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DETERMINATION OF TRYPTOPHAN AND METABOLITES IN RAT BRAIN AND PINEAL TISSUE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

Tryptophan and many of its indole metabolites were separated using reversed-phase high-performance liquid chromatography (HPLC) and determined using electrochemical detection. This was accomplished isocratically using an acetate—citric acid eluent with various amounts of methanol. Brain and pineal tissue was analyzed for several tryptophan metabolites. Tissue preparation required only homogenization in acidic solution and centrifugation prior to application to the HPLC column. Detection limits in the low picogram range were found for those indoles separated.

INTRODUCTION

The involvement of tryptophan (TRP) and its metabolites in a variety of both normal functions and pathological states has been well established [1]. Tryptophan is metabolized by two major pathways, either the "kynurenine" pathway, leading to the formation of NAD, or through a series of indoles, as shown in Fig. 1.

Various techniques have been employed to determine tryptophan and its metabolites in brain and other tissues, including thin-layer chromatography [2, 3], amino acid analysis [4], gas—liquid chromatography with electron-capture detection [5], UV spectrometry [6], fluorescence spectrometry [7, 8], gas chromatography—mass spectrometry (GC—MS) [9, 10] and radio-immunoassay (RIA) [9, 11, 12]. Each of these has its drawbacks, either in sensitivity, selectivity or versatility. Recently, several analyses employing high-performance liquid chromatography (HPLC) with fluorescence detection of tryptophan metabolites have been described [13—17]. HPLC with electro-

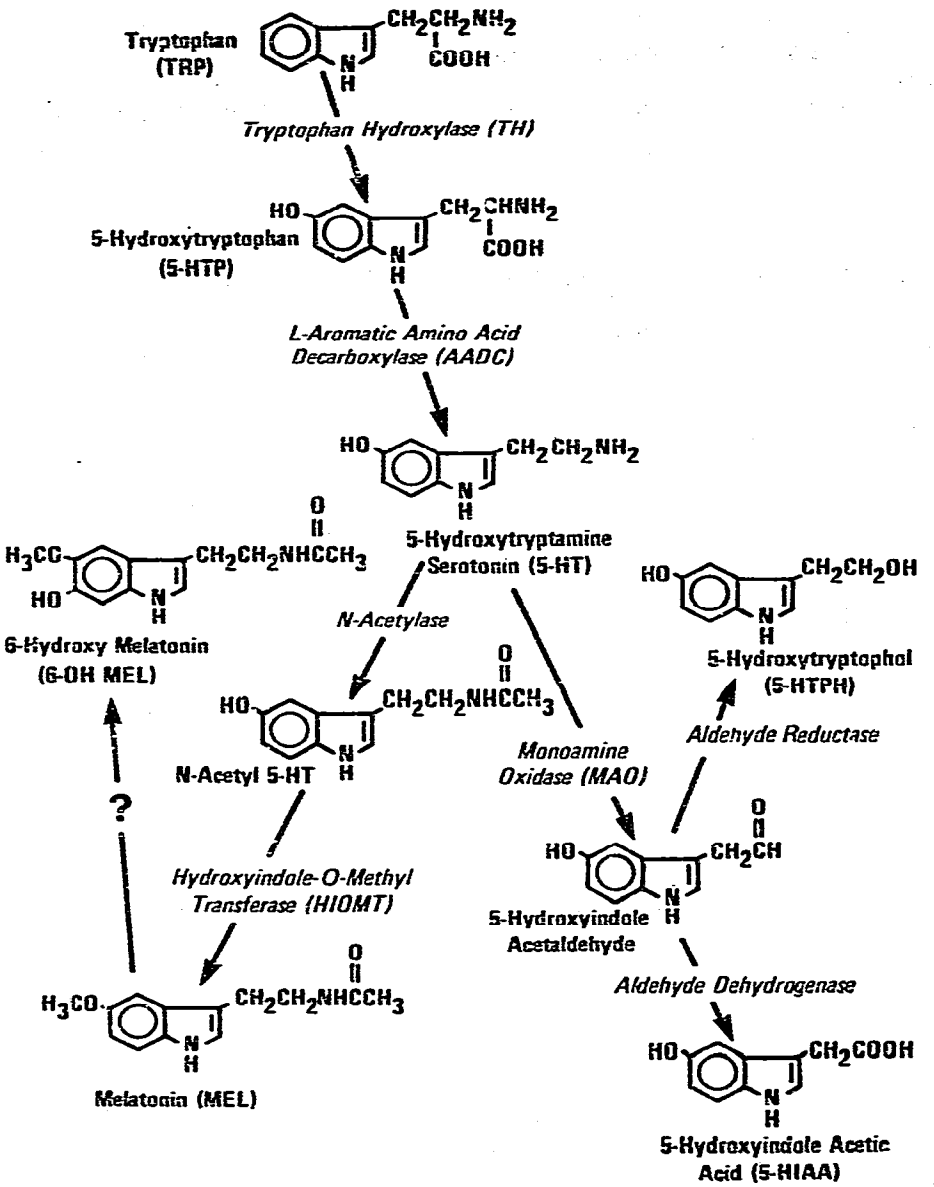

Fig. 1. Proposed synthetic route for tryptophan—indole metabolism in rat brain and pineal.

chemical detection (LCEC) has been employed to determine 5-HT and 5-HIAA in brain tissue [18—20], serum [21] and cerebrospinal fluid [22]. These LCEC methods have employed conventional pellicular (30—50 μm) ion-exchange resins, excluding the simultaneous determination of amine, amino acid and acid metabolites. The use of reversed-phase HPLC allows the simultaneous determination of these with a single injection, but it has not previously been coupled with electrochemical detection for the determination of this series of compounds.

LCEC has been primarily used for the determination of compounds with low oxidation potentials (i.e., <+0.8 V versus Ag/AgCl), such as the catecholamines and their metabolites [23]. However, with proper care in elimination of excess background oxidation current and electrode preparation, compounds of higher oxidation potentials may be determined.

We report here the separation of a variety of tryptophan metabolites [tryptophan, 5-hydroxytryptophan, 5-hydroxytryptamine, 5-hydroxyindoleacetic acid, N-acetyl-5-hydroxytryptamine, melatonin (N-acetyl-5-methoxytryptamine), 5-methoxytryptophan, 5-methoxyindole, 5-hydroxytryptophol and 5-methoxytryptophol] by reversed-phase HPLC and their detection by electrochemical oxidation. These chromatographic separations have been applied to the determination of tryptophan and several metabolites in rat brain and pineal tissue. Detection limits in the low picogram range are reported for all those metabolites analyzed.

EXPERIMENTAL

Apparatus

Chromatography was performed with a Spectraphysics Model 3500 liquid chromatograph equipped with a 25 cm × 3.2 mm I.D. stainless-steel column packed with 10-μm Vydac 201 TP reversed-phase resin (Separations Group, Hesperia, Calif., U.S.A.). Electrochemical detection was accomplished using a Model LC-2A amperometric detector [Bio-Analytical Systems (BAS), West Lafayette, Ind., U.S.A.]. Detector electrodes of two types were employed. For solvents containing methanol, the TL-3 detector electrode (BAS) was packed with a wax–graphite paste, CP-W (BAS). For aqueous solvents containing no methanol, the TL-3 was packed with a silicone grease–graphite (40:60, w/w) paste.

Oxidation potentials of compounds of interest were determined by cyclic voltammetry using the Model CV-1A (BAS) at a scan rate of 100 mV/sec. A carbon paste working electrode and an Ag/AgCl reference electrode were employed.

Reagents

Standards were obtained from Sigma (St. Louis, Mo., U.S.A.). Reagent-grade methanol (J.T. Baker, Phillipsburg, N.J., U.S.A.) was used as obtained. All other chemicals used were of reagent grade. Solvents were filtered through a 0.1-μm Millipore filter and vacuum deaereated. Standard solutions (2 m*M*) were prepared in 0.1 *M* perchloric acid and diluted to appropriate concentrations. Melatonin, N-acetylserotonin, 6-hydroxymelatonin and methoxylated indoles were dissolved in 0.1 *M* perchloric acid–methanol (1:9) and diluted appropriately with 0.1 *M* perchloric acid.

Tissue preparation

Male albino rats of weight ca. 400 g (Sprague-Dawley, Simonsen Labs., Gilroy, Calif., U.S.A.) maintained on a 12-h light–dark cycle were used. The animals were killed at approximately 2400 h under a very dim red light and the pineals and brains removed. The pineals were placed on dry-ice, and the hypo-

thalami, a portion of cerebellum (ca. 50 mg) and a portion of the midbrain containing raphe nuclei [24] were dissected out and also placed on dry-ice. Tissues were stored at −80° until taken for analysis.

Prior to analysis, brain tissues were weighed into 1.5-ml polypropylene tubes and 400 μl of 0.1 *M* perchloric acid added to each. Pineal glands were not weighed, but were placed in a 1.5-ml polypropylene tube and 200 μl of 0.1 *M* perchloric acid added to each. All tissues were thoroughly disrupted by sonication (Sonifier Cell Disruptor Model W 185D, Heat Systems — Ultrasonics Inc., Plainview, N.Y., U.S.A.). The tissue homogenate was then centrifuged at 15,000 rpm for 10 min using a Brinkman Model 3200 microcentrifuge. A 50-μl amount of the clear supernatant was injected into the chromatographic system. Quantitative determinations were made by comparing the peak heights of the samples with those given by known concentrations of standards.

RESULTS

Table I shows the electrochemical characteristics (oxidation potentials), retention times, detection limits and chromatographic conditions used for several indoles and tryptophan metabolites. Although indoles containing phenolic functional groups are considerably more readily oxidized, it is obvious that the lack of the phenolic group does not preclude the determination of many indoles using LCEC. In order to maximize the sensitivity for all indoles chromatographed, the applied potential was maintained at +0.9 V versus Ag/AgCl. Although increasing the potential to +1.0 V increased the sensitivity for tryptophan and some of the methoxylated metabolites, the detection limits were worse, as the baseline noise also increased and electrode lifetime decreased.

TABLE I

CHROMATOGRAPHIC AND ELECTROCHEMICAL PROPERTIES OF INDOLE METABOLITES OF TRYPTOPHAN

Compound	Oxidation potential*	Retention time (min)	Solvent system**	Limit of detection (pg)
Tryptophan	+0.88	8.0	1	15
5-HTP	+0.54	3.2	1	5
5-HT	+0.58	5.2	1	5
5-HIAA	+0.58	10.8	1	10
N-Acetyl-5-HT	+0.48	5.6	2	20
6-Hydroxymelatonin	+0.45	13.6	2	20
Melatonin	+0.70	10.4	3	20
5-Methoxytryptophan	+0.80	4.7	2	20
5-Methoxytryptophol	+0.70	8.8	3	10
5-Methoxyindole	+0.91	17.2	3	25
5-Hydoxytryptophol	+0.52	3.2	1	10

*Determined at a carbon paste electrode by cyclic voltammetry. Scan rate, 100 mV/sec; solvent, 0.1 *M* citric acid—0.1 *M* sodium acetate, pH 4.1. Ag/AgCl reference electrode.

**Flow-rate, 0.7 ml/min. Solvent: (1) 0.1 *M* sodium acetate—0.1 *M* citric acid, pH 4.1; (2) 0.1 *M* sodium acetate—0.1 *M* citric acid—10% methanol, pH 4.1; (3) 0.1 *M* sodium acetate—0.1 *M* citric acid—25% methanol, pH 4.1.

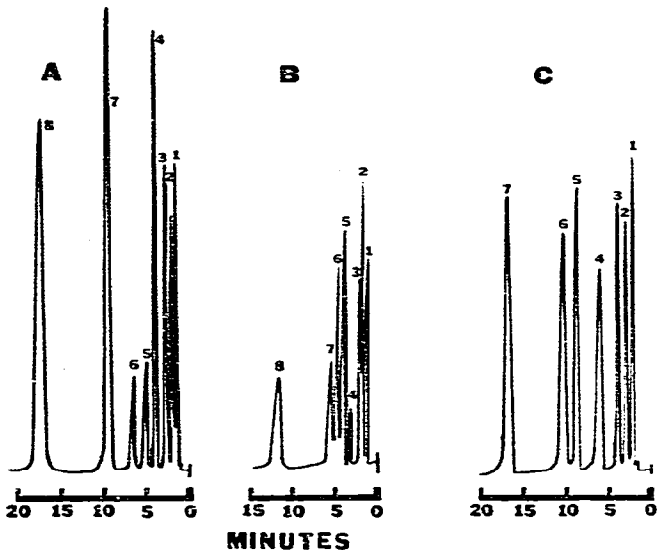

Fig. 2. (A) Solvent system 1 (see Table I). Peaks: 1 = norepinephrine, L-DOPA; 2 = dopamine, epinephrine; 3 = 5-hydroxytryptamine (5-HTP); 4 = serotonin (5-HT); 5 = 3,4,dihydroxyphenylacetic acid (DOPAC); 6 = tryptophan (TRP); 7 = 5-hydroxyindole-3-acetic acid (5-HIAA); 8 = homovanillic acid (HVA). (B) Solvent system 2. Peaks: 1 = solvent; 2 = 5-HTP; 3 = 5-HT; 4 = TRP; 5 = 5-HIAA; 6 = 5-methoxytryptophan; 7 = N-acetylserotonin; 8 = 6-hydroxymelatonin. (C) Solvent system 3. Peaks: 1 = 5-HT; 2 = N-acetylserotonin; 3 = 6-hydroxymelatonin; 4 = tryptophan methyl ester; 5 = 5-methoxytryptophol; 6 = melatonin; 7 = 5-methoxyindole.

Fig. 2 demonstrates the resolution obtained for the tryptophan metabolites and the various chromatographic conditions. Catecholamines and other very polar compounds elute very quickly and do not interfere in the determination of the most polar of the indole metabolites. By increasing the methanol content, one is able to elute the entire series of indole metabolites. Although gradient elution might enable one simultaneously to determine the entire series, this is not yet compatible with electrochemical detection.

Fig. 3 gives examples of the signal-to-noise ratio obtained for the low-level analysis of several of the tryptophan metabolites under the conditions described. The detection limits thus obtained are readily comparable to or better than existing GC—MS [9, 10] and HPLC—fluorescence [13—17] or RIA techniques [9, 11, 12]. Fig. 4 shows sample chromatograms obtained from injection of tissue supernatants. The limits of detection are not approached in the analysis of these samples (ca. 50 mg). This method should therefore be readily applicable to the determination of these metabolites in individual nuclei and tissue samples of the order of 1—5 mg.

Table II shows the results obtained for determination of tryptophan and its metabolites in rat brain and pineal gland. These values compare favorably with values obtained for the same regions by other assay methods.

In summary, we have described the separation of tryptophan and a variety of hydroxylated and methoxylated indole metabolites, N-acetyl-5-hydroxytryptamine, melatonin and 6-hydroxymelatonin. The relative ease of determination of several of these compounds (TRP, 5-HTP, 5-HT, 5-HIAA and melatonin) has been demonstrated in rat brain and pineal tissue. The method as

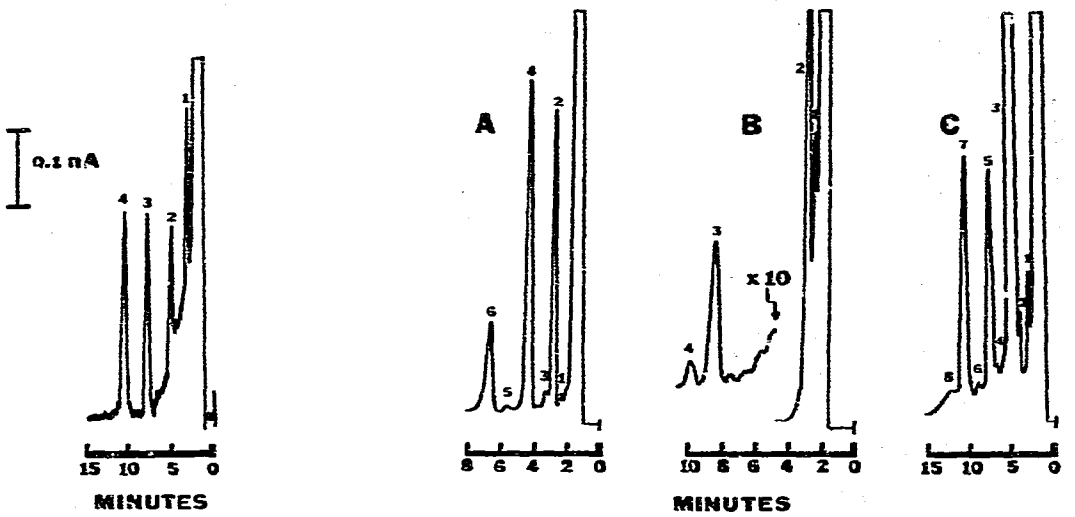

Fig. 3. Chromatogram obtained using solvent system 1 (see Table I). Peaks: 1 = 5-HTP, 39 pg; 2 = 5-HT, 33 pg; 3 = TRP, 105 pg; 4 = 5-HIAA, 93 pg.

Fig. 4. Chromatograms from tissue supernatants. (A) Rat brain cerebellum, solvent system 1 (see Table I). Peaks: 1 = 5-HTP; 2 = 5-HT; 3 = unidentified; 4 = TRP; 5 = unidentified; 6 = 5-HIAA, (B) Rat brain pineal, solvent system 3. Peaks: 1 = TRP; 2 = 5-HIAA; 3 = melatonin; 4 = unidentified. (C) Rat brain pineal, solvent system 1. Peaks: 1 = 5-HTP; 2 = unidentified; 3 = 5-HT; 4 = unidentified; 5 = TRP; 6 = unidentified; 7 = 5-HIAA; 8 = unidentified.

TABLE II

LEVELS OF TRYPTOPHAN AND MAJOR INDOLE METABOLITES IN RAT BRAIN AND PINEAL TISSUE

Values are ng/g wet weight. TRP values are expressed as μg/g.

Region	TRP	5-HTP	5-HT	5-HIAA	Melatonin
Hypothalamus	2.52 ± 0.21	6.5 ± 1.9	841.0 ± 59.0	514.0 ± 57.0	
Raphe	2.09 ± 0.05	2.3 ± 0.5	606.0 ± 14.0	442.0 ± 16.0	
Cerebellum	2.62 ± 0.18	3.5 ± 0.6	70.2 ± 9.1	54.8 ± 6.6	
Pineal*	16.10 ± 2.5	1.02 ± 0.4	82.9 ± 6.7	7.64 ± 0.24	1.35 ± 0.06

*Pineal values are expressed as ng per pineal ($n = 3$); all others, $n = 4$.

described is simple, requiring minimal sample handling and preparation. Although several authors have discussed the instability of tryptophan and metabolites, they appear to be stable at low pH. Storage at −80° for several weeks in 0.1 *M* perchloric acid did not lead to degradation.

Preliminary experiments indicate that the chromatographic conditions described are applicable to the determination of 5-HT, TRP, 5-HTP and 5-HIAA in saliva, serum and urine with only minimal sample pre-treatment to precipitate proteins. Such work is now in progress. Pharmacological manipulations of these systems are also being examined.

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