Column-Chromatographic Analysis of Naturally Fluorescing Compounds: II. Rapid Analysis of Indoleacetic Acid and 5-Hydroxyindoleacetic Acid in Biological Samples

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A previously described [CLIN. CHEM. 18, 778 (1972)] chromatographic separation and fluorometric system has been adapted to determination of 5-hydroxyindole-3-acetic acid and indole-3-acetic acid in body fluids and biological extracts. A strong anion-exchange resin, "Aminex A-27," is used. The indoleacetic acids are eluted at 60°C with a single ammonium acetate-acetic acid buffer. No gradient programming or post-column reaction designed to enhance the fluorescence of the desired compounds is necessary. The sensitivity of the detector allows analysis of 100 ng of each acid in the sample loaded onto the column.

Additional Keyphrases serotonin • anion-exchange chromatography • spectrophotometry • micromethod • serum, urine, tissue extracts

Two indoleacetic acids, 5-hydroxyindole-3-acetic acid (5-HIAA) and indole-3-acetic acid (IAA), are the subject of much scientific investigation, both in plants and in animals. Because 5-HIAA is the metabolic product of serotonin (1), the concentration of 5-HIAA in the milieu of nerve tissue has been carefully studied as an indication of the synthesis of serotonin in those regions (2, 3), especially when the biological system under investigation has been perturbed by the addition of various drugs or chemicals (4-7). Indoleacetic acid, on the other hand, is currently receiving considerable attention as a growth regulator and hormone in plant tissues (8-10). Both compounds are present in human body fluids and are of sufficient interest to justify the investigation of methods for their rapid identification and quantitation.

Several approaches have been taken in an attempt to identify 5-HIAA and IAA. Although thinlinear chromatographic schemes have been devised (2, 11), they possess inherent difficulties with respect to quantitation. A liquid chromatographic system for the determination of 5-HIAA has recently been reported (12). This system consists of a commercially available amino acid analyzer (cation-exchange) with programmable elution and fluorescence-enhancing O-phthalaldehyde reaction in the column effluent; however, the procedure is complex and does not allow the simultaneous determination of IAA. Gas-liquid chromatography has also been used (13), but this approach also has certain undesirable aspects (2) that can be avoided by the technique described here, in which I used a liquid chromatographic system with a strong anion-exchange resin and a single, concentrated eluent. A sensitive fluorometer was used to monitor the compounds in the column effluent.

Materials and Methods

Reagents and Equipment

Reagents. Ammonium acetate-acetic acid buffer, 4.0 molar in ammonia and 5.8 molar in acetate, with a pH of 5.53, was prepared from reagent-grade ammonium acetate and acetic acid. A total of 308.3 g of ammonium acetate was dissolved in 500 ml of distilled water, 107.8 g of acetic acid was added, and the resulting solution was diluted to 1 liter with distilled water.

Standards. The two reference compounds, 5-HIAA (Mann Research Laboratories, New York, N. Y. 10006) and IAA (Calbiochem, San Diego, Calif. 92112), were prepared in stock solutions of approximately 100 ¡g/ml in the 4 molar buffer, and were diluted as necessary.

Column. The jacketed column used in this investigation, 0.22 cm in diameter and 25 cm long, was fabricated from stainless-steel tubing. The resin was supported by a 1/8- to 1/16-in. reducer union containing a 0.5-µm stainless steel frit. The column was slurry packed (14) with the ammonium acetate-acetic acid buffer at a column temperature of 60°C and a volumetric flow rate of 30 ml/h. A Lapp "Microflow Pulsafeeder," Model LS-30 (Process & Instruments Corp., Brooklyn, N. Y. 11207), was used in the column-packing operation and subsequent elution.

Resin. The column was packed with a strong anion-exchange resin, "Aminex A-27" (Batch...
No. 9428), having a particle size range of 12 to 15 \( \mu \)m (Bio-Rad Laboratories, Richmond, Calif. 94804). This resin was cleaned before use by washing successively with acid and alkali (1 mol/liter).

**Sample injection valve.** Samples were introduced onto the column by means of a sample injection valve developed at ORNL (15). The capacity of the sample loop was 95 \( \mu \)l. The loop was flushed with buffer and emptied before sample loading. About 1 ml of sample was passed through the loop to ensure that the 95-\( \mu \)l sample loaded onto the column was not diluted by the buffer used to rinse the loop.

**Spectrophotofluorometric detector.** An Aminco-Bowman scanning spectrophotofluorometer (American Instrument Co., Silver Spring, Md. 20910), fitted with a small flowcell, was used as the fluorescence detector. The excitation wavelength was set at 300 nm and 291 nm, and the emission wavelength at 346 nm and 365 nm for 5-HIAA and IAA, respectively. The fluorometer was operated in such a mode that the output to the recorder was linear in relative intensity. This was verified by measuring the relative intensity of a given sample at different sensitivity settings on the instrument. The fluorometer, which can be operated over a relative intensity scale ranging from 0.1 to 100 (full scale), was normally operated at a sensitivity setting (0.003) corresponding to a relative intensity of 0.3 for full-scale deflection. A Leeds & Northrup “Speedomax Type G” single-pen recorder (Leeds & Northrup Co., North Wales, Pa. 19454) was used to provide a permanent record of the analyses.

The system is like that diagrammed in Figure 1 of reference 17, except that there is a single column rather than two coupled columns.

**Operation**

Urine samples were generally filtered before use, primarily to lengthen column life rather than to improve resolution. The column was maintained at a temperature of 60°C; the flow rate was held at 30 ml/h, with a pressure drop across the column of 18.63 N/m² (2700 psi). The 95-\( \mu \)l sample loop was rinsed and filled with the sample. The pen on the recorder was set to a major division, and the contents of the loop were injected into the column. Except for changing the excitation and emission settings on the fluorometer after the elution of 5-HIAA (to maximize the response of the two compounds), the system required no further attention until the next sample was loaded into the sample loop. Thus a minimal amount of operator time was necessary to maintain the system in an operational mode.

**Results and Discussion**

Since 5-HIAA and IAA are firmly retained on a strong anion-exchange resin (15, 17), liquid chromatography with this type of resin seemed to be a logical system of choice. Such a chromatographic system would require a concentrated buffer to elute the compounds in a reasonable length of time; also, the use of a concentrated buffer would tend to accelerate removal of the less tightly bound compounds that might otherwise interfere with the chromatographic analysis of 5-HIAA and IAA. In addition, a buffer of high ionic strength would elute all (or essentially all) of the compounds in the biological sample loaded onto the column, thus eliminating the need for a column-regeneration step. These considerations provided further support for the feasibility of using a single, concentrated buffer in the chromatographic system, an approach definitely preferable to a programmed buffer system. Because the compounds of interest display a strong native fluorescence, they could be detected with a sensitive fluorometer, obviating the need for a fluorescence-enhancing reaction in the column effluent.

Quantitation of chromatographic peaks generally involves measuring the area of each individual peak of interest in the chromatogram and relating that area to the concentration of compound corresponding to the measured peak. Because peak heights are easier to measure than peak areas, the relationship between peak height (relative intensity) and total amount of compound loaded onto the column was investigated (Figure 1). The log-log plot of peak height vs. the amount

![Fig. 1. Relationship between amount of 5-HIAA of IAA introduced onto the column to relative intensity of the resulting peak at maximum peak height](image)

\( \Delta \) 5-HIAA; O IAA
could be increased about six-fold by increasing the sensitivity of the fluorometer and resetting the excitation and emission wavelengths to optimize the response from IAA. The stable baseline allows reasonable and reproducible analyses at maximum detector sensitivity.

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References