Colorimetric vs Liquid-Chromatographic Determination of Urinary 5-Hydroxyindole-3-acetic Acid
Russell P. Tracy, Lester E. Wold, James D. Jones, and Mary F. Burritt

The popular colorimetric assay for urinary 5-hydroxyindole-3-acetic acid based on the reaction with 1-nitroso-2-naphthol has been criticized for lack of specificity. Goldenberg (Clin. Chem. 19: 38-44, 1973) proposed a modification of this method, in which 2-mercaptoethanol is used to discharge unwanted chromophores. We compared results by both the unmodified and modified colorimetric methods with those by a "high-performance" liquid-chromatographic method, with fluorometric detection. Agreement among all three assays was excellent in the range of 50 to 600 mg/24 h, but there were considerable differences in the range 0 to 30 mg/24 h between results by the unmodified colorimetric assay and the other two. In this range, results by the modified assay (y) correlated well with those by the chromatographic method (x), although the values were slightly lower (y = 0.85x + 0.11, r = 0.9303). Using the modified colorimetric assay to analyze 40 normal specimens, we defined a normal range of 0 to 6 mg/24 h. We recommend the modified colorimetric assay for use in the high-volume laboratory and either this assay or the liquid-chromatographic method for the smaller laboratory.

Additional Keyphrases: reference intervals - carcinoid syndrome - methods for the small laboratory

Udenfriend et al. (1) established the clinical usefulness of the assay for urinary 5-hydroxyindole-3-acetic acid (5-HIAA) and described a colorimetric method that is now widely used. Unfortunately, this method is subject to interference from several drugs, and some 5-HIAA is lost during the procedure (2, 3).

Goldenberg reported a modification of the Udenfriend method, involving the use of 2-mercaptoethanol, which reportedly corrects for drug interference (3).

Several other procedures have been used to measure 5-HIAA, including gas chromatography (4), and "high-performance" liquid chromatography (HPLC) (5). Recently, two groups reported the determination of 5-HIAA in urine. Shihabi and Scaro (6) described an HPLC method with amperometric detection; Draganac et al. (7) reported an HPLC method with ultraviolet detection. Both groups compared their proposed assays to the colorimetric assay (without the 2-mercaptoethanol modification) for evaluation, and found substantial disagreement for many specimens.

In our laboratory we routinely use the 1-nitroso-2-naphthol reaction to assay for 5-HIAA in urine. We find this method, with modification (8), to be sensitive and fast enough for us to assay the 10 to 20 samples per day that we routinely process. Because of the question of interference, however, we under-took to assess the usefulness of incorporating 2-mercaptoethanol into the reaction. An HPLC method was also developed as a procedure for comparison with both the unmodified and 2-mercaptoethanol-modified nitrosonaphthol methods.

Materials and Methods
Colorimetric Assays
Twenty-four-hour urine specimens, collected with 50 mL of an equimolar mixture of acetic acid and water as preservative, were adjusted to pH 3.0 before assay.

Our routine colorimetric assay was essentially that of Udenfriend et al. (1) as modified by MacFarlane et al. (8). Pipet 5.0 mL of urine into a 50-mL glass-stoppered centrifuge tube containing 2 g of NaCl, add 25 mL of diethyl ether, and gently shake the tubes for 1 min. After brief centrifugation, transfer 20 mL of the ether phase into a round-bottom evaporation flask, place the flask in a warm-water bath, and evaporate the solvent. (This step takes about 2 min per flask.) Dissolve the residue in 4.0 mL of distilled water; this is the "urine extract."

Prepare the working nitrosonaphthol reagent by dissolving 0.5 g of 1-nitroso-2-naphthol (Fisher Scientific Co., Pittsburgh, PA 15238) in 500 mL of 95% ethanol. Prepare the nitrous acid solution daily by mixing one part of a 25 g/L solution of NaNO2 and 25 parts of 1 mol/L H2SO4. Prepare the NaNO2 solution fresh daily.

Mix 3.0 mL of urine extract with 1.0 mL of the nitrosop- naphthol solution and 1.0 mL of the nitrous acid solution. In the modified (3) assay, 0.2 mL of a 250 g/L aqueous solution of 2-mercaptoethanol (Eastman Kodak Co., Rochester, NY 14650) was also added, with mixing. Incubate the samples at 60 °C for 15 min, add 10 mL of ethyl acetate, shake the tubes vigorously, and allow them to stand at room temperature for 30 min. Aspirate the ethyl acetate layer, and measure the absorbance of the aqueous layer at either 540 nm (unmodified assay) or 590 nm (assay modified with 2-mercaptoethanol).

Prepare a 1 g/L aqueous stock standard of 5-HIAA (Sigma Chemical Co., St. Louis, MO 63178). Prepare a working 50 mg/L standard in water, and store it at 4 °C. Add 0.5 mL of working standard and 2.5 mL of water to the nitrosonaphthol/nitrous acid solution in place of the urine extracts. Quantitate by comparing sample absorbance with that of the standard and correct for differences in dilution. (In the case of urines expected to have high concentrations of 5-HIAA, dilute the samples.)

HPLC Assay
The HPLC assay was a modified version of that published by Beck et al. (5). We found that 5-HIAA standards degraded when prepared in methanol as suggested, with resulting changes in the standard curve. In contrast, a 5-HIAA standard prepared as described above showed no such degradation.

The internal standard was 5-hydroxyindole-2-carboxylic acid (Aldrich Chemical Co., Milwaukee, WI 53233). A 1 g/L solution in methanol is stable for several months at −20 °C.
The procedure is as follows. Add 200 µL of either urine or the appropriate standard to 0.5 mL of acetate buffer (1.0 mol/L, pH 4.54) containing 0.5 mmol of ascorbic acid per liter. Add 0.4 g of NaCl, 8 mL of ethyl acetate, and 25 µL of internal standard, and shake the mixture for 15 min. Evaporate the organic layer under a stream of nitrogen. Dissolve the residue in 200 µL of mobile phase and inject 10 µL into the chromatographic system. Pump the mobile phase (acetonitrile and 0.1 mol/L sodium phosphate buffer, pH 4.0, 15/85 by vol) at 2.5 mL/min. We used a Model M6000 solvent-delivery system (Waters Associates, Milford, MA 01757) equipped with a Rheodyne injector (Applied Science Division, Milton Roy Co. Laboratory Group, State College, PA 16801). For separation, use a Waters Associates C18 µBondapak column, and monitor the effluent with a Schoeffel FS970 fluorescent detector (Schoeffel Instrument Corporation, Westwood, NJ 07675). As suggested by Beck et al. (5), set the excitation wavelength at 272 nm, and use a 370 nm cut-off filter on the emission side.

Assay a set of standards with each batch of urine samples. Use the peak height ratios to construct a standard curve.

Results

Analytical Variables

Using control pool samples, we found the reproducibility of the 2-mercaptoethanol-modified colorimetric assay to be similar to the unmodified procedure. The between-run CV was 3.9% (X = 4.04 mg/L, n = 25), which compares favorably with within-run and between-run CV's for the HPLC method (X = 3.6 mg/L) of 5.0% (n = 10) and 8.7% (n = 10), and the between-run CV for the unmodified colorimetric assay of 4.5% (X = 3.5 mg/L, n = 30).

For analytical recovery studies we used the HPLC method. Five urine samples with 5-HIAA concentrations ranging from 3.2 to 14.4 mg/L were fortified to either 5 or 10 mg/L higher values with stock 5-HIAA and assayed. Mean recovery in these experiments was 101% (range, 95 to 104%).

The procedure is as follows. Add 200 µL of either urine or the appropriate standard to 0.5 mL of acetate buffer (1.0 mol/L, pH 4.54) containing 0.5 mmol of ascorbic acid per liter. Add 0.4 g of NaCl, 8 mL of ethyl acetate, and 25 µL of internal standard, and shake the mixture for 15 min. Evaporate the organic layer under a stream of nitrogen. Dissolve the residue in 200 µL of mobile phase and inject 10 µL into the chromatographic system. Pump the mobile phase (acetonitrile and 0.1 mol/L sodium phosphate buffer, pH 4.0, 15/85 by vol) at 2.5 mL/min. We used a Model M6000 solvent-delivery system (Waters Associates, Milford, MA 01757) equipped with a Rheodyne injector (Applied Science Division, Milton Roy Co. Laboratory Group, State College, PA 16801). For separation, use a Waters Associates C18 µBondapak column, and monitor the effluent with a Schoeffel FS970 fluorescent detector (Schoeffel Instrument Corporation, Westwood, NJ 07675). As suggested by Beck et al. (5), set the excitation wavelength at 272 nm, and use a 370 nm cut-off filter on the emission side.

Assay a set of standards with each batch of urine samples. Use the peak height ratios to construct a standard curve.

Results

Analytical Variables

Using control pool samples, we found the reproducibility of the 2-mercaptoethanol-modified colorimetric assay to be similar to the unmodified procedure. The between-run CV was 3.9% (X = 4.04 mg/L, n = 25), which compares favorably with within-run and between-run CV's for the HPLC method (X = 3.6 mg/L) of 5.0% (n = 10) and 8.7% (n = 10), and the between-run CV for the unmodified colorimetric assay of 4.5% (X = 3.5 mg/L, n = 30).

For analytical recovery studies we used the HPLC method. Five urine samples with 5-HIAA concentrations ranging from 3.2 to 14.4 mg/L were fortified to either 5 or 10 mg/L higher values with stock 5-HIAA and assayed. Mean recovery in these experiments was 101% (range, 95 to 104%).

The standard curve for the HPLC method was linear up to at least 20 mg/L (Figure 1). Peak-height ratios for the standards were constant from day to day when aqueous 5-HIAA standards were used. Under the chromatographic conditions described, 5-HIAA, 5-hydroxyindole-2-carboxylic acid, and other interfering peaks were separated in less than 8 min per sample (Figure 1, inset).

Method Comparison

We compared values for 45 specimens, in the range of 0 to 30 mg/24 h, as assayed by both the 2-mercaptoethanol-modified (x) and the unmodified (y) colorimetric assays. The least-squares regression equation representing these points is y = 0.222x + 4.5 (r = 0.4380). There is considerable disparity between the results obtained by the two methods, especially in the lower range. When 26 specimens in the 50 to 600 mg/24 h range were compared by the two colorimetric assays, there was excellent agreement. The least squares regression equation is y = 0.965x + 10.16 (r = 0.9908).

We also compared values obtained by unmodified colorimetric assay (y) and HPLC assay (x), for the same experiments as above, in the 0 to 30 mg/24 h range. This scattergram looks very similar to that described above, with least squares regression analysis again indicating poor correlation: y = 0.08x + 6.92 (r = 0.2955, n = 46). As was the case when we compared the two colorimetric assays, a comparison of the results from the HPLC and unmodified colorimetric assay for samples ranging from 50 to 600 mg/24 h gave a much better correlation. The regression line was y = 0.85x + 33.3 (r = 0.9417, n = 14).

A comparison of the 2-mercaptoethanol-modified colorimetric assay and the HPLC assay revealed a different relationship from that seen in the two previous comparisons. Not only was there a correlation between these two assays good in the broad range of 50 to 600 mg/24 h (y = 0.84x + 29.99, r = 0.9451, n = 13) but, as shown in Figure 2, the correlation was also quite good in the 0 to 30 mg/24 h range. The linear regression equation, based on results obtained for the same specimens

Fig. 1. Standard curve for the HPLC assay
Inset: typical chromatogram of patient's sample

Fig. 2. Comparison of modified colorimetric and HPLC assays in the lower range
Regression plus point marked by *: y = 0.85x + 0.11, r = 0.9303; (minus point marked by *). y = 0.87x + 0.45, r = 0.9663. Line drawn by regression analysis.
used in the two previous comparisons was represented by the
equation \( y = 0.85x + 0.11 \) \( (r = 0.9303, n = 44) \).

One specimen (marked with an asterisk in Figure 2) showed
extremely heavy interference, having been originally read as
95.2 mg/24 h in the unmodified colorimetric assay. In the
modified assay, this sample was a distinct pink color, not the
usual blue. The 2-mercaptoethanol modification could not
completely correct for this interference. If this point is elim-
ninated from the analysis, the regression becomes \( y = 0.87x +
0.45 \) \( (r = 0.9663, n = 43) \).

**Normal Range**

Twenty-four-hour urine specimens were collected by 40
healthy volunteers and analyzed for 5-HIAA by the 2-mer-
captoethanol-modified colorimetric assay. The mean result
was 3.8 (SD 2.8) mg/24 h. For clinical purposes we have de-
finite the normal range as 6.0 mg/24 h or less.

**Discussion**

When the unmodified and 2-mercaptoethanol-modified
assays are compared, assuming the values generated by the
modified assay to be correct, interference may range from
slight to substantial. An alternative interpretation of the data,
however, is that 2-mercaptoethanol may interfere with the
color formation. The data for the modified and unmodified
assays in the high range argue against this; correlation was
excellent. This is most likely because all of these samples had
to be diluted before assay: in doing so we were diluting the
interfering substance, thereby enhancing the opportunity for
correct color to form. Because the amount of 2-mercapto-
ethanol remained the same in all assays, it is unlikely that it
was the cause of the discrepancy in the results in the low
range.

Comparison of the colorimetric assays with the HPLC assay
also supports the contention that the unmodified assay suffers
from considerable interference, while the modified assay does
not.

Finally, the colorimetric assay, even with the modification,
is much faster than the HPLC method for a large batch of
samples, about 150 min vs 250 min for a 20-sample batch.

In summary, we conclude that the 2-mercaptoethanol
modification proposed by Goldenberg does in fact correct for
much of the interference commonly seen in the colorimetric
assay for 5-HIAA in urine, and for the high-volume laboratory
would be the method of choice. For smaller laboratories, either
the modified colorimetric assay or the HPLC assay, as out-
lined here, would be acceptable.

We thank Richard Woodruff for his excellent technical assistance
and Dr. Thomas Moyer for the use of his chromatographic equipment
during the early phases of this work.

**References**

1. Udenfriend, S., Titus, E., and Weissbach, H., The identification
   of 5-hydroxy-3-indoleacetic acid in normal urine and a method
2. Fornstedt, N., Determination of 5-hydroxyindole-3-acetic acid
   in urine by high-performance liquid chromatography. *Anal. Chem.* 50,
3. Goldenberg, H., Specific photometric determination of 5-hy-
4. Willliams, C. M., and Sweeley, C. C., A new method for the deter-
5. Beck, O., Palmskog, G., and Hultman, E., Quantitative determi-
   nation of 5-hydroxyindole-3-acetic acid in body fluids by high-per-
   urinary 5-hydroxy-3-indoleacetic acid, with electrochemical detection.
7. Draganac, P. S., Steindel, S. J., and Trawick, W. G., Liquid chro-
   matographic separation of urinary 5-hydroxy-3-indoleacetic acid, with