
<td>5.6 ± 1.0</td>
<td>4.7 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>(4.0 – 10.8)</td>
<td>(4.0 – 7.5)</td>
<td>(3.0 – 8.8)</td>
</tr>
<tr>
<td>HVA, mg/24 h</td>
<td>2.6 ± 0.8</td>
<td>3.6 ± 0.7</td>
<td>4.3 ± 1.6</td>
</tr>
<tr>
<td></td>
<td>(1.4 – 4.3)</td>
<td>(2.1 – 4.7)</td>
<td>(2.4 – 8.7)</td>
</tr>
<tr>
<td>HVA, mg/g creat.</td>
<td>9.9 ± 2.5</td>
<td>8.0 ± 1.9</td>
<td>5.3 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>(5.4 – 15.5)</td>
<td>(4.4 – 11.5)</td>
<td>(3.3 – 10.3)</td>
</tr>
</tbody>
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*a n = no. of children in each age group. b Mean ± standard deviation (and observed range) of concentrations.

The age-related reference ranges are shown in Table 1. The values, expressed both in micrograms (or milligrams) per 24 h and in micrograms (or milligrams) per gram of creatinine, were grouped by age. Three groups could be observed. The range of values for each group correlated rather well with the mean ± 2 SD.

From three to 16 years of age, there was a linear relationship between age and the excretion of NE, DA, VMA, and HVA, expressed either per gram of creatinine or per 24 h (Table 2). For NE, a linear relation with age was observed when values were expressed in micrograms per 24 h but not when expressed in micrograms per gram of creatinine. The excretion of catecholamines and metabolites per 24 h increased with age. On the contrary, the excretion expressed per gram of creatinine decreased with age. Gitlow et al. (5) also found a linear relationship between VMA excretion per gram of creatinine and age (from one to 15 years) and between HVA excretion and age (from four to 15 years). McKendrick and Edwards (4) showed that VMA excretion per 24 h is a linear function of age between one and 12 years. The values we observed for VMA excretion per 24 h agree well with those of McKendrick and Edwards (4) and De Schaepdryver et al. (6). Values we obtained for HVA excretion agree well with the values determined by De Schaepdryver et al. (6). However, our values for VMA and HVA excretion expressed in terms of creatinine were higher than data reported by Gitlow et al. (5); however, the latter were based on untimed urine collection. Moyer et al. (7) and Abeling et al. (9) have found an age-dependence in catecholamine excretion by children. The values observed for urinary excretion of NE, E, and DA were consistent with results obtained by these authors.

We have not measured the excretion of catecholamines and their metabolites in children younger than three years old because it is difficult to obtain complete 24-h urine collections from very young children in their normal activities at home. On account of the higher incidence of neuroblastoma in early childhood, another study will be undertaken with inpatient children younger than three years.

References