Liquid-Chromatographic Separation of Urinary 5-Hydroxy-3-indoleacetic Acid, with Measurement at 254 nm

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A “high-performance” liquid-chromatographic procedure for 5-hydroxy-3-indoleacetic acid is described and compared with a colorimetric method in which 1-nitroso-2-naphthol is used. The analyte and an internal standard, p-nitrobenzoic acid, were extracted into diethyl ether from urine at pH 4.0 (acidified with HCl) to which sodium chloride had been added, and the ether was back-extracted with acetate buffer, pH 9.2. Aliquots of this extract were injected into a reversed-phase liquid-chromatographic column and eluted with pH 3.5 acetate buffer/methanol (95/5 by vol); the effluent was monitored at 254 nm. The precision (CV) of the method was 11.8% at 1.8 mg/L, 5.5% at 92 mg/L. Analytical recovery averaged 84%. The colorimetric method gave higher values for the analyte than did the chromatographic method for all patients’ urines.

Additional Keyphrases: carcinoid tumors • colorimetry compared

Carcinoid tumors, perhaps the most common hormone-producing tumors (1), generally secrete various amounts of indoles. Many of them secrete other substances such as serotonin, bradykinin, corticotropin, and histamine (2), but carcinoid syndrome is generally characterized by an increased urinary excretion of 5-hydroxy-3-indoleacetic acid (5-HIAA), formed metabolically from serotonin (3). Thus, measurement of urinary 5-HIAA assists in the diagnosis of carcinoid tumors (4). The most widely used procedures for doing so are based on a method (5) in which a colored product is formed by the reaction of 1-nitroso-2-naphthol and 5-HIAA in the presence of nitrous acid. Increasing awareness of the lack of sensitivity and specificity (6, 7) of these methods has resulted in procedures that are more specific, but also more complex and often less sensitive and less accurate.

This study was prompted by a case exhibiting the carcinoid syndrome, including increased urinary 5-HIAA (>300 mg/24 h by a colorimetric method), but with no postmortem evidence of carcinoid tumors.

Chromatographic separation of analyte from interfering compounds is a logical attempt to improve the analysis and to eliminate false positives. Other efforts similar to ours have been reported. Graffeo and Karger (8) used reversed-phase liquid chromatography with a fluorescence detector, without extraction; Beck et al. (9) added an ethyl acetate extraction step before chromatography and measured ultraviolet absorption. Use of fluorometry reduced the interference near the beginning of the chromatogram, but a preliminary extraction achieves the same end.

The greater concentration of 5-HIAA found with our ex-
(5) to be the most effective. At pH 4.0 the 5-HIAA and the internal standard were readily extracted into diethyl ether and then back-extracted into a small volume of the pH 9.2 buffer. Addition of NaCl to the urine improved recovery. We used HCl to acify urine samples. If acetic acid was used, its solubility in diethyl ether often exceeded the capacity of the aqueous buffer; thus, for samples so treated, one must be certain that the pH of the aqueous buffer remains near 9.2.

p-Nitrobenzoic acid, used as the internal standard, is extracted along with the 5-HIAA and is eluted satisfactorily. It was added as a 1 mmol/L solution in methanol/water (10/90 by vol).

**Procedure**

Aliquots of 24-h urine specimens were acidified (with HCl to pH 5 or below), and stored in the dark at about 5 °C (or frozen if analysis was to be much delayed). To a glass-stopped centrifuge tube containing 5 g of NaCl and 25 mL of diethyl ether was added a mixture of 10 mL of urine and 2 mL of internal standard solution that had been adjusted with 10 mmol/L NaOH or HCl to pH 4.0. This mixture was shaken for 5 min, centrifuged, and a 10-mL portion of the organic phase was transferred to another tube containing 0.5 mL of the pH 9.2 buffer, shaken for 5 min, and centrifuged. Aliquots (usually 20 µL) of the aqueous (bottom) layer were injected into the chromatograph, and eluted with a mobile phase (acetate buffer/methanol, 95/5 by vol) pumped at 3.0 mL/min (about 20.7 MPa or 3000 lb/in.²). Typically, the retention time for 5-HIAA under these conditions was about 15 min, and a complete chromatogram (Figure 1) required 30 min or less. Standards were prepared by weighing pure 5-HIAA into aliquots of urine that previously contained negligible amounts of the compound (as measured chromatographically).

For comparison, we determined 5-HIAA in all samples by the HyceI method, 3 which was the routine procedure used in the case that prompted this study.

**Evaluation**

A standard curve consists of a plot of 5-HIAA concentration in the standards vs the ratio of the observed HPLC peak areas of 5-HIAA to the internal standard. The curve is linear to 250 mg/L (40.1 µmol/L), then curves slightly from 250 to 500 mg/L (45.1-96.2 µmol/L), the maximum concentration tested. Sample concentrations exceeding 300 mg/24 h (57.7 µmol/24 h) are uncommon.

Quality-control specimens, prepared as were the standards at concentrations approximating 20 mg/L (3.8 µmol/L) and 100 mg/L (19.2 µmol/L), were analyzed by both the HyceI method and the present method. Table 1 summarizes the results. Single-variable analysis of variance, with method as the variable, showed results by these methods to differ significantly (p < 0.001) for the low controls, but not (p > 0.6) for the higher concentrations (72).

Analytical recovery of added 5-HIAA averaged 84% (SD, 8%) for the chromatographic method, with insignificant variation over the range studied. However, the HyceI method gave recoveries of 155% (SD, 27%) for low concentrations (<10 mg/L), 93% (SD, 4%) for intermediate concentrations, and 84% (SD, 4%) for the high concentrations (>100 mg/L).

The regression equation for concentrations of 5-HIAA (ranging from 1 to 16.6 mg/L by HPLC) in urines from 19 patients, as determined by the chromatographic method (x) and the HyceI method (y), was y = 1.94x + 4.37, with poor correlation (standard error of y = 6.47). The only pattern we saw was that the concentration by the colorimetric method always exceeded that by the chromatographic method. In some cases the colorimetric values deviated greatly; e.g., concentrations of 13.4, 19.7, 23.6, 22.4, and 43.8 mg/L by the HyceI method gave, respectively, 1.0, 1.9, 5.1, 6.3, and 16.6 mg/L by the present method. Goodwin et al. (13) came to a similar conclusion in their comparison of a gas-chromatographic method with a fluorometric method for 5-HIAA. A confirmatory test would be a prudent follow-up to colorimetric measurements yielding high 5-HIAA.

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**References**


Corrections
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p 374: Second column, line 1: “designated,” not “design.”

p 457: First line of abstract: correct number is “2.7.3.2”; second paragraph under “Serum Samples”: “mammary artery,” not “mammary vein.”

p 458: Under “Column Chromatography,” line 9 to read “buffer (200 mmol/L NaCl, 50 mmol/L Tris-HCl) . . .” Sublegend to Figure 1: add “a, concentration gel; b, separation gel.” Under “Isoelectric Focusing,” line 6 should read “7 mg of ammonium persulfate, 0.1 mL of pH 4-6 carrier ampholyte, and 0.1 mL of pH 5-7 ampholyte.”

p 485: last paragraph: insert “98” before “(29.4%),” delete “low value,” change “(6.5%),” to “(6.3%),” and change “μ/L” to “μg/L.”