Liquid-Chromatographic Determination of Urinary 5-Hydroxy-3-indoleacetic Acid, with Fluorescence Detection

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We describe and evaluate a procedure for measuring urinary 5-hydroxy-3-indoleacetic acid by "high-performance" liquid chromatography. After a simple organic extraction, the analyte and internal standard are chromatographed on a reversed-phase column and are detected by native fluorescence. The detection limit (3 ng per injection), between-day precision (CV 5.2%), absolute recovery (70%), analytical recovery (99%), and working linear range (up to 15 mg/L) have been determined. Compared with colorimetric results with nitrosonaphthol, values obtained with the chromatographic method are significantly lower. Reference values and clinical experience with the method are reported. The method is simple, free from interferences, and suitable for use in routine analysis in the clinical laboratory.

Additional Keyphrases: fluorometry · carcinoid tumor · reference interval

Measurement of 5-hydroxy-3-indoleacetic acid (5HIAA), the major metabolite of 5-hydroxytryptamine (serotonin) appearing in the urine, is important in detecting carcinoid tumors (1, 2) and the study of neurological disorders (3). The widely used colorimetric methods involving nitrosonaphthol lack specificity for 5-HIAA, and various medications and metabolites can produce false-positive or false-negative results (4). In an attempt to improve analytical specificity, liquid-chromatographic procedures for 5-HIAA have been reported. A chromatographic method with a fluorescence detection was shown to be specific for 5HIAA (5), but in this method the extraction procedure is complicated by sample-matrix effects and the method was not fully evaluated for application in the clinical laboratory. More recently, alternative liquid-chromatographic procedures with electrochemical and ultraviolet detectors have been reported (6, 7). Both methods, however, lack specificity, owing to multiple urinary compounds eluting close to 5-HIAA. Because of problems with sample preparation and detector specificity, practical application of these liquid-chromatographic procedures has been limited.

In the present report we describe an improved method, which is both specific for 5HIAA and simple to perform.

Materials and Methods

We use a liquid chromatograph (Model 870; DuPont Instruments, Wilmington, DE 19898) with a 4.6 × 25 cm CLC-1 column (DuPont), packed with octyl silica and maintained at room temperature. We also use a 4.6 × 5 cm guard column containing Permaphase ODS (DuPont). The flow rate of the mobile phase [methanol/sodium acetate buffer (0.1 mol/L, pH 5.5), 1/4 by vol] is 1.3 mL/min. The native fluorescence of 5-HIAA and the internal standard is monitored with a spectrofluorometer (Model 204-A; Perkin-Elmer Corp., Norwalk, CT 06856) equipped with a fluorescence flow cell. The excitation is set at 300 nm and emission at 350 nm.

A stock standard (0.5 g/L) of 5-HIAA (Sigma Chemical Co., St. Louis, MO 63178) is adjusted to a pH below 3 with glacial acetic acid and is stable for at least one month at 8 °C. Working standards, 2.5 to 15 mg/L, are prepared on the day of assay. For an internal standard we use 5-hydroxy-3-indolepropionic acid (5-HIPA), provided by Dr. Albert Manion, Neuroscience Research Branch, National Institute of Mental Health, Rockville, MD 20857. A stock solution (0.5 g/L) of 5-HIPA is prepared and stored under the same conditions as the stock 5-HIAA solution; working internal standard solution (5 mg/L) is prepared by dilution with water. For the correlation and reference value studies, we collected 24-h urine samples in glass bottles containing 12 mL of HCl (6 mol/L); samples not assayed within 24 h were stored frozen. The colorimetric measurement of 5-HIAA is based upon the method of Udenfriend et al. (2) as modified by Dalgleish (8).

The procedure: Combine a 50-μL aliquot of acidified urine or of working standard solution with 50 μL of working internal standard solution and 150 μL of HCl (0.7 mol/L). Add 1.5 mL of anhydrous diethyl ether (Mallinckrodt, Paris, KY 40361) and vortex-mix for 30 s. After centrifugation, transfer approximately 1 mL of the ether layer to a clean tube and evaporate with a gentle stream of air. (For experiments to determine absolute extraction efficiency, we recovered as much as possible of the ether layer.) Reconstitute the dried extract with 400 μL of mobile phase and inject 50 μL into the liquid chromatograph. Use peak height ratios of 5-HIAA and 5-HIPA for calculation.

Results and Discussion

Figure 1 shows a chromatogram of standard and urine samples. 5-HIAA and 5-HIPA have retention times of 3.6 and 4.9 min, respectively. Indoleacetic acid, observed in some of the urine chromatograms, has a retention time of 7.2 min. No other peaks were observed in the patterns for the more than 300 urine samples we have chromatographed so far. Experiments in which potential interferents were added to urines showed no interference by vanillymandelic acid, homovanillic acid, serotonin, salicylate, or gentisic or salicylic acids.

The absolute analytical recovery of 5-HIAA in aqueous standards (2.5 and 5.0 mg/L) ranged from 66 to 72% (n = 3). That for 5-HIPA at the concentration used in our assay system was 78 to 79% (n = 3). Recovery for 5-HIAA in 10 urine samples to which 5 mg of 5-HIAA per liter had been added ranged from 96 to 105% (mean 99%). These extraction-efficiency data were obtained without the use of a salting-out technique. Contrary to the reports of others (5, 9), we find that salt does not affect the partitioning of 5-HIAA into the extraction solvent. Saturation with salt resulted in an absolute recovery of 65 to 69%. This discrepancy may be explained by the lower pH (1.3) in our extraction system. However, we did find that saturation significantly decreased the partitioning of 5-HIPA into the ether layer (absolute recovery, 47 to 52%). Because of this poor recovery, saturation with salt is not recommended in our procedure.

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We correlated results by this procedure with those by a colorimetric method (8), using 52 24-h urine samples. Comparison with the nitrosonaphthol method (y) gave a regression equation of $y = 3.1x + 0.9$ ($r = 0.91$). The colorimetric assay gave higher results in 46 of the 52 comparisons. To establish a reference range for the chromatographic assay, we assayed 24-h urine samples from 27 individuals who did not have a diagnosis of carcinoid tumor (laboratory personnel and patients under evaluation for other disorders). 5-HIAA excretion ranged from 1.1 to 7.0 mg/24 h (mean 3.9 mg/24 h). In two patients with a diagnosis of carcinoid tumor, urinary 5-HIAA was 82 and 156 mg/L.

The proposed method is sensitive and specific for 5-HIAA and has been fully evaluated for analytical performance. As compared with other chromatographic methods we find it simpler to perform, and the chromatographic separation is clean and efficient. We are currently using the method in our clinical laboratory for routine analysis.

References