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Note

High-performance liquid chromatography of 5-hydroxyindole-3-acetic acid in urine with direct sample injection

VICTOR SKRINSKA*,* and SHINEUI HAHN

Department of Biochemistry, The Cleveland Clinic Foundation, Cleveland, OH 44106 (U.S.A.)

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Early detection of carcinoid tumors occurring in the small intestine is essential for successful surgical treatment. Since the tumors release large amounts of 5-hydroxytryptamine, an elevated level of 5-hydroxyindole-3-acetic acid (HIAA), the major metabolite of 5-hydroxytryptamine, in the urine is a reliable indicator of carcinoid tumors [1—3]. A number of methods have been described for quantitative determination of HIAA in urine. Udenfriend et al. [2] originally described the widely used nitrosonaphthol colorimetric method which was later modified by Goldenberg [4]. This method is subject to error owing to interfering substances in the urine as well as error introduced by incomplete extraction [5]. Several high-performance liquid chromatographic (HPLC) procedures have been described with increased sensitivity and specificity for HIAA with electrochemical or fluorescence detection [6—11]. Most of these procedures require sample extraction prior to analysis to eliminate interfering peaks. Wahlund and co-workers [8, 9] have reported a direct injection method for HIAA in urine; however, a complex chromatographic system which incorporates tributyl phosphate in the stationary phase is required. We are describing a rapid and simple fluorescence HPLC method with direct injection on an unmodified reversed-phase chromatographic system. The sensitivity and reproducibility of the method are sufficient for routine quantitative screening of urine for HIAA.

*Present address: Cleveland Research Institute, 2351 East 22nd Street, Cleveland, OH 44115, U.S.A.

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EXPERIMENTAL

Apparatus

Chromatography was performed with a Waters isocratic liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.). The system included a manual injector, an M6000 pump, a 50 × 3.9 mm guard column packed with Vydac RP (Varian, Synnyvale, CA, U.S.A.), and a 300 × 3.9 mm μBondapak C_{18} analytical column (Waters Assoc.). The effluent was continuously monitored with an Aminco Bowman Model J4-8960A variable-excitation- and -emission-wavelength spectrophotofluorometer (Aminco Bowman, Silver Spring, MD, U.S.A.) with an HPLC flow cell.

Reagents

Anhydrous sodium acetate and glacial acetic acid were reagent grade. Methanol and water were HPLC grade. Acetate buffer was prepared by adjusting a 0.1 *M* sodium acetate solution to pH 4.5 with acetic acid. The acetate buffer and methanol were filtered through 0.45- and 0.5-μm filters, respectively, and degassed prior to use. Standard solutions of HIAA (Sigma, St. Louis, MO, U.S.A.) and the internal standard, 5-hydroxyindole-2-carboxylic acid (HICA) (Aldrich, Milwaukee, WI, U.S.A.) were prepared at 500 μg/ml in water and adjusted to pH < 3 with acetic acid.

Procedure

Urine samples were initially centrifuged to remove solid material. Sample aliquots of 100 μl were mixed with an equal volume of the internal standard and 20-μl aliquots were injected for HPLC. The chromatography was performed at room temperature with a mobile phase of methanol—acetate buffer (14:86), and a flow-rate of 1.4 ml/min. The effluent was continuously monitored at an excitation wavelength of 295 nm and emission wavelength of 345 nm. Positive samples above the calibration range were diluted with water and rerun.

For calibration aqueous dilutions of HIAA at concentrations ranging from 1 to 30 μg/ml were substituted for the urine samples. Peak height ratios relative to the internal standard were used for quantitation.

RESULTS

Fig. 1 is a typical chromatogram of a 10 μg/ml HIAA standard. The retention times for the internal standard and HIAA were 5.5 and 7.5 min, respectively. The retention times for other compounds tested for interference were: 5-hydroxytryptophan, 3.5 min; 5-hydroxytryptamine, 4.8 min; tryptophan, 6.1 min; and indol-3-acetic acid, not detected.

Calibration curves with aqueous standards were linear from 1 to 30 μg/ml with an intercept at 0. The minimum detection limit based on a signal-to-noise ratio of 3:1 was 0.2 μg/ml using the dilutions and injection volume described in the procedure. Calibration curves prepared by spiking negative urine samples with HIAA were identical with those obtained with aqueous standards when the HIAA originally present in the urine was subtracted from each point.

The within-run coefficient of variation at a level of 1.4 μg/ml was 9.2% (n = 10) and 2.3% (n = 10) at a level of 433 μg/ml. The day-to-day coefficient of variation was 6.7% (n = 20) at 1.4 μg/ml and 3.2% (n = 20) at 433 μg/ml.

All samples were finally calculated as μg HIAA per mg creatinine. A level of 3.2 ± 1.7 μg HIAA per mg creatinine (± S.D., n = 47) was obtained for patients without carcinoid tumors. Fig. 2 is an example of the chromatograms obtained for negative samples. The unidentified peaks in Fig. 2 were present in all negative patient chromatograms and varied in intensity. The retention times of these peaks differed from the standard compounds tested. The results for five patients with carcinoid tumors were: 323, 267, 25, 63, and 226 μg HIAA per mg creatinine. Fig. 3 shows the a chromatogram of a positive sample with 433 μg/ml HIAA or 323 μg HIAA per mg creatinine.

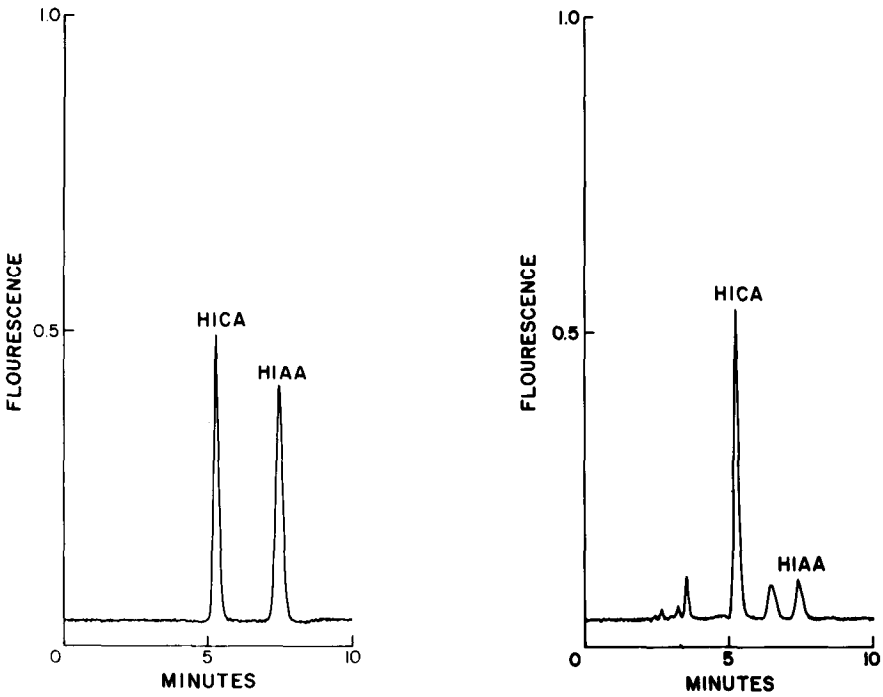

Fig. 1. Chromatogram of an aqueous HIAA standard and the internal standard, HICA. Initial concentrations were: HIAA, 10 μg/ml; HCA, 500 μg/ml. A mixture containing 10 μl of each was injected.

Fig. 2. Chromatogram of a negative patient urine containing a 1.4 μg HIAA/ml.

DISCUSSION

Direct injection of biological fluids offers significant advantages over extraction methods by considerably shortening the sample preparation time and eliminating errors that may arise from variable extraction recoveries. In many cases, however, direct injection is not practical owing to numerous interfering peaks and a decrease in the life of the analytical column. We have minimized

these effects by using a variable-wavelength spectrofluorometer for detection and maintaining a small sample injection volume. The pH of the mobile phase and the excitation and emission wavelengths were optimized to enhance the HIAA and HICA fluorescence relative to other fluorescent substances present in the urine. The retention time of HIAA varied with pH as a single peak with no evidence of interference. The selectivity is significantly improved with a variable-wavelength detector. In comparison, chromatograms under similar conditions with a filter fluorescence HPLC detector contained a number of interfering peaks.

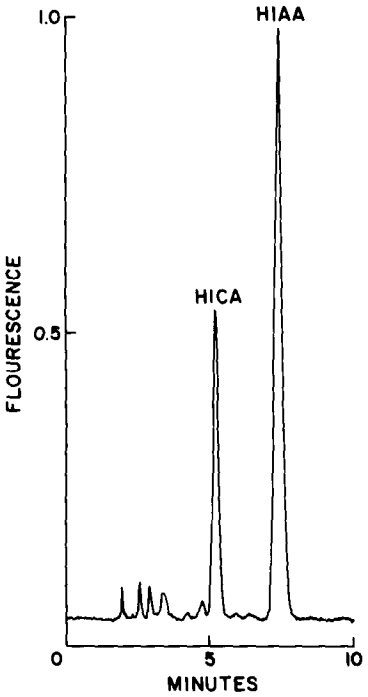

Fig. 3. Chromatogram following a 1:20 dilution of urine from a patient with carcinoid tumor. The undiluted concentration of HIAA was 433 μg/ml.

The guard column was routinely repacked each month when the assay was performed daily. There was no observed change in retention times, increase in column pressure, or any apparent decrease in analytical column life. The procedure described minimizes the problems associated with direct injection an provides a rapid and simple quantitative measurement of HIAA in urine. The procedure has good reproducibility and sufficient sensitivity for routine quantitative screening of urine for HIAA.

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