Quantitation of Urinary Normetanephrine and Metanephrine by Reversed-Phase Extraction and Mass-Fragmentographic Analysis

Claver Canfell, Steven R. Binder, and Hassan Khayam-Bashi

Hydrolyzed urine with added ring-trideuterated normetanephrine and metanephrine is applied to wet C₁₈-reversed-phase minicolumns. The "metanephrines" are eluted, dried, derivatized with pentafluoropropionic anhydride, and analyzed with the gas chromatograph–mass spectrometer. Ions for the nondeuterated and trideuterated compounds are monitored at m/z 458 and 461, respectively. For both normetanephrine and metanephrine, the standard curve is linear over the range 10–2000 μg/L and the procedure has adequate precision both within-run (CV <3%) and between-day (CV <7%). Alkaline pH in the extraction is important for optimal analytical recovery. We have examined the potential value of untimed urine specimens for screening purposes and compared 24-h urine concentrations of these analytes in normotensive and hypertensive persons.

Additional Keyphrases: catecholamines • chromatography, gas–liquid • screening • hypertension • reference interval • pheochromocytoma • timed vs untimed urine specimens

Diagnostic biochemical tests for the presence of pheochromocytoma or other neural crest tumors include plasma and urine catecholamines (epinephrine and norepinephrine), urinary "metanephrines" (metanephrine (M) and normetanephrine (NM)), and vanillylmandelic acid (VMA).¹ The value of urinary total "metanephrine" measurement as a screen for pheochromocytoma is well established (1–3). However, the widely used colorimetric assay of Pisano (4) has several major limitations: it does not distinguish, nor is it entirely specific for, M and NM; and it requires a 24-h urine collection because sensitivity is inadequate for untimed (random) urines having total "metanephrine" concentrations less than 100 μg/L.

Recently, liquid-chromatographic assays with electrochemical (5, 6) and fluorescence (7) detection have been introduced, but these still require time-consuming clean-up steps with recoveries typically less than 70%. In addition, the liquid-chromatographic assay conditions were not selective, and other biogenic amines were eluted after M and NM, limiting the chromatographic throughput to three or four samples per hour. In these procedures untimed or timed collections (5, 6) were used, but results were not compared with those from 24-h collections. Gas-chromatographic–mass-spectrometric (GC-MS) procedures have been published that are more sensitive and selective (8, 9), but only 24-h collections from normotensive subjects have been analyzed.

We have developed a specific and sensitive GC-MS assay for M and NM involving a simple sample preparation. Using ring-trideuterated internal standards and an unmodified commercial mass spectrometer in the selected ion mode, we have achieved adequate precision with a throughput of 10 to 12 samples per hour. Untimed and 24-h excretions of both these metabolites are compared in urines from normotensive and hypertensive persons.

Materials and Methods

Apparatus: We used a Hewlett-Packard 5992A gas chromatograph/quadrupole mass spectrometer/data system (Hewlett-Packard, Palo Alto, CA 94304) equipped with a 2 m × 2 mm (i.d.) glass column packed with 3% OV-1 on 80–100 mesh Supelcoport (Supelco Inc., Bellefonte, PA 16823). The 3-mL Bond-Elut® C₁₈ extraction columns and the Vac-elut® vacuum manifold were obtained from Analytichem International, Inc., Harbor City, CA 90710.

Reagents: Metanephrine and normetanephrine (HCl salts) were obtained from Sigma Chemical Co., St. Louis, MO 63178. Pentafluoropropionic (PFP) anhydride was obtained from Pierce Chemical Co., Rockford, IL 61105. Methanol, dichloromethane, and ethyl acetate were analytical grade. Deuterium oxide (D₂O; 100 atom% D) and deuterium chloride (DCI, 99+ atom% D and 5.33 mol/L D₂O) were obtained from Aldrich Chemical Co., Milwaukee, WI 53233.

Standards and control: We prepared an aqueous stock standard solution of M and NM (500 mg/mL in 1 mol/L HCl) and stored it at 4 °C. Urinary standards covering the range 200–2000 μg/L were prepared by adding known amounts of the aqueous stock standard to freshly filtered (0.45-μm pore-size filter) and acidified normal urine; these were stored at −20 °C. Lyophilized quality-control material (Urine Chemistry Control, Level 1; Fisher Diagnostics, Orangeburg, NY 10962) was reconstituted in 1 mol/L HCl, aliquoted, and stored at −20 °C; this was used daily as a control. 2,5,6-Tri-deuteropropyl-NM (NM-d₃) and 2,5,6-trideuteropropyl-M (M-d₃), prepared according to the procedure of Musket et al. (10) and stored at −20 °C, were used as internal standards.

Specimen collection: Twenty-four-hour urines were collected in 15 mL of 5 mol/L H₂SO₄; untimed urine collections were acidified on receipt in the laboratory. All urines were stored at 4 °C if assayed within a week or at −20 °C if kept longer before analysis. A reference laboratory estimated VMA content by a modification of the spectrophotometric method of Pisano et al. (11) on 24-h collections from hypertensive patients. Creatinine was determined in all acidified urine samples with an Astra-8 (Beckman Instruments, Inc., Brea, CA 92621) by means of a modified Jaffé rate method (12); the CV of this procedure was <2%.

Procedure: Acidify 2 mL of urine to pH 1 ± 0.5 by adding 200 μL of 3 mol/L HCl; hydrolyze at 100 °C for 1 h. After cooling, add to each sample 50 μL of NM-d₃ and M-d₃ (approximately 2 μg of each in 3 mmol/L HCl) as internal standard and adjust the pH to 8–9 with NaOH. Apply the samples to the C₁₈ extraction minicolumns, after preswaking with methanol and water. Wash each loaded column with 2 mL of water, then 300 μL of dichloromethane, then 2 mL of methanol. "Metanephrines" are eluted with the methanol; evaporate...
this eluate at 60 °C, then derivatize by heating with 150 µL of PFP anhydride at 80 °C for 10 min. Evaporate the PFP anhydride, add 50 µL of ethyl acetate to the residue, and inject 1 µL onto the 3% OV-1 column. Set the gas chromatograph at 166 °C and the helium carrier gas flow at 40 mL/min. The temperature of the membrane separator at the GC-MS interface is 166 °C and the ion source is at 250 °C. Monitor major specific ion fragments of PFP-NM and PFP-M (m/z 458) and PFP-NM-d3 and PFP-M-d3 (m/z 461) in the selected ion mode with dwell times of 250 ms. We used peak-area ratios for quantitation and optimized the slope sensitivity for peak area integration at 0.06. Retention times for NM and M under the conditions described are 3.0 and 3.6 min, respectively.

Calibration curve and quantitation: The concentration of endogenous NM and M in the urine used for the standard curve was determined over 10 separate runs. Subsequently, these amounts of NM and M were used to assign actual standard concentrations by addition to the weighed-in amounts. Using linear regression analysis, we determined the calibration curve through the standard points and the origin, and calculated the analyte concentrations in the controls and unknowns from this curve. Over the concentration range 10–2000 µg/L, the accuracy of this procedure is unaffected by either the residual amount of unlabeled internal standard with ion current of m/z 458 or the contribution of ion current m/z 461 from the nondeuterated NM and M.

For statistical analysis we used Student's t-test.

Results and Discussion

Analytical Variables

Recovery: Extraction recoveries of both NM and M from urine in these C18 reversed-phase columns were pH dependent. Absolute recoveries from a normal urine to which M and NM were added at different pHs are shown in Figure 1. In experiments with both aqueous salt solutions and normal urines, recoveries of both NM and M were greatest at pH 8 ± 1. Although C18-reversed-phase column packing deteriorates at pH >8, the short time for which we used this condition in the procedure allowed for good recoveries of NM and M and permitted reuse of the columns. Thus, the procedure described is economical. Dichloromethane was used only to displace water from the column packing before the elution with methanol. Although the reversed-phase columns showed little specificity in the recovery of most urine components, recoveries of NM and M were better than either those achievable with cation-exchange chromatography (5, 9) or those we achieved with ethyl acetate extraction at alkaline pH (8).

Analytical recoveries from urine containing 200 and 1000 µg of added NM and M per liter, carried through the entire procedure, were assessed in 10 replicate samples; recovery of endogenous NM and M was taken into account (Table 1). Because of the potential for isotopic exchange or dilution of the deuterium in the internal standards during hydrolysis, we added the ring-deuterated internal standards to cooled, hydrolyzed samples; loss of NM and M is negligible during this step (9).

Linearity: The standard curve was prepared daily from urine standards carried through the entire procedure; it was linear over the range 10–2000 µg/L. Satisfactory precision was demonstrated as low as 20 µg/L, which permitted quantitation of NM and M well into the subnormal range.

Precision: Within-run and between-day precision of the assay was determined as shown in Table 2. In addition, the variability of the GC-MS determination, assessed by multiple injections of the same sample, was also determined at different analyte concentrations. This variability accounted for more than one-half of the within-run variability of NM but less than one-third of that of M.

Specificity: We monitored the ion current for both NM and M at m/z 458 because of the specificity of a mass-fragmentographic assay, in general, decreases with decreasing relative molecular mass of the fragment ion chosen for monitoring. The only commercially available deuterated analog, α,α-d₂β₃-d₃-NM, fragments to give a more abundant ion current at m/z 460 than at m/z 461 because of the rearrangement of the PFP-amide moiety. We used ring-trideuterated internal standards for both NM and M, to enhance reproducibility and specificity by monitoring the more abundant ion at m/z 461. Potential interfering endogenous and exogenous substances would have to fulfill two criteria: (a) cochromatograph with M and NM on the 3% OV-1 column at the specified temperature, and (b) give fragment ions (after derivatization) of either m/z 458 or 461. Some antihyperten-

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**Table 1. Analytical Recoveries of NM and M Added to Normal Urine**

<table>
<thead>
<tr>
<th>Amount added, µg/L</th>
<th>% NM recovered, mean ± SD</th>
<th>CV, %</th>
<th>% M recovered, mean ± SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>200</td>
<td>9</td>
<td>98 ± 1.6</td>
<td>1.6</td>
<td>103 ± 0.5</td>
</tr>
<tr>
<td>1000</td>
<td>10</td>
<td>104 ± 1.7</td>
<td>1.6</td>
<td>104 ± 2.3</td>
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</tbody>
</table>

**Table 2. Variability of the GC-MS, Within-Run and Between-Day Precision**

<table>
<thead>
<tr>
<th></th>
<th>NM</th>
<th></th>
<th></th>
<th>M</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Conc, µg/L</td>
<td>CV, %</td>
<td>Conc, µg/L</td>
<td>CV, %</td>
</tr>
<tr>
<td>GC-MS variability</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal urine</td>
<td>10</td>
<td>672 (6)</td>
<td>0.9</td>
<td>147 (2)</td>
<td>1.3</td>
</tr>
<tr>
<td>Commercial control</td>
<td>5</td>
<td>127 (2)</td>
<td>1.8</td>
<td>439 (2)</td>
<td>0.4</td>
</tr>
<tr>
<td>Within-run variation</td>
<td>10</td>
<td>124 (4)</td>
<td>2.9</td>
<td>442 (6)</td>
<td>1.4</td>
</tr>
<tr>
<td>Between-day variation</td>
<td>41</td>
<td>128 (8)</td>
<td>6.4</td>
<td>447 (11)</td>
<td>2.5</td>
</tr>
</tbody>
</table>

* Mean (and SD). * Assessed by multiple injections of the same sample into the GC-MS. * Assessed with commercial control. * Over 10 days.
sive drugs and metabolite analogs, derivatized and tested for such interference by GC-MS, are listed in Table 3. None of these compounds tested co-chromatographed with NM or M or gave fragment ions at m/z 458 or 461. Routine selected ion analysis of more than 30 urine collections from patients undergoing antihypertensive therapy revealed no detectible chromatographic interference. Only 3-methoxytyramine, a major metabolite of dopamine, had a retention time similar to that for NM or M on this column (3.3 min), but this compound gives no significant fragment ions at m/z 458 or 461.

Excretion Values

Normotensive persons: We determined NM and M concentrations in untimed urine from 26 healthy normotensive persons; in 17 of these, we also measured concentrations in 24-h urines. These values were normalized in terms of creatinine excretion to allow comparison of timed vs random collections, as well as comparison with values previously reported by specific methods. Comparison of random vs 24-h concentrations (Figure 2) by paired t-test revealed no significant difference for either NM or M (t < 1.5). The mean ± SD of random and 24-h NM and M concentrations in normotensive persons is shown in Table 4. We did not compare our results with those by a method specific for NM and M. However, the 24-h excretion values we obtained for normotensive persons compared well with those previously reported by similar GC-MS selected ion techniques (8, 9) and by a liquid-chromatographic/electrochemical technique (5).

Hypertensive persons: We also assessed 24-h urine collect-

<table>
<thead>
<tr>
<th>Table 3. Drugs and Catecholamine Metabolite Analogs Found Not to Interfere in the Assay*</th>
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<tbody>
<tr>
<td>3-Hydroxytyramine</td>
</tr>
<tr>
<td>5-Methoxytryptamine</td>
</tr>
<tr>
<td>4-Hydroxy-3-methoxymandelic acid</td>
</tr>
<tr>
<td>4-Hydroxy-3-methoxyphenyl lactic acid</td>
</tr>
<tr>
<td>4-Hydroxy-3-methoxyphenyl acetic acid</td>
</tr>
<tr>
<td>Dihydroxyphenylacetic acid</td>
</tr>
<tr>
<td>L-Dihydroxyphenylalanine (L-DOPA)</td>
</tr>
<tr>
<td>α-Methyl DOPA</td>
</tr>
<tr>
<td>Δ1,Δ2-Synephrine</td>
</tr>
<tr>
<td>Hydrochlorothiazide</td>
</tr>
<tr>
<td>Triamterene</td>
</tr>
<tr>
<td>Ethacrynic acid</td>
</tr>
<tr>
<td>Furosemide</td>
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<tr>
<td>Metoprolol</td>
</tr>
<tr>
<td>Propranolol</td>
</tr>
<tr>
<td>Guanethidine</td>
</tr>
<tr>
<td>Reserpine</td>
</tr>
<tr>
<td>Stelazine</td>
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<tr>
<td>Hydralazine</td>
</tr>
<tr>
<td>Spiroloclonate</td>
</tr>
</tbody>
</table>

* Compounds were derivatized with PFP anhydride and chromatographed under assay conditions; they did not elute in the region of PFP-NM and PFP-M.

Table 4. Untimed and 24-h Urinary NM and M Excretion by Healthy, Normotensive Persons

<table>
<thead>
<tr>
<th></th>
<th>NM</th>
<th>M</th>
</tr>
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<tbody>
<tr>
<td>Un timed</td>
<td>µg/24 h</td>
<td>µg/g creatine</td>
</tr>
<tr>
<td>(n = 26)</td>
<td>194 ± 54*</td>
<td>(94–300)</td>
</tr>
<tr>
<td>24-h</td>
<td>231 ± 67</td>
<td>164 ± 64</td>
</tr>
</tbody>
</table>

* Mean ± SD (and range).

The data presented in Figure 3 indicate that the mean values for NM in hypertensive individuals are greater than those in normal persons, expressed as either µg/24 h or µg/g of creatinine, and this difference is probably significant (p < 0.025). This difference, however, may not be clinically significant for the diagnosis of pheochromocytoma, but rather may reflect hypertension and (or) sympathetic nerve activity in response to such factors as stress, physical activity, and antihypertensive medication. There was no significant difference between the mean values for M in both groups studied (p > 0.05). Our values for NM and M in hypertensive patients are also greater than those in untimed specimens from six hypertensive patients reported by Bertani-Dziedzic et al. (6): mean ± SD for NM was 140 ± 20 µg/g creatinine, and for M was 40 ± 10 µg/g creatinine. This difference could be due to the limited number of patients they presented and the lack of internal standard and recovery data in their procedure. Moreover, if aberrations in creatinine excretion occur, owing to age or pathophysiologic or nutritional influences, the ex-

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**Fig. 2.** Comparison of normal 24-h vs untimed urine content of (A) NM and (B) M, expressed as µg/g creatinine

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**Fig. 3.** Comparison of 24-h excretion values for NM and M in normal and hypertensive persons

One hypertensive person (not shown) had NM values of 960 µg/24 h and 1065 µg/g creatinine

CLINICAL CHEMISTRY, Vol. 28, No. 1, 1982
pression of excreted quantities per gram of creatinine may be invalid.

VMA excretion in all the hypertensive patients studied was
within the normal range (1–10 mg/24 h), but comparison of
24-h excretion values of VMA and total NM + M on these
patients showed only moderate correlation (r = 0.502). This
is not unexpected because (a) the secretion rate of NM and
M and VMA output may be different (15), and (b) a com-
parison of nonspecific colorimetric methods and highly spe-
cific methods may not produce valid conclusions.

Clinical correlations: Measurement of “metanephrines”
in untimed and timed urine collections by colorimetric
methods has been validated by other investigators for dis-
tinguishing pheochromocytoma patients (3, 14). The cor-
relation we achieved in normotensive persons by using
this specific method (see Figure 2) is considerably better than
that reported in the hypertensive population studied (3). We
have analyzed untimed samples from two patients undergo-
ing surgery for the removal of a pheochromocytoma: in one
patient, we obtained values ranging from 1089 to 1307 μg/g
creatinine for NM and 1800 to 2104 μg/g creatinine for M,
while for the other patient NM values ranged from 7680 to
14 100 μg/g creatinine and M values from 133 to 450 μg/g
creatinine. These values are clearly above the range observed
in the hypertensive population described in this study. Al-
though measurement of 24-h NM and M output is recom-
manded, determination of these metabolites in untimed urine
collections, especially those obtained during or after a hy-
pertensive episode, can also provide valuable clinical infor-
mation.

The application of simple and specific methodologies should
be of considerable help in the diagnosis of pheochromocytoma.
We have optimized and instituted a rapid (3 to 4 h) and spe-
cific method for measuring normal NM and M excretion
in normal and hypertensive persons, in untimed and 24-h urine
collections.

We are grateful to Dr. C. H. L. Shackleton of the Biomedical Mass
Spectrometry Resource, University of California, Berkeley (NIH
Division of Research Resources, Grant RR70019), for his advice
and assistance in development of the technique described. We thank Dr.
M. Roizen, Departments of Anaesthesia and Surgery, University of
California San Francisco Medical Center, for providing samples from
patients with pheochromocytoma.

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