New Assay for Tryptophan and Its 5-Hydroxyindole Metabolites in Blood

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We describe a new cation-exchange fluorometry procedure for separation and measurement of three 5-hydroxyindole metabolites of tryptophan in blood. The metabolites are detected and measured via the fluorescence emitted in the visible region when the separated metabolite is added to a hydrochloric acid–ascorbic acid mixture. The assay requires only 30 min and is both selective and sensitive. The normal concentration of 5-hydroxytryptamine (serotonin) in serum is 98 μg/liter; 5-hydroxyindole acetic acid and 5-hydroxytryptophan are normally present in concentrations of less than 20 μg/liter.

Additional Keyphrases: normal values • 5-hydroxytryptamine (serotonin) • 5-hydroxyindole acetic acid • monitoring the dialyzed patient • cation-exchange chromatography • fluorometry

The abnormal metabolism of tryptophan has been observed in patients suffering from a wide variety of diseases. Rose (1), Davis et al. (2), and Bell et al. (3) have shown that patients afflicted with various forms of cancer metabolize tryptophan to yield higher than normal amounts of several intermediate metabolites along the kynurenine pathway. Uremic patients have lower than normal concentrations of tryptophan in their serum; presumably its metabolites are likewise altered (4).

Tryptophan is metabolized via two major pathways, the kynurenine pathway and the serotonin pathway. Several serum-assay procedures for metabolites along the kynurenine pathway have been reported, but few procedures exist for metabolites along the serotonin pathway (see references 5 and 6, for examples).

We have developed a rapid, selective, and sensitive assay procedure, with which blood concentrations of 5-hydroxytryptophan, 5-hydroxytryptamine (serotonin), and 5-hydroxyindole acetic acid can be measured. These three metabolites may be assayed in about 30 min by use of a cation-exchange column for separation and fluorescence analysis for detection and quantitation. This assay, used in conjunction with our recently reported assay for tryptophan (7), should prove useful for investigation of the metabolism of tryptophan.

The need for rapid, sensitive, and selective assay procedures for tryptophan and its metabolites is perhaps best exemplified by consideration of the use of dialysis equipment on patients suffering from chronic uremia. The dialysis procedure normally requires 8 h; hence, a rapid analytical procedure might make it feasible to monitor the blood of the patient, to follow the progress of the treatment. Selective assay procedures are required, because structural differences among the various metabolites that may be present are often subtle.

Materials and Methods

Reagents

1-Tryptophan, 1,5-hydroxytryptophan, 1,5-hydroxytryptamine (creatinine sulfate complex), and 5-hydroxyindole acetic acid were obtained from Sigma Chemical Co., St. Louis, Mo. 63178, and were used as received.

Sodium borate (certified ACS reagent), sodium hydroxide (certified ACS reagent), trichloroacetic acid (certified ACS reagent) and formaldehyde (12 mol/liter, certified ACS reagent) were obtained from Fisher Scientific Co., Fairlawn, N. J. 07410.

L-Ascorbic acid (Baker analyzed reagent) and Dowex 50W-X8 cation-exchange resin (100–200 mesh, H⁺ form) were obtained from J. T. Baker Chemical Co., Phillipsburg, N. J. 08865. Ascorbic acid was used as received. The resin was converted
to the Na\(^+\) form by washing 100 g five times with 300-ml portions of 0.2 molar NaOH. Excess NaOH was removed by washing the resin with doubly distilled water until the wash water was neutral. A chromatographic column was prepared by packing the resin in a burette (1 cm i.d.) to a height of 3 cm.

All fluorometric measurements were made with an Aminco-Bowman Spectrofluorometer, Model 4-8202 (American Instrument Co., Silver Spring, Md. 20910).

Methods

Two milliliters of blood was shaken well for 10 min. The serum was separated and was then mixed with 1 ml of water and 3 ml of trichloroacetic acid solution (100 g/liter) and shaken occasionally for 5 min to ensure total deproteinization. The precipitate was then removed by centrifugation. One milliliter of the decanted supernate was used to determine tryptophan as described earlier (7), and two further milliliters was added to the ion-exchange column, allowed to drip slowly through the resin bed, and collected.

One milliliter of the collected liquid, which contains part of the 5-hydroxyindole acetic acid was added to 2 ml of a solution of ascorbic acid in concentrated HCl (1 g/liter), shaken, and placed in the fluorometer. The fluorescence observed at 540 nm (upon excitation at 290 nm) is due to 5-hydroxyindole acetic acid. The column was eluted with 5-ml portions of water until the eluate, when treated with the ascorbic acid hydrochloric acid reagent, did not fluoresce. The fluorescence intensities of the eluates were added to yield the total fluorescence intensity for 5-hydroxyindole acetic acid. The column was then washed with 5-ml portions of a saturated sodium borate solution. The eluates (1 ml) were treated with 2 ml of the ascorbic acid-hydrochloric acid reagent and the fluorescence intensity measured. The process was repeated until the eluate indicated no fluorescence, and the fluorescence intensities were added to yield the total fluorescence intensity for 5-hydroxytryptophan. The column was then washed with NaOH, 0.1 mol/liter, and the eluates, which contain 5-hydroxytryptamine, were treated with ascorbic acid-HCl as described above, to yield the total fluorescence intensity for this metabolite.

Preparation of Calibration Curve

Ten solutions of each of the three metabolites studied were prepared and treated as described in the assay. The calibration curve for each metabolite was linear between 20 and 250 \(\mu g/liter\) within this range of concentration. Figure 1 shows the calibration curve for 5-hydroxytryptophan (slope = 0.61 fluorescent unit per milligram per liter). Similar lines were obtained for 5-hydroxyindole (slope = 0.56 units per milligram per liter) and 5-hydroxyindole acetic acid (slope = 0.53 units per milligram per liter).

![Fig. 1. Calibration curve for 5-hydroxytryptophan](image)

Twenty-five blood samples were treated as described above. The average 5-hydroxytryptamine content was found to be 98 (SD, ±8) \(\mu g/liter\). Ascroft et al. (8) determined the 5-hydroxytryptamine concentration in about 100 blood samples; it averaged 120 \(\mu g/liter\), with a large interindividual variation. We were able to detect 5-hydroxyindole acetic acid and 5-hydroxytryptophan in only two of the samples, the observed fluorescence intensities being barely above the experimental error. Hence we conclude that the concentration of these metabolites is less than 20 \(\mu g/liter\) in normal sera.

The concentration of tryptophan in the blood was 10.5 (SD, ±0.6) \(\mu g/liter\), roughly 100-fold the concentration of 5-hydroxytryptophan.

Each of three blood samples was assayed five times. Data for one of these are typical: 5-hydroxytryptamine—107, 113, 116, 109, 116 \(\mu g/liter\), tryptophan—10.4, 10.7, 10.4, 10.8, 10.6 \(\mu g/liter\), and 5-hydroxytryptophan and 5-hydroxyindole acetic acid <20 \(\mu g/liter\).

Standard addition methods were used to ensure the reliability of the assay procedure. Various amounts of a 5-hydroxytryptamine solution (101 \(\mu g/liter\)) were added to the blood samples to alter the concentration of this metabolite. The 5-hydroxytryptamine solution was added in place of the corresponding amount of water used in the assay. For increments of 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 ml of the 5-hydroxytryptamine solution the observed fluorescence intensities were 45, 50, 56, 63, 67, and 71, from which the recovery is calculated to be 93%. (Complete recovery of the added 5-hydroxytryptamine would demand fluorescence intensities of 45, 51, 56, 62, 67, and 73.) The original sample contained 80 \(\mu g/liter\), and addition of the 5-hydroxytryptamine solution produced increments of 20.2 \(\mu g/liter\) of the metabolite for each 0.2 ml of solution. Similar experiments were performed for 5-hydroxyindole acetic acid. With similar increments, the respective fluorescence intensities were 0.4, 11, 14, 19, and 24 (recovery, 89%). For 5-hydroxytryptophan, the respective fluorescence intensities were 0, 6, 11, 19, 23, and 29 (recovery rate, 86%).
We could not measure the reproducibility of the assay of 5-hydroxyindole acetic acid and 5-hydroxytryptophan in blood because of the low concentrations of these metabolites. We did add 0.5 ml of a 5-hydroxytryptophan solution (103 µg/liter) to the blood (the concentration of this metabolite was 25 µg/liter, leading to an expected fluorescence intensity of 15 units) replacing of 0.5 ml of water, and measured the fluorescence intensity of the eluate for six such samples. They were 14, 15, 14, 15, 15, and 16. The assay procedure is able to detect concentrations of the tryptophan metabolites as low as 20 µg/liter. 5-Hydroxytryptophan and 5-hydroxyindole are normally present in the blood at lower concentrations; but we have not attempted to determine whether our separation procedure would lead to quantitative recovery at concentrations lower than our fluorometric detection limits.

A supplemented sample was prepared by adding 0.3 ml of a tryptophan solution (20.5 mg/liter), 0.3 ml of a 5-hydroxyindole acetic acid solution (306 µg/liter), 0.2 ml of a 5-hydroxytryptophan solution (503 µg/liter), and 0.2 ml of a 5-hydroxytryptamine solution (299 µg/liter) to a 2-ml blood sample. The fluorescence intensities of the various eluents from the chromatographic separation were: for the water eluate, 24; sodium borate eluate, 28; and sodium hydroxide eluate, 64. Eluate fluorescence intensities for the blood with 1 ml of water in place of the various added metabolites were: water, 0; sodium borate, 0; and sodium hydroxide, 47. The addition of tryptophan to the sample caused an increase in the rate of fluorescence decay equivalent to 97% of that expected. These experiments indicate that the tryptophan metabolites can be successfully separated from each other when present in a clinical sample.

Discussion

Fluorometric methods are well suited for the analysis of indoles, because the ring system is a good fluorophore. The excitation and emission maxima lie in conveniently used regions of the spectrum and relatively few compounds in biological systems have similar fluorometric characteristics.

Indoles fluoresce in the ultraviolet region of the spectrum (λmax = 360 nm, on excitation at 275 nm); this emission is useful for the assay of tryptophan and its metabolites if they can be separated from each other or a specific reaction for a given metabolite can be developed. We have recently reported (7), for example, on a selective assay for tryptophan in serum, based on the decrease in fluorescence when the amino acid reacts with formaldehyde. In contrast, in the present assay the various metabolites are separated column chromatographically.

The fluorescence spectra of 5-hydroxyindoles are quite similar to the spectrum of indole in the ultraviolet region. In the presence of hydrochloric acid, however, 5-hydroxyindoles emit radiation in the visible \( (λ_{max} = 560 \text{ nm}, \text{ upon irradiation at 290 nm}) \). The intensity of this emission decreases with time, but in the presence of ascorbic acid the fluorescence intensity remains constant. It is believed that the phenolic group is involved in a charge transfer process in the excited state, or an excited state ionization takes place (9).

Assay procedures for 5-hydroxyindoles based on the emission in the visible range obviate the need to separate tryptophan, tryptamine, and other indoles that do not have the 5-hydroxy group from the sample. About 100-fold as much tryptophan as 5-hydroxytryptamine is normally present in serum; hence the utility of the emission in the visible is clear in these analyses.

A number of fluorometric assay procedures for tryptophan metabolites have been reported in recent years. An excellent review of this area may be found in Udenfriend’s monographs (9, 10); hence we will consider previously reported assays for the 5-hydroxyindoles in general terms. The assay of Udenfriend et al. (11) for 5-hydroxytryptamine in tissues, by measuring emission in the visible, involves a fairly tedious extraction procedure in which other 5-hydroxyindoles are removed from serum. If 5-hydroxytryptamine is the only 5-hydroxyindole present, as usually is the case, such an assay is satisfactory. The assay of 5-hydroxytryptamine may also be performed via the procedure developed by Davis et al. (12), in which the amine is separated from interfering species by an ion-exchange resin. Ashcroft et al. (8) have reported an assay for 5-hydroxytryptamine in blood, which is useful in those situations where other 5-hydroxyindoles are absent.

Useful assay procedures for 5-hydroxytryptophan in urine and tissues have been reported by Udenfriend et al. (11), which could easily be adapted to use in serum. Similarly, there are reliable assay procedures for 5-hydroxyindole acetic acid in biological media (13).

The assay of these metabolites in matrices containing more than one of these species is complicated by the separation procedure required to isolate the various moieties. The three tryptophan metabolites of interest in this study have been separated from tissues by Fischer and Aprison (14) via a solvent extraction procedure that is quite tedious, involving seven extraction steps and 1.5 to 2 h of analytical time.

Bakri and Carlson (15) separated 16 tryptophan metabolites by use of Sephadex QAE-A25 and carboxymethyl cellulose columns; this method might be used with blood samples (the authors used the procedure with urine), but massive quantities of eluate are required (≈2.5 liters) for each assay, which also suggests that it is time-consuming.

Wiegand and Scherfling (16) used a cation-exchange resin (Dowex 50W-X4, 200-400 mesh, Na+) to separate 5-hydroxytryptophan and 5-hydroxytrypt-
taminate from homogenized brain tissue solutions.

Although the same ion-exchange resin is used in our procedure as in that of Wiegand and Scherfling (16), we have made several significant modifications so that the three tryptophan metabolites could be rapidly assayed. The advantages of using the emission in the visible range have been mentioned; tryptophan does not emit at 540 nm. Our column is longer than that of Wiegand and Scherfling (16), to allow the separation of 5-hydroxyindole acetic acid from the other moieties by elution with water. The borate solution we use as an eluent is a buffer at pH 9.2 than the phosphate buffer they use, and is easier to prepare and store. 5-Hydroxytryptamine is eluted more rapidly with 0.1 molar NaOH than with more dilute solutions. More concentrated NaOH solutions tend to discolor the resin and lead to erroneous values for 5-hydroxytryptamine concentration.

The concentration of 5-hydroxyindoles in blood is quite low; reliable data are available only for 5-hydroxytryptamine, the concentration being about 100 μg/liter (8). It is believed that almost all of the blood 5-hydroxytryptamine is normally present in the platelets (17) and that the small quantity present in the blood is due to breakdown of platelets.

The amines under study are of extreme interest in the study of brain tissues and other parts of the central nervous system. We believe that our assay has use in this area, following pretreatment of the tissue. Likewise, after suitable pretreatment, urine samples might well be assayed for these biogenic amines by the procedure discussed herein.

Our assay procedure is rapid, selective, and sensitive. Patients afflicted with chronic uremia could be monitored via this assay during dialysis to determine the effectiveness of the treatment. Present methods of analysis, where they exist, are quite time-consuming and are not as useful in monitoring dialysis, which normally requires about 8 h.

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References


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