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Note

Determination of urinary 5-hydroxyindole-3-acetic acid using solid-phase extraction and reversed-phase high-performance liquid chromatography with electrochemical detection

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5-Hydroxyindole-3-acetic acid (5-HIAA), a metabolite of serotonin (5-hydroxytryptamine) measured in urine, has been documented as a biochemical marker of carcinoid tumor [1, 2]. 5-HIAA analysis has also been suggested as an aid in the diagnosis of certain neurological [3] and psychiatric [4] disorders. Traditionally, this compound is assayed by diazotization with nitrosonaphthol to form a purple color [2, 5, 6]. However, it is well known that many medications and their metabolites interfere in this reaction yielding incorrect 5-HIAA results [7—10].

Recently, liquid chromatographic procedures for 5-HIAA with either ultraviolet (UV) [11—13] or fluorometric [14—18] detection have been described. Most of these procedures involve solvent extraction. In addition, some procedures have been referred to as lacking selectivity [18]. Various electrochemical detection procedures have been published [19—23]. These procedures generally involve extensive specimen preparation. Most of the reported electrochemical detection procedures lack an internal standard to monitor extraction efficiency and injection variation. The only exception is the work reported by Petruccelli et al. [23].

The present system is designed to simplify the extraction procedure and incorporate an internal standard to improve the analytical precision.

EXPERIMENTAL

Instrumentation

A high-performance liquid chromatographic (HPLC) pump (Model 8800;

0378-4347/85/$03.30

DuPont, Wilmington, DE, U.S.A.) was used to deliver the solvent (flow-rate 1 ml/min) through a 250 × 4.6 mm column of Biophase ODS, 5-μm spherical C_{18} particles (MF6017; Bioanalytical Systems, West Lafayette, IN, U.S.A.) at room temperature. Samples were introduced through an automatic sample injector (Model 710B; Waters Assoc., Milford, MA, U.S.A.) and the effluent was monitored by an electrochemical detector with a glassy carbon electrode cell (Model LC4B; Bioanalytical Systems). The oxidation potential was maintained at 0.55 V (versus Ag/AgCl reference electrode), while the detector and recorder ranges were adjusted to 20 nA and 1 V full scale deflection, respectively. A Baker-10 extraction system (No. 70180; J.T. Baker, Phillipsburg, NJ, U.S.A.) equipped with a vacuum gauge was used for the extraction.

Reagents

The 5-HIAA (H8876, Sigma, St. Louis, MO, U.S.A.) stock standard solution concentration was 0.5 g/l. 5-HIAA working standard solution was prepared daily by diluting the stock standard solution 100-fold with deionized water.

The 5-hydroxyindole-3-propionic acid (5-HIPA, provided by Dr. J. Stephen Kennedy, Neurosciences Research Branch, National Institute of Mental Health, Rockville, MD, U.S.A.) stock standard solution concentration was 0.5 g/l. Working standard solution was prepared by diluting the stock standard solution 100-fold with deionized water.

The HPLC mobile phase was prepared by adding 50.7 ml of concentrated ammonium hydroxide, 64.7 ml of glacial acetic acid and 0.2 g of disodium EDTA to 1760 ml of deionized water and adjusting the pH to 5.1 with either 6 *M* acetic acid or 6 *M* ammonium hydroxide. To this mixture 325 ml of methanol were added and the solution was filtered and degassed.

Procedure

The Baker C_{18} extraction columns should be conditioned prior to the addition of specimens. The columns were washed with two column volumes of HPLC-grade methanol under a vacuum. As the level of methanol approached the top of the packing, the vacuum was discontinued. Following the methanol wash, two column volumes of deionized water were added. The vacuum was discontinued before the solution had been totally aspirated through the columns. Reconditioning was performed if drying occurred before sampe introduction.

Centrifuged urine (100 μl) together with 400 μl of the working internal standard solution were added to 4 ml of acetate buffer (0.1 *M*, pH 5.0). Of this solution 1 ml was allowed to pass through the C_{18} disposable extraction column (7020-1; J.T. Baker). The column was washed three times with 1 ml of mobile phase. The void volume and eluates were collected in a 10 × 75 mm test tube. Of this mixed solution 50 μl were introduced into the HPLC system. Standards were prepared by adding 200, 400, and 600 μl of working 5-HIAA standard solution to 3.9, 3.7, and 3.5 ml of acetate buffer (0.1 *M*, pH 5.0). After the addition of 400 μl of working internal standard solution, all standards were treated in the same manner as the urine specimens.

RESULTS AND DISCUSSION

The percentage cumulative recovery for the extraction of 5-HIAA and 5-HIPA is depicted in Fig. 1. These results demonstrate that in addition to the void volume, 3 ml of the eluate must be collected in order to maximize the recovery of both components.

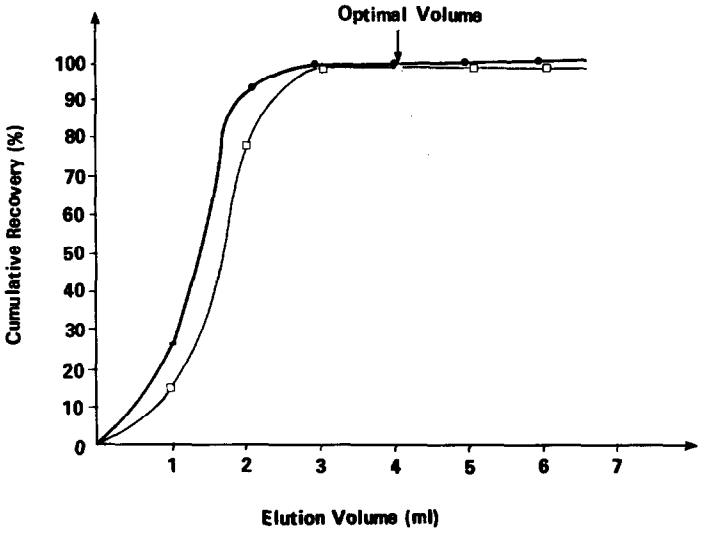

Fig. 1. Percentage cumulative recovery of 5-HIAA (•) and 5-HIPA (□) obtained from a solid-phase extraction column. "1" ml represents the fraction containing the void volume.

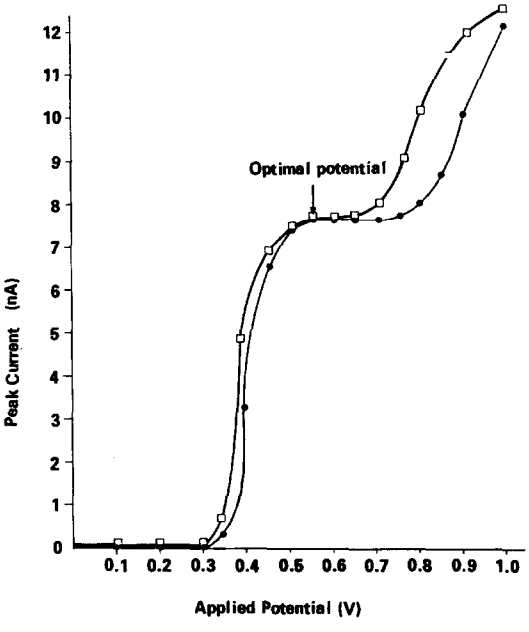

Fig. 2. Hydrodynamic voltammograms of 5-HIAA (•) and 5-HIPA (□). The potential readings are oxidizing voltage versus a Ag/AgCl reference electrode.

Fig. 2 illustrates the voltammograms for both 5-HIAA and 5-HIPA. In order to maximize the signal strength and minimize the variation from amperometric measurements, a potential of 0.55 V versus a Ag/AgCl reference electrode was selected.

The intra-assay and inter-assay coefficients of variation (C.V.) at a level of 6.7 mg/l were 4.8% (n = 15) and 7.1% (n = 9), respectively, while the minimum detection limit was 1 mg/l (signal-to-noise ratio is 2.5). Specimens spiked with various amounts of 5-HIAA when analyzed by this method resulted in an analytical recovery ranging from 97% to 102% (Table I) with absolute recovery ranging from 90% to 98%. A reference range of 1.9—10.4 mg per 24 h

TABLE I

RESULTS OF RECOVERY STUDY

Number of measurements, n = 4.

Standard added (μg)	Amount measured (μg)	Standard recovered (μg)	Percentage recovery
0	0.12	0	—
1	1.14	1.02	102
2	2.11	1.99	99.5
3	3.04	2.92	97.3

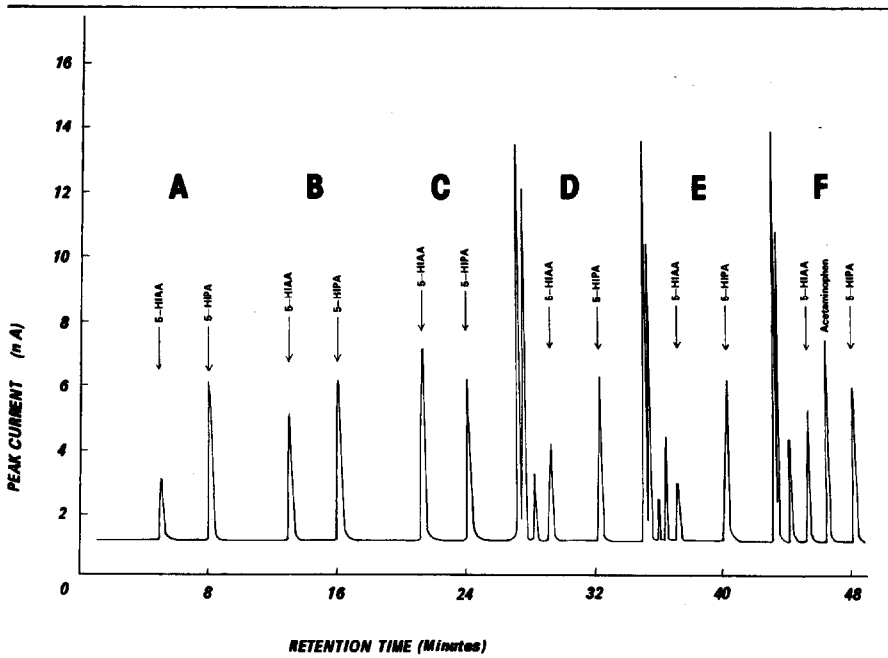

Fig. 3. Chromatograms of standard solutions and three urine extracts. A, B and C represent 1, 2 and 3 μg of 5-HIAA in 200, 400 and 600 μl of working 5-HIAA standard solution, respectively (quality sufficient to 4.1 ml with acetate buffer followed by the addition of 400 μl of working 5-HIPA standard solution and solid phase extraction). D and E represent two normal urine specimens while F demonstrates one urine specimen containing acetaminophen.

was established by analyzing specimens collected from 23 apparently healthy control subjects.

Thirty-one specimens demonstrating no apparent interferences in a colorimetric procedure [2] (x) when analyzed by the present method (y) yielded a slope of 0.992, an intercept of —0.35 mg per 24 h and 0.990 for the correlation coefficient.

The linearity of the present procedure has been established as 0—10 μg (absolute quantity). Chromatograms obtained from a typical analysis are demonstrated in Fig. 3.

A common interference for urinary 5-HIAA determination by colorimetric procedures is the presence of acetaminophen [6]. A chromatogram of a specimen containing acetaminophen is presented in Fig. 3. The separation between 5-HIAA and acetaminophen will not cause identification difficulty with the present method.

In the current procedure, the HPLC separation provides high selectivity while the electrochemical detector improves the minimum detection limit. However, there are numerous electrochemical active compounds present in urine specimens which will prolong the chromatographic separation time. Therefore, the specimen preparation step is very important. With the use of solid-phase extraction columns, the average retention time can be reduced from 20 to 8 min.

In summary, the proposed method is sensitive, selective and reduces the time required for analysis when compared to many existing procedures. Ten specimens can be processed within 1 h. This procedure is adaptable for routine analysis.

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