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Note

Determination of melatonin by high-performance liquid chromatography with electrochemical detection

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The pineal gland has a wide phylogenetic distribution and varied function in vertebrates. In lower vertebrates for example, it acts as a photoreceptor whereas in higher vertebrates it controls certain reproductive functions[1,2]. In 1958, Lerner *et al.*[3] isolated melatonin as one of the active compounds of the pineal gland. At night when animals are in the dark, the pineal enzyme serotonin N-acetyltransferase is activated and converts serotonin (5-hydroxytryptamine) to N-acetylserotonin which is then O-methylated to melatonin by hydroxyindole-O-methyltransferase[4,5]. Alternatively, serotonin may be reduced to 5-hydroxytryptophol then O-methylated to 5-methoxytryptophol. Melatonin is hydroxylated by the liver forming 6-hydroxymelatonin[6].

Many analytical methods have been developed for the quantitation of melatonin including fluorimetry[7], gas chromatography–mass spectrometry (GC–MS)[8], bioassay[9], and radioimmunoassay (RIA)[10–12]. There are several limitations of these methods. Fluorimetry lacks sensitivity and specificity and requires a lengthy extraction process. Although GC–MS has more than adequate sensitivity and selectivity its use is limited due to the great expense and frequent maintenance of the necessary equipment and the need to form derivatives of the compounds. Bioassay methods are extremely laborious procedures and require animal breeding facilities. RIA methods are widely used; however, there is evidence that the antibodies directed against melatonin can cross-react with unknown substituents of some biological samples[13].

High-performance liquid chromatography (HPLC) with electrochemical detection is becoming increasingly popular as an analytical tool for determination of endogenous substances of the body such as catecholamines and serotonin[14,15]. The equipment is relatively inexpensive and little sample preparation is generally required. Since this system allows high resolution and sensitive detection it was felt that HPLC with electrochemical detection could be employed for the separation and detection of indole compounds related to serotonin.

A system employing a citrate–acetate buffer and a cation-exchange resin was used to provide baseline resolution of melatonin from other indoles and is described in this report.

MATERIALS AND METHODS

The indole compounds and citric acid monohydrate were purchased from Sigma (St. Louis, Mo., U.S.A.). Distilled-in-glass grade methanol was obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). All other reagents were obtained from Fisher Chemical (Fair Lawn, N.J., U.S.A.) and were of analytical reagent grade. Water was deionized and double-distilled in glass. A glass chromatographic column (50 cm × 2.0 mm) dry-packed with Vydac SCX cation-exchange resin (Separations Group, Hesperia, Calif., U.S.A.) was employed as the stationary phase. The mobile phase contained 5.30 g sodium acetate (anydrous), 5.75 g citric acid (monohydrate), 2.40 g sodium hydroxide and 1.05 ml glacial acetic acid per liter (pH 5.3)[14]. The mobile phase was maintained at a flow-rate of 50 ml/h which yielded a column pressure of approximately 550 p.s.i.

HPLC system

The LC-40 system manufactured by Bioanalytical Systems (BAS; West Lafayette, Ind., U.S.A.) was employed. It consists of a Milton-Roy (Riviera Beach, Fla., U.S.A.) reciprocating pump, Rheodyne (Berkeley, Calif., U.S.A.) Model 70-10 injection port with a 20-μl loop, a carbon-paste (wax impregnated oil binder) electrochemical detector (BAS), a LC-2A potentiostat (BAS) and a Houston Instruments (Austin, Texas, U.S.A.) stripchart paper recorder. The entire system with the exception of the paper recorder was enclosed in a copper faraday cage. The optimal working potential was determined for melatonin by cyclic voltammetry (scan-rate = 50 mV/sec) and found to be + 0.7 V. All determinations were carried out at a cell potential of + 0.90 V *versus* a saturated silver–silver chloride electrode.

Standard solutions

All stock solutions were prepared fresh daily at a concentration of 0.1 mg/ml in either 0.1 *M* perchloric acid or methanol. All solutions were diluted with 0.1 *M* perchloric acid.

Chromatography

To determine if each indole was separated from the solvent front and oxidized at the electrode, a 10-ng sample was injected and the HPLC was run for 1 h at a sensitivity of 1 nA full scale. This sensitivity is great enough to yield a 150 mm peak from a 500-pg sample of norepinephrine. Reactive compounds were then diluted so that retention times could be determined.

RESULTS AND DISCUSSION

Table I demonstrates that out of fourteen compounds tested, only six were retained by the column and electroactive at the + 0.90 V potential. When the three major indole compounds present in the pineal are injected together they were resolved

TABLE I

SPECIFICITY OF THE HPLC-ELECTROCHEMICAL DETECTION METHOD FOR MELATONIN AND RELATED INDOLES

Compound	*Retention time of electroactive compounds (min)*
Melatonin	6.2
N-Acetylserotonin	2.2
Serotonin	26
6-Hydroxymelatonin	3.8
5-Hydroxytryptophol	4
5-Methoxytryptophol	2.7
Tryptophan	NE*
Tryptamine	NE
N-Acetyltryptophan	NE
N-Acetyltryptamine	NE
5-Methoxytryptamine	NE
5-Methoxytryptophan	NR**
5-Hydroxyindole acetic acid	NR
5-Methoxyindole acetic acid	NR

* Not electroactive at + 0.90 V.
** Possibly electroactive but not retained by the column.

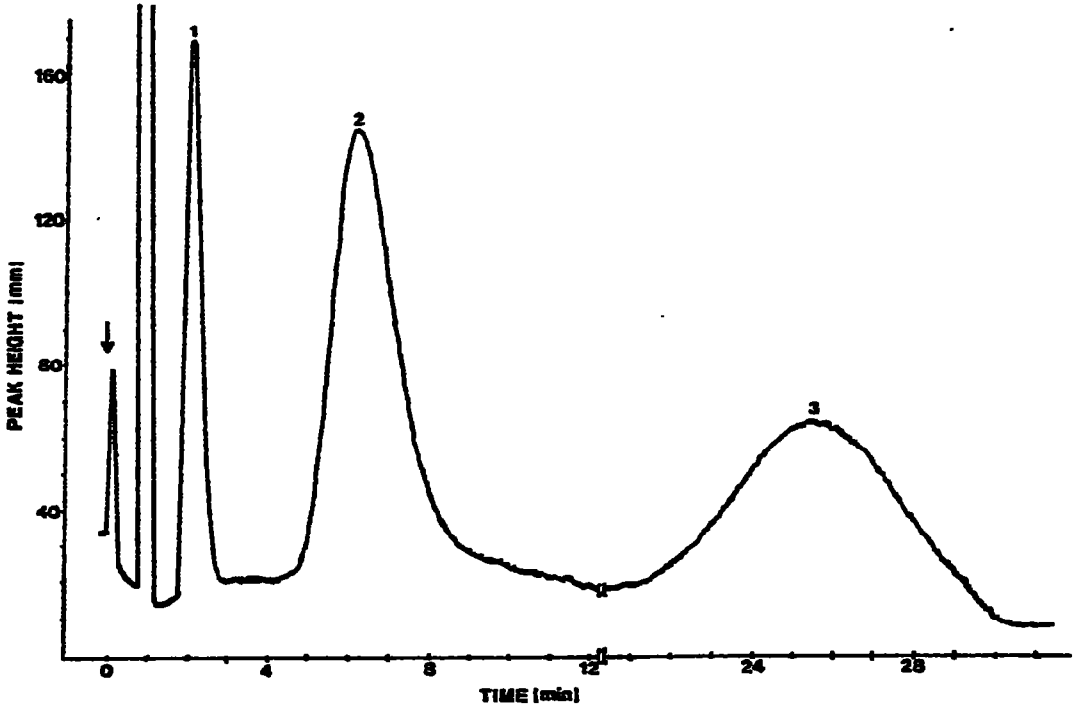

Fig. 1. HPLC separation of melatonin and related indoles. Peaks 1 = N-acetylserotonin (250 pg); 2 = melatonin (1 ng); 3 = serotonin (2 ng).

from each other (Fig. 1). The other electroactive indoles however do interfere with the N-acetylserotonin peak but not the melatonin peak.

The standard curve was linear for melatonin concentrations of 100 pg to 2.0 ng. The line had the following characteristics: correlation coefficient = 0.999, slope = 0.110 and y-intercept = −1.15. The lower limit of sensitivity was calculated to be 50 pg for a 3:1 signal-to-noise ratio.

In conclusion, the results demonstrate that melatonin can be adequately separated and quantitated by HPLC with electrochemical detection. This system possesses more than sufficient sensitivity to analyze melatonin in biological tissues and fluids[7–13]. Investigations are presently in progress to develop a method of extraction and quantitation of melatonin in biological samples.

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