Urinary Metanephrine Radioimmunoassay: Comparison with the Colorimetric Assay

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A radioimmunoassay involving an $^{125}$I ligand has been developed and applied to the measurement of urinary metanephrine. To validate the clinical usefulness of this assay, we compared measurement of metanephrine by radioimmunoassay and of total urinary metanephrines by the Pisano colorimetric method. The radioimmunoassay is specific for metanephrine, whereas the colorimetric method measures both metanephrine and normetanephrine. We used both methods to determine urinary metanephrine or total metanephrines in subjects with essential hypertension, pheochromocytoma, the syndrome of multiple endocrine adenomatosis type 2, and normotensive volunteers. The mean and upper limit of normal (3 SD) for metanephrine by radioimmunoassay in our normotensive volunteers was 94.2 µg/24 h and 229 µg/24 h, respectively, which compares well with reported values of 87.6 µg/24 h and 319 µg/24 h by non-radioimmunoassay methods. Both radioimmunoassay and colorimetry accurately identified five patients with known pheochromocytoma. Good correlation ($r = 0.993$) was demonstrated between the two assays in a comparison of patients with essential hypertension and pheochromocytoma. We conclude that the radioimmunoassay is at least equivalent to the colorimetric methods in distinguishing pathological and normal catecholamine secretion, and is faster, more precise, and 1000-fold more sensitive.

Additional Keyphrases: Pisano method for metanephrines compared • essential hypertension • pheochromocytoma • multiple endocrine adenomatosis type 2 • neuroblastoma • adrenal tumors • pediatric chemistry

Spectrophotometric, gas-chromatographic, and fluorometric procedures have been used to measure urinary metanephrine and normetanephrine, but the Pisano colorimetric technique (1) has probably received the widest application in clinical laboratories. Because this method does not distinguish between metanephrine and normetanephrine, results are reported as combined urinary "metanephrines." In addition, the colorimetric method is somewhat nonspecific and involves a rather lengthy extraction with ion-exchange resin.

To overcome some of the deficiencies of the spectrophotometric method, we have developed a rapid, sensitive, and specific $^{125}$I radioimmunoassay for metanephrine, adapted from an $^{125}$I radioimmunoassay for catecholamines (2).

Because our radioimmunoassay is specific for metanephrine and does not cross react with normetanephrine, we undertook to compare both the colorimetric method and the radioimmunoassay for their ability to distinguish between hypertensive patients who did not have pheochromocytoma and patients with documented pheochromocytoma.

In an additional application, we used the radioimmunoassay as a screening test for pheochromocytoma in a family with the hereditary syndrome of multiple endocrine adenomatosis type 2. Patients with this syndrome develop various combinations of pheochromocytoma, medullary carcinoma of the thyroid, and hyperparathyroidism. We collected 24-h urine specimens from 13 members of this family. Results of their urinary metanephrine excretions by radioimmunoassay and by colorimetric assay were compared with results obtained for 14 normotensive volunteers.

Materials and Methods

Materials

Synephrine, metanephrine, normetanephrine, epinephrine, dopamine, 3-methoxytyramine, tyramine, octopamine, vanillylmandelic acid (VMA), homovanillic acid, and 3-methoxy-4-hydroxyphenylglycolic acid were obtained from Sigma Chemical Co., St. Louis, MO 63178. a-Methyldopa (Merck, Sharpe and Dohme), Nordefrin (Sterling-Winthrop), metaprotenerol (Boehringer-Ingelheim), methoxyamine (Burroughs-Wellcome), isoxsuprine (Mead-Johnson), and phenylephrine (Winthrop) were all kindly donated by their respective manufacturers. $^{3}$H]Metanephrine (spec. act. 5.29 kCi/mol) and $^{3}$H]methoxytyramine (spec. act. 22.1 kCi/mol) were obtained from New England Nuclear, Boston, MA 02118.

Crystallized bovine serum albumin and Freund's complete and incomplete adjuvant were obtained from Miles Labs, Inc., Elkhart, IN 46514. Rabbits (females, New Zealand strain) were maintained on Purina Rabbit Chow and water ad lib. Carrier-free $^{125}$I was obtained from Industrial Nuclear Co., St. Louis, MO 63114.

Immunization Procedure

About 2 mg of synephrine–albumin conjugate, prepared by the method of Grota and Brown (3), was emulsified in 2 mL of complete Freund's adjuvant and injected into the rabbits intradermally, in 20 to 30 sites in the back and neck. Booster injections of conjugate (1 mg in 2 mL of incomplete Freund's adjuvant) were given subcutaneously every four weeks. Blood was collected just before the booster injection and the antisemum was assayed for antibodies to metanephrine.

Of the four rabbits immunized, all produced titratable antiserum by the fourth booster immunization, but the highest-titer antiserum (final dilution 10 000) was the antiserum we used in this study.

Iodination

The radioactive ligand, prepared by the Chloramine-T
oxidation reaction, is [125I]3-iodosynephrine. Synephrine, 0.1 ng in 25 μL of 0.2 mol/L phosphate buffer, pH 8.0, was combined with 2.0 mCi of Na[125I] and 50 μL of Chloramine-T in 10 μL of the same buffer. The reaction was allowed to proceed for 90 s while air was bubbled through the solution (with a Drummond microcap). The reaction was stopped by adding 50 μg of sodium metabisulfite in 10 μL of the same buffer. The [125I]3-iodosynephrine was separated from unreacted synephrine, Na[125I], and [125I]3,5-diiodosynephrine by electrophoresis on polyacrylamide gel (4).

Antibody Detection

We tested the immunological potency of the metanephrine antisera by measuring the binding of [3H]metanephrine produced by various dilutions of antisera. We incubated 10⁴ cpm of [3H]metanephrine and 50 μL of various dilutions of rabbit antisera at 37 °C for 1 h in phosphate buffer (50 mmol/L, pH 7.5, and containing, per liter, 9 g of NaCl, 1 g of sodium azide, and 1 g of gelatin) in a total volume of 500 μL. At the end of the incubation, the antibody-bound [3H]metanephrine was precipitated, along with 50 μL of a 250 mL/L solution of normal rabbit serum, by adding 500 μL of a saturated solution of ammonium sulfate. After centrifugation at 2400 rpm for 15 min, the supernatant fluid was aspirated and the pellet was redissolved in 2 mL of water and transferred to a scintillation vial. We added 15 mL of Scintiverse (Fisher Scientific, Fair Lawn, NJ 07410) scintillation fluid to the vial and counted the radioactivity in the sample with a Beckman LS 3000 liquid scintillation spectrometer (counting efficiency of 42% for [3H]) (Beckman Instruments, Fullerton, CA 92634). Controls and blanks were treated exactly as above, but with normal rabbit serum substituted for antimanephrine antiserum (nonspecific binding, NSB), or with buffer and [3H]metanephrine that were not precipitated (total). The percent binding (%B) was calculated as follows:

\[ \frac{(A - NSB)/total} \times 100 = %B \]

where A is the cpm precipitated at the various selected antisera dilutions, NSB is the nonspecific binding in cpm, and total is the total number of counts of [3H]metanephrine added to each tube (usually 10 000 cpm). When the %B reached 30% at 500-fold dilution, the antisera was characterized by determining sensitivity and cross reactivity with the 125I radioligand.

With use of excess antibody (100-fold final dilution), 80% of the [125I]3-iodosynephrine (10 000 cpm) could be bound within seven days after radioliodination. Figure 1 shows the difference in binding titers between [3H]metanephrine and [125I]3-iodosynephrine.

Radioimmunoassay Procedure

Because the [125I]3-iodosynephrine has much higher specific activity (1000–2000 kCi/mol) than the [3H]metanephrine, the %B vs antisera dilution (binding check) is repeated before each radioimmunoassay. In general, a %B (or B₀ in the competitive inhibition radioimmunoassay) of 30% can be obtained with a 10 000-fold final dilution of antisera.

Unlabeled synephrine, metanephrine, or other analogs were incubated for 30 min with 150 μL of diluted rabbit antisera and 50 μL of 0.1 mol/L EDTA at 37 °C in the pH 7.5 phosphate buffer mixture described above, in a total volume of 1450 μL; then 10 000 cpm of [125I]3-iodosynephrine was added in 50 μL of the buffer mixture and the incubation continued at 37 °C for another 30 min. Antibody-bound [125I]3-iodosynephrine was then precipitated, along with 100 μL of a 250 mL/L solution of normal rabbit serum, by adding 1.5 mL of a solution of saturated ammonium sulfate. After centrifugation at 2400 rpm for 15 min, the supernate was aspirated and radioactivity in the pellet counted with a gamma counter.

Extraction and Hydrolysis Procedure

To monitor extraction efficiencies, we added 10 000 cpm of [3H]methoxytyramine in 50 μL of 0.1 mol/L HCl to 500-μL aliquots of urine. The pH of samples to be hydrolyzed was adjusted to 0.9 to 1.3 with 1 mol/L HCl; the mixture was heated in a boiling water bath for 30 min, then cooled in an ice bath; and 500 μL of borate buffer (1.0 mol/L, pH 11) was added. The hydrolyzed metanephrine and the [3H]methoxytyramine were extracted into 10 mL of toluene/isoamyl alcohol (3/2 by vol) by vortex-mixing for 30 s. The organic and aqueous phases were separated by low-speed centrifugation and 9.5 mL of the organic phase was transferred to tubes containing 500 μL of 50 mmol/L HCl. The metanephrine and [3H]methoxytyramine were extracted into the aqueous phase by vortex-mixing, after which the mixture was centrifuged. After the organic (top) phase was discarded, the aqueous phase was washed twice with 3 mL of water-saturated diethyl ether and the ether aspirated. The small amount of ether remaining after aspiration was evaporated by exposing the aqueous phase to a stream of nitrogen at room temperature for 5 min. A 100-μL aliquot of the aqueous phase was placed in a scintillation vial, mixed with 10 mL of Scintiverse, and its radioactivity counted with a scintillation counter. Five- to 50-μL aliquots were used directly in the radioimmunoassay.

The recovery of extracted [3H]metanephrine is not significantly different from that of [3H]methoxytyramine. Because methoxytyramine does not cross react in this assay, the number of counts added could be generous without causing concern that the quantity of compound added would inhibit binding. This latter is a problem with the [3H]metanephrine commercially available, which has a low specific activity. The recovery of [3H]methoxytyramine and immunoreactive [3H]metanephrine was 60.2 (SD 5.1)% and 61.8 (SD 5.8)%, respectively.

Assay Specificity, Accuracy, and Precision

The specificity of the antibody was evaluated by determining the percent inhibition of binding of the 125I-labeled synephrine in a series of 10 sequential dilutions of the various
Table 1. Specificity of Anti-Metanephrine Antibodies

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cross reaction, %</th>
</tr>
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<tbody>
<tr>
<td>Metanephrine</td>
<td>100</td>
</tr>
<tr>
<td>Synephrine</td>
<td>20</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>12.8</td>
</tr>
<tr>
<td>Normetanephrine</td>
<td>4.8 \times 10^{-3}</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>9.7 \times 10^{-4}</td>
</tr>
<tr>
<td>Dopamine</td>
<td>&lt;10^{-5}</td>
</tr>
<tr>
<td>3-Methoxytyramine</td>
<td>&lt;10^{-5}</td>
</tr>
<tr>
<td>Octopamine</td>
<td>&lt;10^{-5}</td>
</tr>
<tr>
<td>Tyramine</td>
<td>&lt;10^{-5}</td>
</tr>
<tr>
<td>Vanillylmandelic acid</td>
<td>&lt;10^{-5}</td>
</tr>
<tr>
<td>Homovanillic acid</td>
<td>&lt;10^{-5}</td>
</tr>
<tr>
<td>3-Methoxy-4-hydroxyphenylglycol</td>
<td>&lt;10^{-5}</td>
</tr>
<tr>
<td>Isoproterenol</td>
<td>&lt;10^{-5}</td>
</tr>
<tr>
<td>( \alpha )-Methyldihydroxyphenylalanine</td>
<td>&lt;10^{-5}</td>
</tr>
<tr>
<td>Noradrenalin</td>
<td>&lt;10^{-5}</td>
</tr>
<tr>
<td>Methoxamine</td>
<td>&lt;10^{-5}</td>
</tr>
<tr>
<td>Metaproterenol</td>
<td>&lt;10^{-5}</td>
</tr>
<tr>
<td>Phenylephrine</td>
<td>&lt;10^{-5}</td>
</tr>
<tr>
<td>Isoxsuprin</td>
<td>&lt;10^{-5}</td>
</tr>
</tbody>
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The percent cross reaction is determined by dividing the mass of metanephrine at 0.5 \( B/B_0 \) by the mass of the cross-reacting compound at 0.5 \( B/B_0 \) and multiplying by 100.

compounds tested (Figure 2). These data were then converted by logit transformation to produce a linear regression line. To calculate cross reactivity, we divided the concentration of the compound being tested that produced a 50% inhibition by the concentration of metanephrine that produced a 50% inhibition and multiplied by 100.

To assess analytical recovery, we added 10, 100, or 250 pg of metanephrine standard (representing 80, 50, and 20% \( B/B_0 \)) to 500 \( \mu \)L of a low-value pool of normal urine, and made four determinations at each concentration. These pools were hydrolyzed, then extracted and quantified by radioimmunoassay, with regression analysis of the logit-transformed data. Within-assay error was calculated from the four replicates at each concentration.

Urine pools with previously determined high, medium, and low concentrations of metanephrine (representing 20, 50, and 80% \( B/B_0 \)) were used to determine between-assay error; the pools were hydrolyzed and extracted before each determination. We assayed 100-\( \mu \)L aliquots of each extracted pool in seven different complete radioimmunoassay procedures and calculated between-assay error from the results.

The within-assay coefficients of variation (CVs) at 80, 50, and 20% \( B/B_0 \) were 13.6, 9.9, and 7.3%, respectively. The between-assay CVs at 80, 50, and 20% \( B/B_0 \) were 37.0, 14.1, and 12.7%, respectively.

Most assays involved a 10,000-fold final dilution of antibody, producing a percent total binding of 33% (\( B_0 \)), which was assigned a value of 100% (\( B/B_0 \)). The inhibition of binding of \( ^{125}I \)-labeled synephrine to the antibody, as related to increasing concentrations of added metanephrine to a low urine pool sample or decreasing volumes of high pool urine sample (a pheochromocytoma patient), is shown in Figure 3. Metanephrine either added to urine before extraction and hydrolysis or in diluted pheochromocytoma urine was quantitatively recovered and displayed inhibition curves parallel to the standard buffer curve.

The relative cross reactivities of the various compounds tested are listed in Table 1. The only significant displacement occurred with metanephrine, synephrine, and epinephrine. The 20% cross reactivity of synephrine was decreased to 5%
with the extraction step, and the 12.8% cross reactivity of epinephrine was decreased to less than 10–5%.

Results

We compared the concentrations of metanephrine as determined by radioimmunoassay in hydrolyzed urines from hypertensive (non-pheochromocytoma) and five known pheochromocytoma subjects with the hydrolyzed total urinary metanephrines as determined with the Pisano colorimetric technique (Figure 4). On the average, the Pisano method measured 8.7-fold more metanephrines (metanephrine and normetanephrine) than the metanephrine radioimmunoassay. The correlation between the two methods was \( r = 0.993 \). The mean of 13 nonpheochromocytoma hypertensive subjects by the colorimetric method was 412 \( \mu \text{g/L} \) with a SD of 459. Three standard deviations gives an upper limit of normal of 1789 \( \mu \text{g/L} \). Values for all five known pheochromocytoma patients exceeded this value; and one patient (undiagnosed) gave a result well above this limit. The mean by radioimmunoassay of the nonpheochromocytoma hypertensive subjects was 56.6 (SD 47.4) \( \mu \text{g/L} \). The five pheochromocytoma patients and one hypertensive subject had values by radioimmunoassay that exceeded the upper limit for normal of 198.8 \( \mu \text{g/L} \) (mean + 3 SD).

The urinary concentration of metanephrine (\( \mu \text{g/L} \)) will vary on the basis of volume of urine excreted and is not a measure of the rate of secretion. To determine the discriminatory power of the two assays to detect pathologic secretion of catecholamines as reflected in the urinary metanephrine, volume is factored out and the results are reported in \( \mu \text{g/} \text{24 h} \).

By radioimmunoassay, the mean urinary metanephrine for the normotensive subjects (Figure 5) was 92.4 (SD 45.7) \( \mu \text{g/} \text{24 h} \). The mean + 3 SD delineates an upper limit of normal of 229 \( \mu \text{g/} \text{24 h} \), compared with a mean of 87.6 \( \mu \text{g/} \text{24 h} \) and an upper limit of 319 \( \mu \text{g/} \text{24 h} \) reported for non-radioimmunoassay methods (5). Of the hypertensive non-pheochromocytoma patients, only one had a value significantly above (346 \( \mu \text{g/} \text{24 h} \)) the reported normal limit (229 \( \mu \text{g/} \text{24 h} \). Unfortunately, this patient has been lost to follow-up. All five pheochromocytoma patients had values significantly above the normal limits.

Urinary metanephrine measurements by radioimmunoassay were determined in a family with suspected-multiple endocrine adenomatosis type 2 (Figure 5). Two members of the 14 family members tested had values above normal limits. One subject is hypertensive and was being treated with \( \alpha \)-methyldopa. His urine collection was repeated after stopping \( \alpha \)-methyldopa for 14 days and although his blood pressure remained high, his urinary metanephrine (as determined by radioimmunoassay) decreased from 336 to 182 \( \mu \text{g/} \text{24 h} \). The second subject had significantly above-normal metanephrine excretion, 668 \( \mu \text{g/} \text{24 h} \), and was near the range of several of the patients presented with proven pheochromocytoma. Additional testing is in progress on this subject.

Discussion

The radioimmunoassay described is significantly more sensitive than colorimetric methods. The radioimmunoassay can detect urinary metanephrine in as little as 10 \( \mu \text{L} \) of normal human urine, whereas the colorimetric technique requires as much as 2.5 \( \text{mL} \). The greatly increased sensitivity will allow quantitation of metanephrine in very small samples, such as might be obtained from neonates or small research animals.

The radioimmunoassay is less time consuming and simpler than the colorimetric assay. By radioimmunoassay, one can easily analyze 400 samples per day, whereas more than 100 samples per day would be difficult by the colorimetric method, which requires multiple extractions, incubations, and manual quantitation with a spectrophotometer.

The radioimmunoassay was at least equivalent to the Pisano method in its ability to differentiate patients with pheochromocytoma from hypertensive nonpohochromocytoma patients. Although the colorimetric method gave values almost ninefold higher than the radioimmunoassay, the former also measures normetanephrine, whereas the latter does not. The correlation between the two assays was excellent \( r = 0.993 \) for samples analyzed in parallel (Figure 4).

Several substances interfere in the measurement of total urinary metanephrine by the colorimetric technique. Some interact directly or are metabolized and converted to vanillin, which is the actual compound being measured colorimetrically. These compounds include isoprotrenorol, octopamine, and 4-hydroxybenzaldehyde (5). We tested a large number of sympathomimetic amines and anti hypertensive agents, none of which cross reacted in our assay (Table 1). One patient (Figure 5), while being treated with \( \alpha \)-methyldopa, had an increased value for metanephrine, but this returned to normal when the patient ceased taking \( \alpha \)-methyldopa. Although neither \( \alpha \)-methyldopa nor any of its conceivable metabolites interfere with the radioimmunoassay itself, its effect in increasing endogenous metabolism of catecholamines can produce increased concentrations of metanephrine (6). This drug-induced increase in urinary metanephrine may also be produced by other drugs that increase catecholamine turnover, including reserpine, phenoxybenzamine, and monoamine oxidase inhibitors (6, 7). Therefore, we recommend that patients be as drug free as possible during testing.

The radioimmunoassay’s specificity for metanephrine has both advantages and disadvantages. Unlike the colorimetric method, the radioimmunoassay does not measure normetanephrine, and therefore may not detect pathologic states in which normetanephrine secretion is increased and metanephrine is not. However, being able to distinguish metanephrine by radioimmunoassay from normetanephrine may allow better characterization of catecholamine secretion. Other currently available methods capable of measuring
metanephrine require extensive extractions with ion-exchange resins or elaborate equipment such as a gas chromatograph. Because of this difficulty in differentiating metanephrine from normetanephrine, few clinical studies have been performed to determine how these metabolites may differ in various pathophysiologic states.

The measurement of urinary metanephrines is made, almost exclusively, to distinguish essential hypertension from pheochromocytoma and to aid in the diagnosis of a rare, usually malignant, tumor of childhood: neuroblastoma. Ninety-three percent of pheochromocytomas are of adrenal origin. All of our cases were adrenal and had above-normal values for urinary metanephrine by radioimmunoassay. The enzyme phenylethanolamine N-methyltransferase, required for the synthesis of metanephrine, is maintained by high concentrations of glucocorticoids from the adrenal cortex (8). This would suggest that nonadrenal pheochromocytoma, which accounts for only 7% of these tumors, may not be associated with increased metanephrine (epinephrine) secretion because of the lack of local, highly concentrated glucocorticoids. This reasoning has, however, not been supported by clinical testing, because tumors from the organ of Zuckerkandl and intrathoracic pheochromocytomas commonly produce epinephrine (metanephrine) (9). Therefore, it would be an extremely rare tumor that would not be detected by the metanephrine radioimmunoassay.

Fifty percent of neuroblastomas are also of nonadrenal origin. Because of the tendency of neuroblastoma to secrete more dopamine and its metabolites rather than normetanephrine, total urinary catecholamine measurements are more likely to detect this tumor than either the colorimetric method or the radioimmunoassay.

As is standard medical practice, when attempting to diagnose pheochromocytoma or neuroblastoma, a series of three urinary tests is usually performed, for metanephrine, VMA, and total catecholamines. It is our contention that if the radioimmunoassay is used to replace the colorimetric method, the few cases of false negatives will not be overlooked if measurements of VMA and total catecholamines are also obtained.

The specificity of the metanephrine radioimmunoassay may be advantageous as an aid in distinguishing an adrenal from a nonadrenal pheochromocytoma. Although most all of these tumors secrete increased amounts of epinephrine (metanephrine), the ratio of urinary metanephrine to VMA by radioimmunoassay may be significantly higher in an adrenal tumor than in a nonadrenal tumor. This theoretical advantage of the metanephrine radioimmunoassay is totally beyond the scope of the colorimetric, VMA, or total catecholamine methods, alone or in combination.

Stress-induced secretion of catecholamines in patients with essential hypertension can be difficult to distinguish from pheochromocytoma. A technique of collecting urine for metanephrines from sleeping patients, to avoid stress-induced and postural increases in norepinephrine (normetanephrine) from the autonomic nervous system, has been reported (10). Because epinephrine is not secreted by the noradrenergic neurons of the autonomic nervous system, urinary metanephrine values (by radioimmunoassay) may be less affected by stress and postural changes and therefore possibly better distinguish essential hypertension from pheochromocytoma. We have begun to study this possibility, but only one patient has been investigated completely. We found in split 12-h day and night urines that metanephrines (by colorimetry) and VMA, both exceeding normal limits during the day, decreased by 50–60%, to normal values at night. Total urinary catecholamines in the high normal range decreased by 30% at night. Urinary metanephrine by radioimmunoassay was in the normal range during the day and decreased only 19% at night. Although this patient has had multiple laboratory tests and clinical studies over the past five years, no pheochromocytoma has been found, in agreement with the findings for metanephrine by radioimmunoassay.

We conclude that the metanephrine radioimmunoassay is a reliable aid in the diagnosis of pheochromocytoma. Technically, the assay is significantly more rapid and 1000-fold more sensitive than the colorimetric method. The marked specificity of the assay may help differentiate pheochromocytoma from essential hypertension, and help localize tumors.

References


