Fluorometric Determination of Urinary Metanephrine and Normetanephrine

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A detailed procedure for the quantitative determination of metanephrine and normetanephrine is given. Epinephrine and norepinephrine are eliminated by selective adsorption on an alumina column. Metanephrine and normetanephrine are isolated with a cation-exchange resin. Fluorescence is developed by a two-step ferricyanide oxidation at two different pH values followed by tautomerization. The concentrations of both metanephrine and normetanephrine are quantitated by differential fluorometry.

The metabolism of the catecholamines has been extensively studied in the last several years and Axelrod (1) has recently published an excellent review. Also a symposium on catecholamines, resulting from a meeting at the National Institutes of Health, has provided a number of papers on catecholamine measurement, synthesis, metabolism, and physiology (2). Paper chromatography methods are available for measuring metanephrine and normetanephrine (3), but have not found wide application. Ion-exchange methods for the separation of metanephrine and normetanephrine of urine have been devised (4), however, these methods were utilized for study of exogenously administered, radioactively tagged catecholamines and did not include a chemical method for measuring the amount of metanephrine and normetanephrine. Bertler et al. (5) described a fluorometric method for the estimation of metanephrine and normetanephrine, quantitating
them by differences in their fluorescence properties. This method was not applied to urine. Randrup (6) has described the separate determination of metanephrine and normetanephrine by differences in the stability of fluorescent products of the two compounds. However, he was unable to obtain satisfactory results using hydrolyzed urine because of high blanks. von Studnitz (7) described a method for separation and determination of metanephrine and normetanephrine by high-voltage paper electrophoresis, but did not describe a method for isolating the metabolites from urine.

Yoshinaga et al. (8, 9) have recently described a solvent separation and high-voltage electrophoresis method for the separate determination of urinary metanephrine and normetanephrine. Their normal values indicate that there is more metanephrine than normetanephrine in normal urine. Yoshinaga et al. (10) later indicated that their values for metanephrine also include N-methylmetanephrine, which could not be distinguished by high-voltage paper electrophoresis, but could be separated by paper chromatography.

Pisano (11) has determined metanephrine and normetanephrine by oxidizing them to vanillin which is measured colorometrically. This method does not differentiate between metanephrine and normetanephrine. Using this method, Crout et al. (12) have found the determination of the total 3-methoxy catecholamine metabolites to be of value in the diagnosis of pheochromocytoma. However, this method is not sensitive enough to measure excretion in the normal or low normal range. Smith and Weil-Malherbe (13) have described a fluorometric method for metanephrine and normetanephrine, differentiating them by oxidations at different pH levels.

Because of the great interest in metanephrine and normetanephrine, there is a need for a practical procedure for their separate determination. We feel that a practical urinary metanephrine and normetanephrine procedure should be quantitative, including preferably an adequate column isolation method. It should allow the separate determination of metanephrine and normetanephrine, be sensitive enough to measure low normal or subnormal values, and it should be a relatively simple procedure. We believe that the procedure described here and previously described in an abstract (14) meets these criteria.

Principle

Urine is hydrolyzed with heat and acid. Epinephrine and norepinephrine are removed by adsorption on alumina, and metanephrine
and normetanephrine are adsorbed on and eluted from a cation exchange resin. Using a two-step ferricyanide oxidation at two different pH values followed by tautomerization, fluorescence is developed and the metanephrine and normetanephrine are quantitated by differential fluorometry. Internal standards and an unoxidized blank are used for each determination.

**Method**

**Reagents**

1. *Distilled water*  
   Redistill in glass if necessary.
2. 4*N* HC1 and 0.5*N* HC1
3. 0.5*N* acetic acid
4. 5*N* NaOH
5. 0.2*M* sodium acetate  
   Adjusted to pH 8.5 with sodium hydroxide
6. 0.25% potassium ferricyanide (Merck) (stock) and 0.050% potassium ferricyanide (working).
7. *Zinc catalyst solution*  
   0.5% zinc sulfate in 0.02 N hydrochloric acid
8. *Eluate diluting solution*  
   1500 ml. 0.5 N acetic acid and 500 ml. 0.5 M sodium acetate. This solution has buffer characteristics similar to the eluate from the column.
9. *Buffer solution*  
   To give a pH of 5.5 during second part of the oxidation: dilute 4.75 ml. of 10 N NaOH to 100 ml. with 1 M sodium acetate.
10. *Tautomerizing solution*  
    Prepare immediately before use: 8.7 ml. 5.0 N NaOH, 0.3 ml. ethylenediamine (Matheson, Coleman & Bell), and 1.0 ml. of 2% ascorbic acid (G. T. Baker Chemical Co.). Prepare the ascorbic acid solution daily.
11. *Preservative powder (mixed dry)*  
    Sodium metabisulfite 2 parts by weight and sodium fluoride 1 part
12. *Aluminum oxide*  
    Heat Alcoa activated alumina—grade F-20 60-200 mesh (Aluminum Corporation of America)—with 2-3 volumes of 2 N HC1 in a boiling water bath for 20 min. with stirring. Wash and resuspend repeatedly in several volumes of distilled water until the wash fluid has a pH of 4.0 or greater. This requires about 20 washings. Keep the alumina covered with distilled water in the same Erlenmeyer flask in which it was washed.
13. *Amberlite CG-50, 100-200 mesh. (Rohm & Haas)*  
    Rinse with

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*All reagents are Mallinckrodt AR unless otherwise specified.*
0.2 M ammonium acetate, pH 6.7, until pH 5.5 is reached. Then wash repeatedly with 0.2 M ammonium acetate at pH 8.5 until a stable pH of 6.2 is reached. Store the resin in 0.2 M ammonium acetate at pH 6.2. (For each washing use three times as much volume of solution as there is resin.)

14. Stock standards 50 µg./ml. as the free base in 0.1 N hydrochloric acid. (a) dl-normetanephrine HCl (Winthrop), 6.0 mg./100 ml. of 0.1 N HCl (refrigerate). Solution is stable for 6 months at 4°. (b) dl-metanephrine HCl (Winthrop), 5.9 mg./100 ml. of 0.1 N HCl (refrigerate).

15. Working standards with zinc catalyst Prepare weekly. These give 0.2 µg./ml. (a) Normetanephrine—1.00 ml. of stock normetanephrine standard diluted to 250 ml. with zinc catalyst solution (No. 7). (b) Metanephrine prepared as described above using stock metanephrine standard.

16. Quinine fluorescence standard (a) Stock—100 mg. of quinine sulfate in 1 L. 0.1 N H₂SO₄, (b) Working standard—1 to 500 dilution of stock with 0.1 N H₂SO₄.

Equipment


2. Fluorometer (Farrand Model A) for measurement of fluorescence. The filters used are, the A set primary, a 390-mµ Bausch and Lomb interference filter and a 5-58 Corning filter, the secondary in the A set being a 500 mµ Bausch and Lomb interference filter and a 3-72 Corning filter. The B set is a 430-mµ Bausch and Lomb interference filter and a 3-73 and a 5-59 Corning filter. The secondary B filter is a 520-mµ Bausch and Lomb interference filter and a 3-70 Corning filter.

Procedure

Collection and Preservation

Timed, measured specimens are collected so that the excretion, in micrograms per hour, may be determined. Single-voiding timed specimens are preserved by adding 0.15 gm. of the preservative powder for each 30 ml. of urine. If possible, these specimens should be kept refrigerated. Twenty-four-hour urine specimens are collected in a gallon jug containing 5 gm. of the preservative powder, the urine being added to the refrigerated jug after each voiding.
Hydrolysis

A 4-ml. aliquot of the specimen is diluted to 8 ml. with distilled water and acidified to pH 1 with 4 N HCl, using a pH meter. The acidified urine is diluted to 10 ml. and heated for 1 hr. at 100°. After cooling and centrifuging, the supernatant is adjusted to pH 8.5 with 5 N NaOH, using a pH meter.

Chromatography

At pH 8.5 catecholamines are removed by passing the diluted urine through a short (7 mm. diameter, 15 mm. long) alumina column. The alumina column is prepared from a water suspension of previously conditioned alumina and buffered at pH 8.5 with 5 ml. of 0.2 M sodium acetate buffer (pH 8.5). After the urine has passed through the alumina, the column is washed with 15 ml. of water. The 25 ml. of specimen plus wash is then acidified to a pH of 6.2 with 0.5 N HCl, using a pH meter. Metanephrine and normetanephrine are adsorbed on a cation exchange resin column (7 × 60 mm.) of CG-50. This column is filled from a suspension in 0.2 M ammonium acetate buffer, pH 6.2, and the excess buffer drained off. The combined urine and washings are passed through the column and the column washed with an additional 15 ml. of water. The specimen and washings are discarded. The metabolites are eluted from the column with two 10-ml. portions of 0.5 N acetic acid. The eluates are mixed and may be kept overnight at 4° if desired.

Oxidation and Fluorescence Development

Oxidation and tautomerization are carried out on a carefully timed schedule which includes blanks and also standards. Internal standards are used to allow for variations in fluorescence intensity produced in different specimens.

Table 1 shows the order of addition of reagents and the time schedule. Duplicates are prepared for blanks, unknowns, and standards, using 10 × 75-mm., acid-washed Pyrex test tubes, which have been previously selected by checking with a quinine fluorescence standard. The oxidation is started in successive pairs of tubes at 30-sec. intervals to maintain a 4-min. initial oxidation time for each pair of tubes.

Measurement of Fluorescence

The outside of the tubes used for the oxidations is washed, dried with a clean towel, and then read. The readings are in arbitrary units.
**Table 1. Time Schedule and Order of Addition of Reagents**

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Reagent</th>
<th>Blank</th>
<th>Unknown</th>
<th>Normetanephrine + unknown</th>
<th>Metanephrine + unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zinc catalyst solution</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Working normetanephrine standard</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Working metanephrine standard</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eluate</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>H₂O*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.050% Potassium ferricyanide*</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>4</td>
<td>Buffer solution*</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>8</td>
<td>Tautomerizing solution*</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*Mix by inversion, using a Parafil cap.

The fluorometer is set to read 6 using diaphragm 4, the A filter set and the quinine working standard. The fluorescence of all eight tubes is read at the A and the B frequencies, in the same order that they were oxidized. If the reading of the unknown is much higher than the reading of the added standards, the eluate is diluted with the eluate diluting solution and the oxidation is repeated.

**Calculation**

All of the paired readings are averaged. If there is a significant difference between the two readings, this oxidation is repeated. The blank readings are subtracted from the unknown readings at each frequency. \( U_a \) designates results with the A filters and \( U_b \) results with the B filters. The unknown readings are subtracted from both the metanephrine plus unknown and normetanephrine plus unknown readings. The results with the A filters are designated \( M_a \) and \( NM_a \) and, with the B filters, \( M_b \) and \( NM_b \). The amounts of metanephrine and normetanephrine are calculated by solving the following pair of equations:

\[
U_a = xM_a + yNM_a \\
U_b = xM_b + yNM_b
\]

\( U_a \) and \( U_b \) are the net fluorescence of the unknown with the A and B filters, and similarly \( M_a, NM_a, M_b \) and \( NM_b \) are the net fluorescence of the internal standards with the A and B filters; \( x \) is proportional to the amount of metanephrine and \( y \) is proportional to the amount of normetanephrine present in the sample. The actual values are dependent on the original volume of urine used, the portion of the total eluate present in each tube, and the amount of standard used. For the pro-
procedure as outlined, if \( x \) and \( y \) are multiplied by a factor of 50, the concentration of metanephrine and normetanephrine in \( \mu g./100 \) ml. of urine is obtained. We use a form which outlines a simplified method of calculation in steps (15).

Comment

Most of the reagents and equipment used for this procedure are identical with those used for our procedure for determining epinephrine and norepinephrine (16).

Recoveries have been evaluated through the various steps, all by adding mixtures of metanephrine and normetanephrine before a given step and the same amount to another aliquot after the step.

Preservation

The preservation method is the same that we use for epinephrine and norepinephrine. The use of acid without refrigeration may hydrolyze catecholamines, but this is not a problem with metanephrine and normetanephrine, as they must be hydrolyzed before determination. Twenty-four-hour urines are collected at the patient’s bedside with continuous refrigeration (17).

Hydrolysis

Most of the metanephrine and normetanephrine in human urine are excreted as conjugates—principally glucuronides and sulfates. The optimum time for hydrolysis at \( pH \) 1.0 is 1 hr. Longer hydrolysis or a lower \( pH \) results in excessively high blanks and a shorter time, and a higher \( pH \) produces incomplete hydrolysis. The hydrolysis step gives a recovery of 94.7% ± 4.7% of added standards.

Chromatography

Our oxidation procedure will also produce fluorescence from epinephrine and norepinephrine. Epinephrine, norepinephrine, metanephrine, and normetanephrine are all adsorbed on amberlite CG-50. Therefore, epinephrine and norepinephrine must be either removed or measured independently and their fluorescence subtracted from the total fluorescence. As epinephrine and norepinephrine are much better adsorbed on alumina than metanephrine and normetanephrine, passing the hydrolyzed urine first through an alumina column removes the epinephrine and norepinephrine. We have found that amberlite CG-50 in the sodium ion form produces variable recoveries. Amberlite
in the ammonium ion form yields recoveries of 88.9 ± 3.7%. Dilution of the urine with distilled water is essential for good recoveries; 96 ± 1.7% of the epinephrine and norepinephrine are adsorbed on the alumina column and 98.7 ± 2.3% of the metanephrine and normetanephrine pass through the column without being absorbed. As there is usually much more metanephrine and normetanephrine in urine than there is epinephrine and norepinephrine, this does not cause a significant error.

Oxidation

Metanephrine readily forms a fluorescent indole when oxidized with ferricyanide at a low pH (3.5 or lower) in the presence of a zinc catalyst. With somewhat greater difficulty, it is possible to form an indole from normetanephrine at a higher pH (5.0 or higher). It is desirable to oxidize them both simultaneously and differentiate them by their fluorescent properties, as Price and Price (18) and Cohen and Goldenberg (19) have done with epinephrine and norepinephrine. However, none of the standard methods of oxidation utilized for catecholamines were satisfactory for both metanephrine and normetanephrine. There is a difference in the optimum pH of oxidation of metanephrine and normetanephrine. Therefore, it seems feasible to initiate the oxidation with ferricyanide at a low pH, oxidizing the metanephrine and then, after adjusting the pH to a higher value, to continue the oxidation, oxidizing the normetanephine in the same test tube. This not only proved successful, but resulted in higher fluorescence readings from normetanephrine than can be obtained with ferricyanide at any single pH of oxidation. It has been possible to produce higher yields of metanephrine by altering the conditions of oxidation, but the procedure has been adjusted to maximize the fluorescence from normetanephrine.

The mechanism of this oxidation or the end product are not known. Possibly the oxidation at two different pH values favors successive steps of the reaction. Lund (20) in his systematic stepwise evaluation of a formation of adrenolutin from epinephrine has shown that at least five steps are involved. If his postulated intermediates are correct, an indole cannot be formed in the presence of a methyl group at the No. 3 position. Perhaps this methyl group is removed when the indole is formed. If so, the indoles formed from metanephrine and normetanephrine may be identical with those formed from epinephrine and
norepinephrine. The shape of the excitation and fluorescence curves of metanephrine and normetanephrine are identical to those of epinephrine and norepinephrine, respectively. With a reduction of the sensitivity setting of the spectrophotofluorometer, the epinephrine curves can be directly superimposed on the metanephrine curves and, similarly, the norepinephrine curve on the normetanephrine curve. This favors the hypothesis that these indoles are identical.

Metanephrine and normetanephrine produce approximately one half as much fluorescence as the same concentration of epinephrine or norepinephrine when they are all oxidized by our standard procedure for metanephrine and normetanephrine. Oxidation of metanephrine and normetanephrine requires a zinc catalyst; epinephrine and norepinephrine do not. By oxidizing without zinc, it is possible to measure the small amount of epinephrine and norepinephrine present in the eluate. This is not done routinely, as more than 95% of the epinephrine and norepinephrine is removed on the alumina column.

Tautomerization

von Euler (21) has suggested the use of ethylenediamine in the tautomerizing solution in the determination of epinephrine and norepinephrine to keep the blank from rising spontaneously. We have found that the ethylenediamine stabilizes the readings in the metanephrine and normetanephrine determination by preventing the formation of a fluorescent breakdown product from ascorbic acid, which is usually unstable in alkaline solution.

Blanks

There are many possible ways of producing a blank and most of them have been tried. A common method of producing blanks has been the omission of ascorbic acid. However, this produces a blank which is too low and which yields an erroneously elevated value after it is subtracted from the unknown. We have found that an unoxidized blank (no ferricyanide) gives the most accurate results.

Fluorometry

The Amino-Bowman spectrophotofluorometer with an XY recorder proved useful in setting up and evaluating this procedure. Excitation curves of metanephrine and normetanephrine at two different fluorescence frequencies are illustrated in Fig. 1. Note that excitation at 390
m\(_\mu\) produces equal fluorescence from metanephrine and normetanephrine at 500 m\(_\mu\). Excitation at 430 m\(_\mu\) yields much more fluorescence at 520 m\(_\mu\) from metanephrine than from normetanephrine. Once the correct set of excitation and fluorescence frequencies for the two

![Excitation curves of the fluorescent oxidation products produced from metanephrine and normetanephrine. (Left: fluorescence set at 500 m\(_\mu\). Right: fluorescence set at 520 m\(_\mu\).)](image)

sets of measurements have been determined, a filter instrument is more satisfactory for routine use. The Farrand Model A fluorometer modified to hold a four-tube cuvet holder and for automatic switching between two sets of filters has been used in this procedure. It is fairly stable, quite sensitive, and, using interference filters, the separation of metanephrine from normetanephrine is as good as that with the spectrofluorometer.

The "net" readings of metanephrine and normetanephrine added to the eluate vary moderately from one eluate to another, as they do in most catecholamine procedures. Therefore, internal standards are used. When the fluorometer is set to read 6 with the quinine working standard, typical net readings of our internal standards with the A filters are: metanephrine, 2.4 ± 0.20, and normetanephrine, 1.8 ± 0.25. Similarly, with the B filters typical readings are: metanephrine, 3.4 ±
.11, and normetanephrine, .74 ± .14. The readings from added amounts are proportional as long as amounts present are not excessive. When large amounts of metanephrine and normetanephrine are present, as in urine from pheochromocytoma patients, the eluate has been diluted so as to keep the readings in the same range of magnitude as the usual added standards. Over-all recoveries are 88% ± 6.8%. All of the preceding recoveries were not done at the same stage of development of the procedure and the three most recent over-all recoveries ranged from 90 to 92%.

Most of our determinations have been done on 2- or 3-hr. timed, measured, urine specimens rather than on 24-hr. urine specimens. In 24 normal individuals the mean and standard deviation respectively were: metanephrine, 4.6 ± 2.7 μg./hr.; normetanephrine, 8.0 ± 3.6 μg./hr.; and total (metanephrine plus normetanephrine), 12.6 ± 5.4 μg./hr. The mean percentage of the total which is metanephrine is 36% with a standard deviation of ±14.6%. In pheochromocytoma patients, the values have ranged from 150 to 600 μg./hr., with 0-70% metanephrine.

Although we feel this procedure will be useful in screening hypertensive patients for a possible pheochromocytoma, it does not offer major advantages over the determination of catecholamines for this purpose. We believe the major usefulness will be in studying the excretion of catecholamine metabolites in various metabolic diseases.

Addendum

With 9 months more experience, we have found that an incomplete oxidation occasionally occurs. This can be prevented by routinely diluting the eluate with an equal volume of the eluate-diluting solution and using 0.075 per cent potassium ferricyanide for the oxidation. If this is done x and y should be multiplied by a factor of 100 instead of 50.

References