Determination of Urinary Normetanephrine and Metanephrine by Radial-Compression Liquid Chromatography and Electrochemical Detection

Paul J. Orsulak,1 Patricia Kizuka, Edwin Grab, and Joseph J. Schildkraut

A procedure has been developed for determining the O-methylated catecholamine metabolites, normetanephrine and metanephrine, in urine by use of radial-compression liquid chromatography followed by electrochemical detection. Normetanephrine and metanephrine are isolated from hydrolyzed urine by ion-exchange on small, commercially available, disposable columns and preconcentrated by solvent extraction. They are then separated by reversed-phase ion-pair chromatography, with use of a radial compression cartridge and radial compression module, and quantitated with 3-methoxy-4-hydroxybenzylamine as internal standard. Normetanephrine, metanephrine, and the internal standard are separated from interfering peaks in about 15 min. The method is applicable to the relatively low amounts of normetanephrine (100–600 µg/24 h) and metanephrine (50–400 µg/24 h) found in normal subjects and patients with depressive disorders or hypertension. Within-day CVs ranged from 1.1 to 2.2% for normetanephrine and 1.2 to 6.9% for metanephrine; the corresponding between-day CVs were 4.9 and 5.7% over these ranges.

Additional Keyphrases: chromatography, reversed-phase ion-pair • metanephrines • hypertension • depressive disorders • reference interval

Assay of the catecholamines (epinephrine and norepinephrine), metanephrines (normetanephrine and metanephrine), and vanillylmandelic acid in urine for the diagnosis and characterization of pheochromocytoma or other neural crest tumors is well established (1–3). More recently, however, determination of urinary normetanephrine and metanephrine has been found useful in disorders in which the amounts excreted are much lower than usually occur in these disorders. For example, Canfell et al. (4) showed that the mean rate of excretion of normetanephrine by hypertensive individuals is greater (100–600 µg/24 h) than that of normal persons (100–350 µg/24 h). They found no significant difference between mean values for metanephrine when hypertensive patients and normal subjects were compared. Other studies (5, 6) have shown that determinations of urinary normetanephrine and metanephrine (taken in conjunction with data on other catecholamine metabolites) may also be valuable in differentiating patients with various subtypes of affective (depressive) disorders. In these latter cases, it is essential to quantify concentrations of normetanephrine and metanephrine in the "normal" range reliably and to detect relatively small changes in the concentrations of these metabolites.

Widely used colorimetric assays for normetanephrine and metanephrine (7) have several major limitations. They do not distinguish nor are they entirely specific for normetanephrine and metanephrine, and they are not sufficiently sensitive to quantify samples in which the total normetanephrine plus metanephrine concentrations are <500 µg/L. Recently, "high-performance" liquid-chromatographic assays for measuring normetanephrine and metanephrine in urine, with electrochemical detection (8, 9) or fluorescence detection (10), have been developed. Methods involving gas chromatography–mass spectrometry have also been published (4, 11, 12) that are highly specific and sensitive, but they require relatively expensive equipment, whereas liquid-chromatographic procedures can be performed with equipment that is now readily available and familiar to clinical laboratory personnel.

Here we describe modifications of one of the "high-performance" liquid-chromatographic procedures (8) incorporating the radial-compression liquid-chromatography cartridge system (Waters Associates Radial-PAK C8) after isolation of normetanephrine and metanephrine with a commercially available cation-exchange column developed for determination of catecholamines and metabolites (13). In the original procedure for determination of urinary normetanephrine and metanephrine (8) a C18 reversed-phase column was used and 30–45 min of chromatography time was required for each sample, because of late-eluting peaks. The present procedure, by virtue of the radial compression system, requires only about 15 min for chromatography of each sample, permitting more specimens to be analyzed per day. Moreover, with the C4 radial-compression cartridge, the order of elution is favorably altered.

Materials and Methods

Apparatus

For development and evaluation of this method, we used a high-pressure liquid-chromatography system consisting of a Model 710B sample processor, Model U6K injector, Model M6000 pump, and Model RCM-100 radial compression module (all from Waters Associates Inc., Milford, MA 01757).

The electrochemical detector was a Model LC4A (Bioanalytical Systems, Inc., West Lafayette, IN 47906) equipped with a TL5 glassy-carbon electrode. Data were collected with a Houston Instruments Omniscrbe Model B5217-5 recorder. We made injections either manually, with the U6K injector, or automatically, with the Model 710B sample processor.

Reagents

All chemicals used in this procedure were "HPLC" grade, where available, or reagent grade. All aqueous solutions were prepared in Type 1 reagent water ("NANOpure" water purification system; Barnstead Co., Boston, MA 02132).

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Hydrochloric acid, 6 mol/L, 3 mol/L, and 0.75 mol/L.
Ethylendiaminetetraacetate, disodium salt (EDTA), 1 g/L.
Boric acid, 40 g/L.
Ammonium hydroxide buffer, 4 mol/L, pH 10. This is prepared by diluting to 1 L 138 mL of stock ammonium hydroxide (14.5 mol/L) and sufficient 6 mol/L hydrochloric acid to adjust to pH 10.0. This reagent should be freshly prepared each week.

Toluene/isooamyl alcohol, 3/2, by vol.
Ethyl acetate/acetone, 2/1, by vol.
Sodium hydroxide, 3 mol/L, 0.5 mol/L, and 0.25 mol/L.
Saline, NaCl, 9 g/L.

Normetanephrine and metanephrine (cat. no. N7127 and M8825; Sigma Chemical Co., St. Louis, MO 63178). Prepare a standard solution containing, per liter, 5 mg of normetanephrine free base and 2.5 mg of metanephrine free base.

3-Methoxy-4-hydroxybenzylamine hydrochloride (cat. no. H3,660.5; Aldrich Chemical Co., Milwaukee, WI 53233), internal standard solution containing 5 mg of free base per liter.

Catecholamine isolation columns. Columns containing a cation-exchange resin (cat. no. 1892202; Bio-Rad Laboratories, Richmond, CA 94804) are used as provided by the manufacturer.

Standards. A standard urine pool was prepared by combining urine specimens from several healthy human volunteers. The urine was collected over sodium metabisulfite, 500 mg/L final concentration, aliquoted, and stored frozen until use. We measured the concentration of normetanephrine and metanephrine in this urine by the standard-addition technique. The urine pool, divided into 10-mL aliquots, was stored at −20 °C, in plastic vials.

Chromatography

For chromatographic separation of normetanephrine, metanephrine, and the internal standard, 3-methoxy-4-hydroxybenzylamine, we use the Radial-PAK C8 cartridge, 10-μm particle size (Waters Associates).

One liter of the mobile phase consists of 60 mL of acetonitrile and 940 mL of triethylamine/phosphoric acid buffer, pH 3.0, prepared as follows. Each liter of buffer contains 1.10 g (5 mmol) of heptane sulfonic acid, sodium salt (cat. no. 403027; Regis Chemical Co., Morton Grove, IL 60053); 15 mL of triethylamine (cat. no. W635; Baker Chemical Co., Phillipsburg, NJ 08865); and sufficient phosphoric acid (cat. no. A260, HPLC grade; Fisher Scientific Co., Bedford, MA 01730) to adjust the pH to 3.0. Before the mobile phase is prepared, the buffer is filtered through a Millipore type HA filter (cat. no. HAWP 04700; Millipore Corp., Bedford, MA 01730), and acetonitrile (glass-distilled HPLC grade; Burdick and Jackson Laboratories, Muskegon, MI 49442) is filtered through a Millipore type FH filter (cat. no. FHUP 04700). The mobile phase is degassed by stirring at low speed for 2 h before use.

The flow rate is 2 mL/min, and the detector potential is set at +850 mV vs an Ag/AgCl reference electrode supplied with the electrochemical detector.

To calculate the concentrations of normetanephrine and metanephrine, we measure the peak heights of each component as well as the peak height of the internal standard and plot the ratio of the height for each component to that of the internal standard. We determine concentrations by comparison with normalized peak heights obtained with the standard urine pool or by comparison with a standard curve prepared from the standards in the saline solution, which we found give equivalent results, as discussed later.

To maintain optimal performance of the Radial-PAK cartridge, we purge the injector, pump, and column with acetonitrile/water (8/2 by vol) for 15 min, at a flow rate of 2 mL/min, at the end of each day.

Other Procedures

Urine collection. Like the pooled urines, urine specimens for this procedure were collected over sodium metabisulfite and stored frozen until used. Alternatively, urine samples for determination of catecholamines and metabolites can be collected over 20 mL of acetic acid/water (33/67 by vol); after the urine collection is completed, the pH is adjusted to 4–4.5 with additional acetic acid. The samples may then be stored at −20 °C for as long as several months, without loss.

The amount of urine to be analyzed is determined by the total volume of the 24-h collection. In general, when a 24-h collection exceeds 800 mL, use a 5-mL aliquot of urine. If the 24-h urine volume is <800 mL, dilute 2.5 mL of urine to 5 mL with reagent-grade water before analysis.

Sample preparation. Normetanephrine and metanephrine are purified according to the following procedure (essentially the same as that used for purification of these compounds in ref. 13).

Because metanephrines are mostly excreted in conjugated form, either as glucuronides or sulfates, they must be acid hydrolyzed before they can be absorbed on the resin column. To hydrolyze the conjugated compounds in urine, place a 5-mL aliquot of urine in a 20 × 150 mm screw-cap or centrifuge tube. Add 300 mL (3 μg) of the internal-standard solution and adjust the pH to between 0.50 and 0.99 with 3 mol/L hydrochloric acid. Hydrolyze by placing the tubes in a dry bath at 95 to 105 °C for 30 min (Isotemp Dry Bath Model No. 145; Fisher Scientific Co., Fair Lawn, NJ 07410). Then cool the samples and add 5 mL of the tolune/isooamyl alcohol. Shake the tubes for 3 min on a flat-bed shaker, centrifuge to separate the layers, and aspirate and discard the organic (upper) layer.

Transfer the lower layer to a 30- or 50-mL beaker containing 15 mL of the EDTA solution. Adjust the pH of the sample carefully to 6.5 (±0.05) with sodium hydroxide while mixing the sample with a magnetic stirrer. (Because it is important not to repeatedly overrun this pH by sequential additions of acid or base, the pH can conveniently be adjusted by sequential dropwise addition of 3 mol/L sodium hydroxide to pH 3, then 0.5 mol/L sodium hydroxide to pH 6, and finally by addition of 0.25 mol/L sodium hydroxide to pH 6.5.)

After the pH has been adjusted, pour each sample onto a new Bio-Rad catecholamine isolation column, which has been prepared by gently shaking it to resuspend the resin, allowing the resin to settle, and draining the column just before use. After the sample has entered the column, make two 10-mL washes with reagent-grade water, followed by 10 mL of the boric acid solution, and finally by 10 mL of reagent-grade water.

After the columns have drained completely, place them over 15-mL (16 × 125 mm) screw-cap centrifuge tubes containing 2.5 g of sodium chloride. Elute the normetanephrine and metanephrine with 5 mL of the ammonium hydroxide buffer, collecting the eluate over the sodium chloride. Then discard the columns.

Extract the NaCl-saturated aqueous phase with two 6-mL portions of the ethyl acetate/aceton mixture, using a horizontal shaker for 5 min each time to ensure equilibration. Centrifuge to separate the layers, and transfer the organic phase each time with a disposable pipette to a 15-mL (16 × 125 mm) centrifuge tube. Evaporate it to dryness. We use a

2 Columns contain a proprietary Biorex-70 packing of wide mesh size (~50–300 μm)
vortex-type evaporator (Model 3-2200; Buchler Instruments, Ft. Lee, NJ 07024) at 37 °C for this, but the extracts can be evaporated under nitrogen in a water bath at 45 °C (8).

Reconstitute the residue with 500 μL of mobile phase and vortex-mix for 20 s, allow to stand for about 30 min, and mix again, to ensure that all of the residue is in solution. (Samples may be stored frozen at this point in the assay for several days.) Then inject 20 to 50 μL of each concentrated extract onto the chromatographic column.

Results and Discussion

This assay is based on modifications of the method of Shoup and Kissinger (8). The reagents and procedures are essentially the same as those for the "catecholamine by column" test procedure currently used in many laboratories for determination of total metanephrines (13).

Figure 1 illustrates typical chromatograms obtained for urine specimens from apparently healthy human subjects and compares the chromatograms obtained by use of the μBondapak-C18 column evaluated by Shoup and Kissinger (8) with the results obtained by using the Waters Radial-PAK C8 cartridge. The overall quality of the chromatograms is equivalent, but the Radial-PAK C8 has several advantages. Normetanephrines, metanephrine, and the internal standard can be resolved from other substances on both columns, but separation is complete in about half the time with the Radial-PAK C8 cartridge. In addition, the order of elution differs for the two columns. The internal standard is eluted after tyramine on the μBondapak-C18 column; because tyramine can be present in substantially higher concentrations than any of the analytes or the internal standard, it sometimes interferes with complete separation of metanephrine from the internal standard on this column. With the Radial-PAK cartridge, tyramine is eluted after normetanephrine, metanephrine, and the internal standard, so there is less chance of interference from high concentrations of tyramine.

Linearity of the assay was verified by adding known amounts of normetanephrine and metanephrine to saline solution and to the standard urine pool over the range of 0.05 to 0.60 mg/L for normetanephrine and 0.025 to 0.30 mg/L for metanephrine, and carrying these samples through the entire procedure. As Figure 2 shows, the plotted data were linear over the entire range tested for both analytes and, more importantly, there was no difference in the curves obtained when results for a standard urine pool (corrected for contribution of endogenous normetanephrine or metanephrine) were compared with the standard curves obtained when normetanephrine and metanephrine were added to saline and taken through the entire extraction procedure. Evidently the assay can be simplified by preparing standards in saline rather than using a standardized urine pool and correcting for the endogenous contribution of the analytes. This we did, without problems.

Table 1a gives details on the linearity of the assay and comparability of the standard curves, obtained on four days during a week. Table 1b summarizes other data we obtained for standard curves obtained by use of aqueous-based standards, analyzed on 22 separate days during two months. These data indicate not only that the procedure is highly reproducible over long periods of time, but also that the Radial-PAK C8 cartridge and the electrochemical detector can be maintained under stable operating conditions during these intervals.

Table 2 summarizes our data on analytical precision. Within-run precision was determined by analyzing five aliquots of the same urine pool specimen on five separate

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Table 1. Statistical Analysis of Some Results

<table>
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<th>Slope</th>
<th>Intercept</th>
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<td></td>
<td>N</td>
<td>S</td>
<td>M</td>
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<tr>
<td>a. Linearity and precision of assay and comparison of curves for standards in pooled urine or saline solution (n = 4 each)</td>
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<tr>
<td>Normetanephrine</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Urine pool</td>
<td>3.39 ± 0.10</td>
<td>-0.03 ± 0.05</td>
<td>0.9974 ± 0.0043</td>
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<tr>
<td>Saline solution</td>
<td>3.38 ± 0.02</td>
<td>+0.01 ± 0.01</td>
<td>0.9999 ± 0.0001</td>
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<tr>
<td>Metanephrine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine pool</td>
<td>1.99 ± 0.17</td>
<td>+0.01 ± 0.02</td>
<td>0.9985 ± 0.0010</td>
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<tr>
<td>Saline solution</td>
<td>1.92 ± 0.15</td>
<td>+0.01 ± 0.01</td>
<td>0.9996 ± 0.0003</td>
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<tr>
<td>b. Linearity and precision of standard curves over extended period of analyses (n = 22 each)</td>
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<td></td>
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<td>Normetanephrine</td>
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<tr>
<td>Saline solution</td>
<td>3.35 ± 0.06</td>
<td>+0.02 ± 0.007</td>
<td>0.9998 ± 0.0002</td>
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<tr>
<td>Metanephrine</td>
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<tr>
<td>Saline solution</td>
<td>1.85 ± 0.13</td>
<td>+0.01 ± 0.004</td>
<td>0.9995 ± 0.0004</td>
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</table>

All data expressed as mean ± SD.

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Fig. 1. Chromatogram of urine extract

The specimen was from a physically healthy depressed patient. Use of a 30 cm × 4 mm μBondapak-C18 column is compared with the chromatograms obtained with a Radial-PAK C8 cartridge. NMN, normetanephrine; MN, metanephrine; TYR, tyramine; MHBA, 3-methoxy-4-hydroxybenzylamine (internal standard)
occasions during three weeks. The coefficients of variation (CVs) ranged from 1.1 to 2.2% for normetanephrine and 1.2 to 6.9% for metanephrine. Between-run CVs were obtained by analyzing 20 separate aliquots of a urine pool during six weeks. This "control" specimen was included with each of 20 analytical runs, each of which included up to 15 specimens from patients. The CVs were 4.9 and 5.7% for normetanephrine and metanephrine, respectively.

Using the procedures outlined here, we were able to inject between 300 and 500 specimens (either urine specimens or aqueous-based standards and controls) before we noted substantial deterioration of the Radial-PAK C8 cartridge. However, we found it essential to use reagents of the highest purity in preparing mobile phases, to avoid rapid deterioration of the cartridge. For example, we noted that use of reagent-grade heptane sulfonic acid, sodium salt (cat. no. 10583; Eastman Kodak Co., Rochester, NY 14650), resulted in rapid deterioration of the Radial-PAK C8 cartridge, and so it could not be used for this assay.

With this procedure one can routinely measure as little as 25 μg of either normetanephrine or metanephrine per liter of urine. The practical detection limit of the method, as governed by losses and recovery, sample size, and injection volume, is about 10 μg/L for each metabolite.

We now have analyzed more than 180 urine specimens from depressed patients. Data from these patients are summarized in Table 3, in which, for comparison purposes, we also show data obtained by Shoup and Kissinger (8) from a small sample population of graduate students and data reported by Canfell et al. (4) on urinary excretion of normetanephrine and metanephrine by healthy normal persons and hypertensive patients. Data are also provided for urinary excretion of normetanephrine and metanephrine in 24-h urine specimens from depressed patients, determined by a differential spectrophotofluorometric method used previously in our laboratory (5). Evidently these methods exhibit good agreement for both normetanephrine and metanephrine.

Table 3. Urinary Excretion of Normetanephrine and Metanephrine (μg/24 h) by Normal Subjects and Patient Populations

<table>
<thead>
<tr>
<th>Reference</th>
<th>State</th>
<th>n</th>
<th>x ± SD</th>
<th>Range</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normetanephrine</td>
<td>Normal</td>
<td>15</td>
<td>238 ± 80</td>
<td>105–354</td>
<td>HPLC-EC</td>
</tr>
<tr>
<td>Shoup and Kissinger</td>
<td>Normal</td>
<td>17</td>
<td>231 ± 67</td>
<td>117–340</td>
<td>GC-MS</td>
</tr>
<tr>
<td>Canfell et al. (4)</td>
<td>Normal</td>
<td>22</td>
<td>—</td>
<td>~115–650</td>
<td>Fluorometric</td>
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<tr>
<td>Schildkraut et al. (5)</td>
<td>Depression</td>
<td>46</td>
<td>265 ± 119</td>
<td>97–628</td>
<td>HPLC-EC</td>
</tr>
<tr>
<td>This study</td>
<td>Depression</td>
<td>81</td>
<td>291 ± 109</td>
<td>98–632</td>
<td>GC-MS</td>
</tr>
<tr>
<td>Metanephrine</td>
<td>Normal</td>
<td>15</td>
<td>174 ± 79</td>
<td>74–297</td>
<td>HPLC-EC</td>
</tr>
<tr>
<td>Shoup and Kissinger</td>
<td>Normal</td>
<td>17</td>
<td>149 ± 56</td>
<td>79–258</td>
<td>GC-MS</td>
</tr>
<tr>
<td>Canfell et al. (4)</td>
<td>Hypertension</td>
<td>22</td>
<td>—</td>
<td>~70–400</td>
<td>Fluorometric</td>
</tr>
<tr>
<td>Schildkraut et al. (5)</td>
<td>Depression</td>
<td>46</td>
<td>156 ± 68</td>
<td>32–330</td>
<td>HPLC-EC</td>
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<tr>
<td>This study</td>
<td>Depression</td>
<td>81</td>
<td>132 ± 44</td>
<td>54–263</td>
<td>GC-MS</td>
</tr>
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</table>

HPLC-EC, "high-performance" liquid chromatography with electrochemical detection; GC-MS, gas chromatography–mass spectroscopy.

Among the specimens we analyzed by this procedure were 20 urine specimens for which we also had determined urinary normetanephrine and metanephrine concentrations by our previous differential fluorescence method. Figure 3 summarizes the correlation between these two methods. Although these 20 specimens that had been stored at ~20 °C were four to seven years old, reanalysis by the present procedure gave high correlations with previously determined values for normetanephrine (r = 0.99) and the sum of normetanephrine plus metanephrine (r = 0.98); the correlation for normetanephrine alone was somewhat weaker (r = 0.81). Moreover, examination of the data and its distribution as evidenced by the standard deviation and median of these 20 samples indicates that these methods yield comparable results. The new procedure did result, however, in values for both normetanephrine and metanephrine that were approximately 12% lower than were originally obtained some years ago with the fluorometric procedure. Such a modest decrease is usually expected when "high-performance" liquid-chromatographic procedures are compared with older spectrophotometric or fluorometric methods, which were
often subject to interferences from "gross contamination," usually resulting in abnormally high fluorescence in both the blank and the test samples. The correspondence between these two analyses is extremely good when one considers the age of the specimens at the time of analysis by the present procedure.

"High-performance" liquid chromatography and the electrochemical detector are rapidly becoming common equipment in the clinical laboratory. The application of relatively simple and more specific methods for determination of the catecholamine metabolites normetanephrine and metanephrine may be useful, not only in diagnosis of pheochromocytoma and other neural crest tumors, but also in the differential diagnosis of hypertension (4). Current studies indicate that very small changes in the excretion of these metabolites (as well as other catecholamine metabolites) may provide valuable information for the diagnosis and classification of patients with affective (depressive) disorders (5, 6).

Recently, methods for determination of urinary catecholamines including norepinephrine, epinephrine, and dopamine by use of the Bio-Rad catecholamine isolation column, followed by "high-performance" liquid chromatography, with either electrochemical (14) or fluorescence detection (15) have also been published. The availability of these procedures, coupled to the method reported here, can provide the basis for a procedure for comprehensive determination of catecholamines and metabolites, not only in the high concentrations found in patients with pheochromocytoma or hypertension, but also at the relatively low concentrations found in normal subjects or in patients with psychiatric disorders (5, 6).

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References