Journal of Chromatography, 164 (1979) 441—455
Biomedical Applications

CHROMBIO. 421

DETERMINATION OF TRYPTOPHAN AND SEVERAL OF ITS METABOLITES IN PHYSIOLOGICAL SAMPLES BY REVERSED-PHASE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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(First received April 27th, 1979; revised manuscript received August 16th, 1979)

SUMMARY

A new method for the concurrent assay of three tryptophan metabolites at the picomole level is described. The method has been developed for blood, urine, cerebrospinal fluid, and tissue samples such as whole brain, brain parts, and endocrine glands. Tryptophan itself, serotonin, and 5-hydroxyindoleacetic acid are isolated initially on extraction columns, eluted with a suitable solvent, and injected onto a liquid chromatograph with an amperometric detector. This general approach may be applicable to a variety of other tryptophan metabolites and should be useful in both research and clinical investigations.

INTRODUCTION

Many analytical procedures have been devised for tryptophan (TRP), serotonin (5-HT), and serotonin's major metabolite, 5-hydroxyindole-3-acetic acid (5-HIAA). The most popular methods employ ultraviolet absorption, colorimetric, or fluorometric determinations, and these have been thoroughly reviewed [1]. More modern techniques such as radioenzymes, mass spectrometry, and gas chromatography—mass spectrometry have all been used with varying degrees of success [2, 3]. The need for chromatographic separation has led several investigators to choose liquid chromatography (LC). Many of these methods employ fluorescence detection [4—6] but generally require a derivatization step for greater sensitivity. Other LC methods using UV detection suffer from a lack of sensitivity [7, 8].

The present method allows concurrent assay of all three compounds from the same biological sample. Alternately, each can be measured alone, if so desired. Applicability to urine, serum, plasma, cerebrospinal fluid (CSF), and tissue homogenates arising from whole brain, brain parts, and endocrine glands is demonstrated. The method is based on the use of reversed-phase liquid chromatography with electrochemical detection (LCEC). Briefly, the selectivi-

ty arises from three processes: (a) an initial isolation step on small extraction columns, (b) the separation effected by the liquid chromatography, and (c) the electrode used as the detector. The basic isolation procedure has been adapted from the scheme of Hery et al. [9]. LCEC has been successfully applied to the determination of many drugs and metabolites [10—13], most notable being the measurement of tyrosine metabolites in brain tissue [14, 15]. Amperometric detection provides excellent sensitivity, more than adequate for most samples of interest. This method also has the advantages of relative simplicity and low cost. A recent report reviews the principles and methodology of liquid chromatography with electrochemical detection [16].

EXPERIMENTAL

Reagents

Hydrochloric acid, 6 *M*, 3 *M*, and 0.1 *M*. Sodium hydroxide, 3 *M* and 0.1 *M*. Perchloric acid, 4 *M* and 0.1 *M*. Acetate buffer, 0.1 *M* and pH 4.75: Dilute 82.04 g anhydrous sodium acetate and 58.0 ml of glacial acetic acid to 2 l for 1 *M* buffer; then dilute tenfold. Acetic acid, 1 *M*. Ammonium acetate, 1 *M*: Dilute 135 ml concentrated NH_4OH and 115 ml glacial acetic acid to 2 l. Disodium phosphate, 0.2 *M*: Dissolve 56.8 g anhydrous disodium phosphate in 2 l. Citric acid, 0.1 *M*: Dissolve 42.0 g citric acid in 2 l water plus 2 drops of toluene. Phosphate buffer A, 0.2 *M* and pH 6.50: Dissolve 4.46 g KH_2PO_4 and 2.44 g Na_2HPO_4 in 250 ml. Phosphate buffer B, 0.1 *M* and pH 6.80: Dissolve 1.86 g KH_2PO_4 and 1.98 g K_2HPO_4 in 250 ml. Ammonium acetate, 3 *M*: Dilute 50.7 ml concentrated NH_4OH and 43.1 ml glacial acetic acid to 250 ml. Ammonium hydroxide, 0.1 *M*: Dilute 3.4 ml concentrated NH_4OH to 500 ml. Phosphoric acid, 1.0 *N*: Dilute 4.51 ml concentrated H_3PO_4 to 200 ml. Acetate buffer, 0.1 *M* and pH 5.0: Dissolve 1.50 ml 6 *M* acetic acid and 1.31 g sodium acetate in 250 ml.

Authentic standards

5-Hydroxyindole-3-acetic acid (Sigma, St. Louis, Mo., U.S.A.), stock solution, 50 ng/μl, in 0.1 *M* $HClO_4$; store refrigerated and prepare fresh every 4 weeks. Serotonin (creatinine sulfate complex; Aldrich, Milwaukee, Wisc., U.S.A.) and tryptophan (Sigma) stock solutions, 50 ng/μl, in acetate buffer; store refrigerated and prepare fresh every 4 weeks. All concentrations of serotonin are reported as the free base.

Biological controls

Urine pool: Collect approximately 1 l of urine from healthy humans, acidify to pH 2 with 6 *M* HCl, and store 15-ml aliquots at —35° in glass scintillation vials. Serum (plasma) pool: Collect serum (plasma) from blood taken by venipuncture from healthy humans, mix, and store frozen at —35° in 3-dram vials. Plasma samples are drawn into Vacutainer tubes containing citrate to prevent clotting. Brain homogenate pool: Homogenize at least 3 rat brains in 10 ml 0.1 *M* $HClO_4$, collect homogenate and store at —35° in 3-dram vials.

Apparatus

Tissue samples are homogenized with a Brinkmann polytron homogenizer and centrifuged at 12,000 *g* for 20 min in a centrifuge refrigerated at 4° (Sorvall Model RC2-B). Small plasma and serum samples were centrifuged using a Brinkmann Eppendorf centrifuge. The isolation columns (Bio-Rad) are filled with the appropriate resin using a slurry of a weighed amount of dried resin and 0.1 *M* HCl. Amberlite CG-50 II cation-exchange resin (200—400 mesh, p.a. grade, Atomergic Chemetals Co., Plainview, N.Y., U.S.A.) was used for isolation of serotonin, Dowex AG-50 W-X2 cation-exchange resin (200—400 mesh, BioRad Labs., Richmond, Calif., U.S.A.) was used for tryptophan, and Sephadex G-10 gel filtration resin (40—120-μm particle size, Pharmacia, Uppsala, Sweden) extracted 5-HIAA. Dry-packing these columns is not recommended due to the air pockets and inhomogenous beds which sometimes result. Two Model LC-50 (Bioanalytical Systems) liquid chromatographs with carbon paste amperometric detectors (model TL-3) were equipped with stainless-steel columns slurry-packed with microparticulate reversed-phase packing material (μBondapak C_{18}, particle size 10 μm; Waters Assoc., Milford, Mass., U.S.A.). For each system two 15 cm × 4.0 mm I.D. columns were joined together with a short length of 1/16 in. stainless-steel tubing. Two identical chromatographs were used in order to minimize the time necessary to determine all three compounds and to utilize the considerable advantage gained by being able to use a lower electrode potential for some of the assays. The method is flexible enough that one chromatographic system would serve for many applications. The following mobile phases were used:

chromatograph 1: 0.5 *M* ammonium acetate, pH 5.1, 15% methanol. Mix 150 ml 1 *M* ammonium acetate, 150 ml water, 75 ml 1 *M* acetic acid, and 65 ml methanol. NOTE: Ammonium acetate is not stored below 1 *M* concentrations due to noticeable bacteria growth.

chromatograph 2: McIlvaine buffer, pH 4.0, 20% methanol. Mix 200 ml 0.2 *M* Na_2HPO_4, 350 ml 0.1 *M* citric acid, and 140 ml methanol.

The mobile phases were filtered through 0.22-μm (average pore size) filters (Millipore, Bedford, Mass., U.S.A.) and then thoroughly mixed with the appropriate amounts of methanol. Both instruments were used with a flow-rate of 1 ml/min and a rotary injection valve fitted with a 20-μl sample loop (Model 70-10, Rheodyne). The detector potential for chromatograph 1 was set at +500 mV vs. a Ag/AgCl reference electrode, while that for chromatograph 2 was +1.00 V. All glassware used in the procedure was silanized with trimethylchlorosilane [17].

Procedure

Column preparation. The isolation columns were packed as indicated above with the following amounts of resin: Amberlite, 0.15 g; Dowex, 0.30 g; Sephadex, 0.55 g. Once prepared, these columns could be recycled and used for at least 25 samples. Their performance was slightly better when wet, therefore, on the day the samples are to be run, the columns are washed with 12 ml of the appropriate initial solution. For Amberlite, this is phosphate buffer A; for Dowex, phosphate buffer B; and Sephadex, 0.1 *M* HCl. When the liquid stops flowing, the columns are ready for use. After eluting the

sample, a recycling sequence for each column of 3 *M* HCl, 3 *M* NaOH, 0.1 *M* HCl, 0.1 *M* NaOH, and 0.1 *M* HCl (10 ml of each) prepares them for the next sample.

Sample preparation. 1. Biological fluids. Urine, which can be analyzed fresh or from frozen samples, is adjusted to a pH of 5.0 before application to the first isolation column. This is usually possible by titration with a few drops of 1 *M* HCl and/or 3 *M* NaOH per 10 ml of urine. Serum and plasma are deproteinized by the addition of 100 μl of 4 *M* $HClO_4$ to every 1 ml of sample. After shaking the acidified sample and centrifuging at 15,000 *g* for 5 min, the clear supernatant is poured into a beaker and 1 ml of the pH 5, 0.1 *M* acetate buffer is added, along with 250 μl of NaOH to bring the pH to 5.0. Thus, the dilution factor used in calculating the original concentration of the sample is 40/99.

2. Tissue. Rats (Sprague-Dawley) were sacrificed ca. 10:00 a.m. each day by cervical dislocation; their brains were removed as rapidly as possible and frozen on dry ice, weighed to the nearest mg, and either stored frozen, dissected according to Konig and Klippel [18], or homogenized in 3 ml 0.1 *M* $HClO_4$. After centrifugation, the supernatant from the brain homogenates is adjusted to a pH of 5.0 by a method similar to that for plasma: 1 ml of the acetate buffer and 0.5 ml NaOH are added to every 2 ml of homogenate, with a resulting dilution factor of 4/7.

Isolation and determination of the compounds. A flow chart of the basic

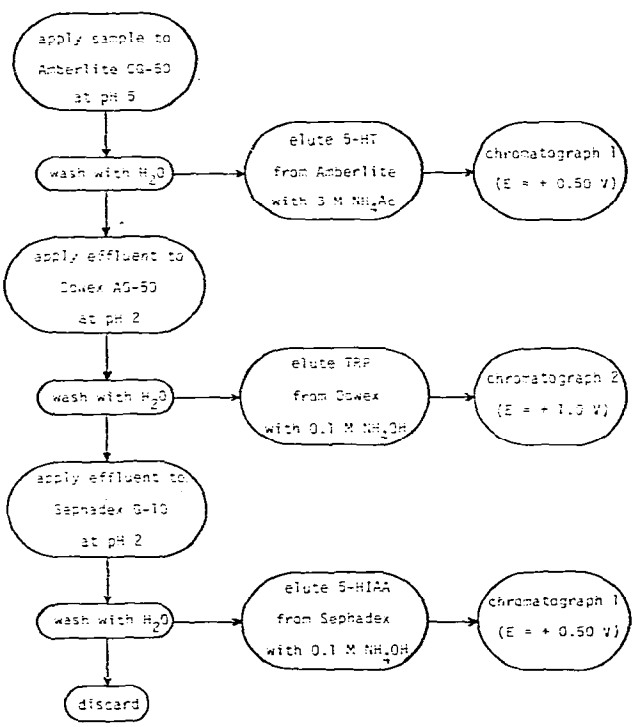

Fig. 1. Flow chart of the steps in the isolation procedure for the tryptophan metabolites. Each of the three compounds is injected individually.

steps in the isolation scheme is shown in Fig. 1. Two milliliters of the sample, now properly adjusted to pH 5.0, are passed through a column of Amberlite CG-50 to isolate the 5-HT. Within each group of 5 to 10 samples one from the standard pool should also be used. Wash the columns with water (5 ml) and elute with 3 *M* ammonium acetate (1.5 ml). Filter the eluent through a Millipore Swinnex assembly and inject a 20-μl portion onto chromatographic system 1 for quantitation of serotonin.

The effluents from each Amberlite column are adjusted to pH 2.0 and applied to a column of Dowex AG-50 resin, on which tryptophan is bound. A consistent method of adjusting the pH at this stage (and before the Sephadex) is to use aliquots of 1.0 *N* phosphoric acid. The columns are washed with water (5 ml) and eluted with 0.1 *M* NH_4OH. Generally 3.0 ml is used to elute the tryptophan, but larger amounts may be necessary, as described in the discussion. As the eluent begins to collect from the column, a drop of 6 *M* HCl is added to lower the pH and minimize oxidative decomposition of the sample. A 20-μl portion of the NH_4OH eluent is injected onto chromatographic system 2 for the quantitation of tryptophan.

Isolation of 5-HIAA from the effluents of the Dowex column is accomplished by adsorption on Sephadex G-10. The effluents are passed through at a pH of 2.0 (adjusted as for the Dowex), the columns washed with water (5 ml), and eluted with 0.1 *M* NH_4OH, 3.0 ml in the case of urine, and 1.0 ml in the case of plasma or brain after washing first with 500 μl. Again, a drop of 6 *M* HCl is added as the eluent begins to collect. Quantitation of 5-HIAA is accomplished after injecting 20 μl of the filtered eluent onto chromatographic system 1.

Calculations. The concentration of each compound is determined by measuring their respective peak heights and comparing with the peak heights obtained for the standard pool for the same sample type. The pool is calibrated using a standard addition method, adding sufficient volume of a standard solution of the three metabolites to make the added concentration fall within the desired levels. It is advantageous, of course, to employ different

TABLE I

STANDARD ADDITIONS FOR METHOD CALIBRATION

Urine			
5-HT:	50 ng/ml	100 ng/ml	150 ng/ml
5-HIAA:	1.5 μg/ml	3.0 μg/ml	4.5 μg/ml
TRP:	3.0 μg/ml	6.0 μg/ml	9.0 μg/ml
Serum and plasma			
5-HT:	100 ng/ml	200 ng/ml	300 ng/ml
5-HIAA:	10 ng/ml	20 ng/ml	30 ng/ml
TRP:	3.0 μg/ml	6.0 μg/ml	9.0 μg/ml
Brain homogenate			
5-HT:	150 ng/ml	300 ng/ml	450 ng/ml
5-HIAA:	150 ng/ml	300 ng/ml	450 ng/ml
TRP:	1.0 μg/ml	2.0 μg/ml	3.0 μg/ml

spikes for each compound in each pool, due to the great variability in concentration expected. The standard additions used in this work are shown in Table I.

Determine the peak heights for each compound in the pool and pool standard additions using the procedure described above. Plot the peak heights vs. the concentration of metabolite added and extrapolate to zero peak height to obtain the concentration in the original pool.

RESULTS

The present method has been optimized for the study of tryptophan, serotonin, and 5-hydroxyindoleacetic acid in urine, plasma, serum, CSF, and various regions of the brain. The assay is based on the use of small gravity-fed extraction columns to isolate the compounds prior to injection onto a reversed-phase liquid chromatograph. Final detection depends upon the compound's oxidation at a carbon electrode. Serotonin, 5-hydroxyindole-3-acetic

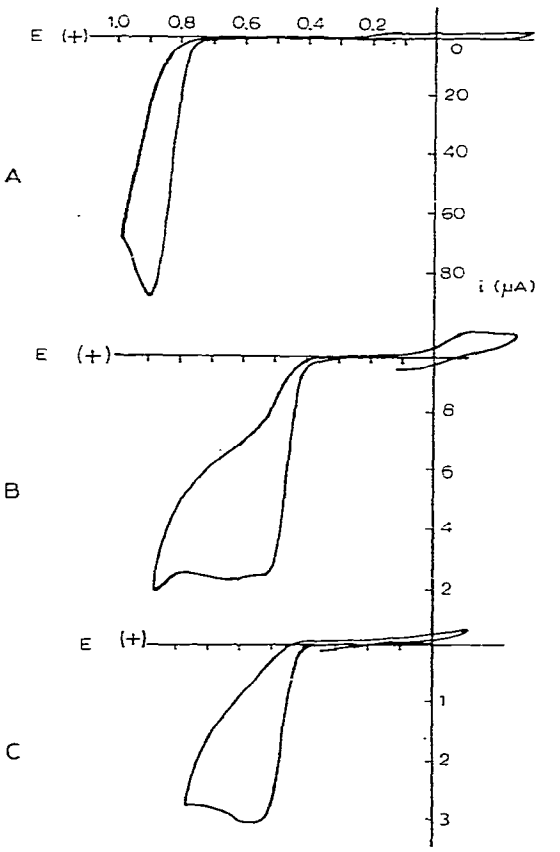

Fig. 2. Cyclic voltammograms of 50 μg/ml solutions of tryptophan (A), serotonin (B), and 5-HIAA (C). All solutions made from 0.1 *M* acetate buffer, pH 4.75. The working electrode was a 1.9 mm^2 disk packed with carbon paste, potentials are vs. a Ag/AgCl reference, and the scan rate was 250 mV/sec.

acid and tryptophan all can be oxidized at an accessible potential, as indicated by the cyclic voltammograms in Fig. 2. Typical chromatograms obtained for each compound are shown for human urine (Fig. 3) and plasma (Fig. 4), and rat brain homogenate (Fig. 5).

Our data from each of these matrices are reported in Tables II—IV, where a compilation of recent values is also presented. In each case, our data are in reasonable agreement with those obtained previously. The wide range in the levels may indicate methodological problems such as endogenous interferences or lack of sensitivity, difficulties which are surmounted in the present work by the isolation and detection scheme used.

Detection limits from an aqueous solution carried through the entire procedure were 1.0 ng/ml for 5-HT and 5-HIAA and 6.0 ng/ml for TRP. Practical detection limits for quantitation of each component in samples with a coefficient of variation of 5—8% were less than 2.0 ng/ml in the case of 5-HT and 5-HIAA, and 20 ng/ml for TRP. Absolute recovery, relative recovery, and reproducibility were adequate (see Table V) for each of the three compounds. Serotonin recovery can be improved by using larger volumes of Amberlite

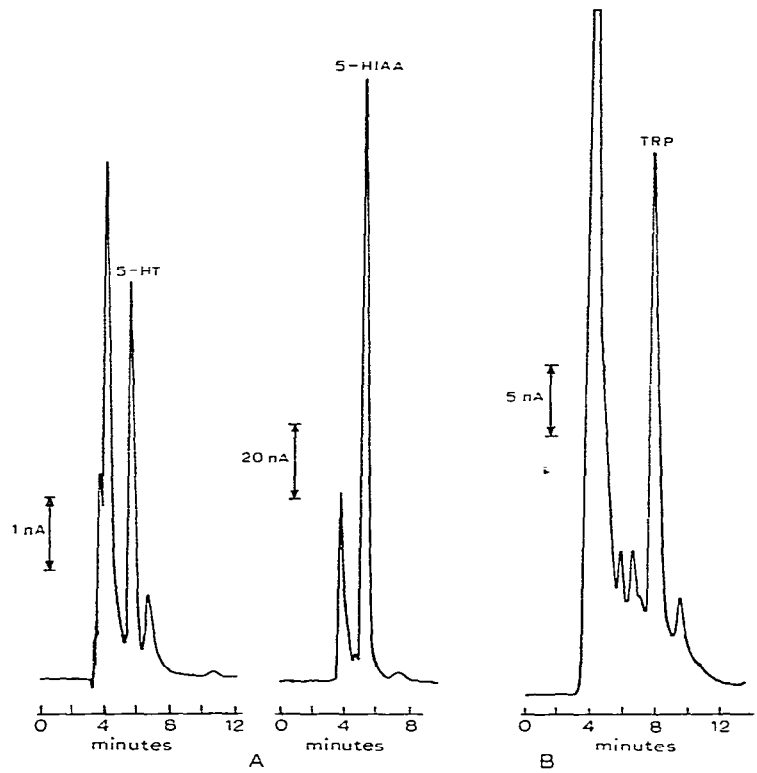

Fig. 3. Determination of urinary 5-HT, 5-HIAA, and TRP by LCEC. Chromatographic conditions: (A) 30 cm × 4 mm I.D. Waters μBondapak C_{18} mobile phase, system 1; flow-rate, 1 ml/min; 0.50 V detector potential vs. Ag/AgCl reference. (B) 30 cm × 4 mm I.D. Waters μBondapak C_{18}; mobile phase, system 2; flow-rate, 1 ml/min; 1.00 V potential. Typical concentrations and injected amounts: 5-HT: 103 ng/ml, 2.2 ng; 5-HIAA: 2.6 μg/ml, 33 ng; TRP: 12.3 μg/ml, 95 ng.

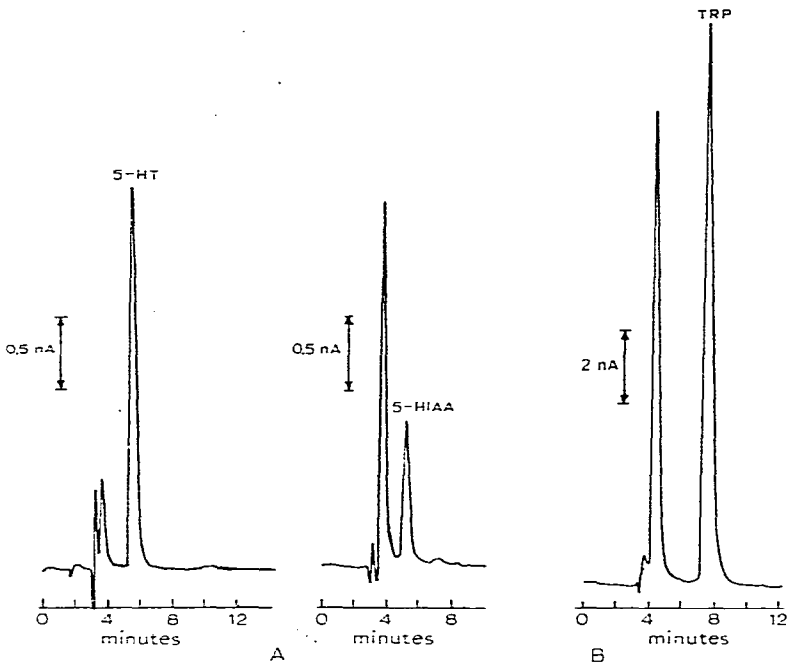

Fig. 4. Typical plasma sample. Chromatographic conditions as in Fig. 3. Typical concentrations and injected amounts: 5-HT: 28.3 ng/ml, 750 pg; 5-HIAA: 15.5 ng/ml, 620 pg; TRP: 12.1 μg/ml, 93 ng.

TABLE II

RAT WHOLE BRAIN VALUES

5-HT*	5-HIAA*	TRP**	Reference
710 ± 30	360 ± 10	3.31 ± 0.31	19
640 ± 30	530 ± 40	5.31 ± 0.06	20
450 ± 10	390 ± 10	4.10 ± 0.20	21
567 ± 25	446 ± 17		22
444 ± 79	349 ± 21	3.18 ± 0.47	23
521 ± 7	323 ± 11	7.91 ± 0.34	24
390 ± 70	580 ± 30	5.58 ± 0.24	25
510 ± 12	533 ± 21	3.75 ± 0.08	26
450 ± 50	870 ± 120	2.04 ± 0.19	27
510 ± 50	420 ± 50	2.0 ± 0.5	28
460 ± 30	503 ± 36		29
526 ± 81	442 ± 24	4.16 ± 0.23	3
238 ± 19	294 ± 7	6.92 ± 0.45	***

*ng/g wet weight, mean values ± S.D.
**μg/g wet weight, mean values ± S.D.
***This work, values ± S.D. One sample was repeated 4 times.

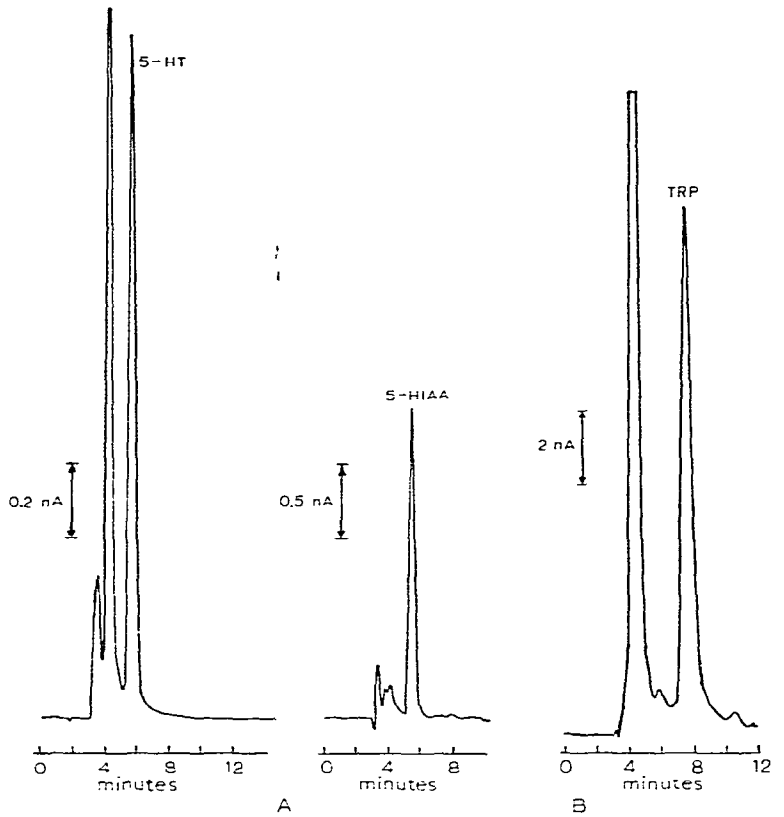

Fig. 5. Rat brain homogenate. Chromatographic conditions as in Fig. 3. Typical concentrations and injected amounts: 5-HT: 238 ng/g, 4.4 ng; 5-HIAA: 294 ng/g, 5.5 ng; TRP: 6.92 μg/g, 48 ng.

eluent, but the resulting dilution is undesirable. Both the recovery and precision of the method, for all three compounds but particularly for 5-HIAA, are aided by silanization of all glassware used in the procedure, presumably by avoiding the likelihood of losses due to adsorption onto the glass. The detector was found to be linear over the ranges of 0.010—80 ng of 5-HT or 5-HIAA injected and 0.10—100 ng of TRP injected. The linearity of the overall method was verified each time a standard addition calibration of a sample was performed. Demonstration of this linearity is given by the plot for 5-HT in urine (Fig. 6).

The identity of the peaks in each of the samples was confirmed by both their chromatographic and electrochemical behavior. Retention times for the three components in each sample were identical with aqueous standards, either injected directly or taken through the procedure. Also, retention times for all samples were compared with standards on LiChrosorb RP-18 (E. Merck, Darmstadt, G.F.R.) and Partisil PXS 5/25 ODS (Whatman, Clifton, N.J., U.S.A.) packing materials, both using two different mobile phases. In every case, co-chromatographic agreement resulted. Such consistency would not be expected unless the substances were in fact identical.

TABLE III

HUMAN PLASMA AND SERUM VALUES

5-HT*	5-HIAA*	TRP**	Reference
Plasma			
		9.8	30
	18 ± 5		31
		11.6 ± 4.7	32
8.5 ± 3			33
		1.57 ± 0.8	7
28.3 ± 2.1	15.5 ± 0.9	12.1 ± 0.4	†
Serum			
98 ± 8	<20***	10.5 ± 0.6	34
		11.1 ± 5	35
144 ± 46			36
84.1 ± 6.4	10.2 ± 0.5	9.82 ± 0.3	†

*ng/ml, mean values. In some cases the standard deviations are given, while in others, the range of data is indicated.

**μg/ml, total concentration, mean values. Standard deviations or ranges given. In one case, no standard deviation or range was included.

***Below detection limit.

†This work, values ± S.D. One sample was repeated 4 times. The range of data for 7 healthy males age 23—28 years was 13.3 ± 7.9, 16.5 ± 7.5, and 13.7 ± 5.5 in plasma and 39.5 ± 41.0, 10.2 ± 3.5, and 9.91 ± 3.1 in serum for serotonin, 5-hydroxyindole-3-acetic acid and tryptophan, respectively.

TABLE IV

HUMAN URINE VALUES

5-HT*	5-HIAA**	TRP**	Reference
		13.9	30
		1.7 ± 1.7	35
250 ± 20	1.1 ± 0.09		37
		4.2 ± 2.5	38
	10.0 ± 2.8		31
	4.3 ± 1.5		8
		27.9 ± 1	7
103 ± 8.2	2.6 ± 0.07	12.3 ± 0.80	***

*ng/ml, mean values ± S.D.

**μg/ml, mean values ± S.D. In a few cases, the range is shown. Where the data were given per 24 h or per g creatinine, the values reported here were calculated using 1200 ml urine per day and 1.5 g creatinine per day.

***This work, values ± S.D. One sample pool was repeated 7 times.

TABLE V

PRECISION OF THE ASSAY

	Absolute recovery (%) standards	Absolute recovery (%) samples *	Within-run coefficient of variation (%)
5-HT	77.8 ± 1.66	65.7 ± 6.7	6.6
5-HIAA	95.6 ± 7.5	82.8 ± 3.1	4.5
TRP	89.0 ± 2.6	58.7 ± 2.9	4.9

*These data are from 10 urine samples.

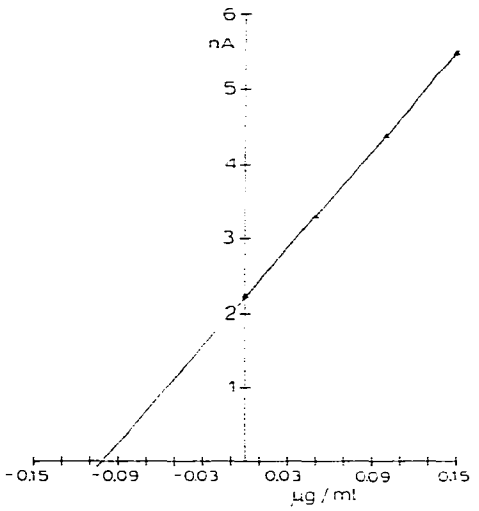

Fig. 6. Standard addition calibration for 5-HT in urine pool. Plot added concentration vs. peak height in nA.

Electrochemical confirmation was based on comparison of the hydrodynamic voltammograms of the actual sample eluent with aqueous standards for each compound. These curves are constructed by making repeated injections of a sample or standard at different electrode potentials, starting where the response is maximal and decreasing the potential, continuing until there is no response. The current (peak height) at each potential is divided by the current at the most positive potential to obtain the relative current ratio (ϕ) which is plotted vs. potential for both the sample and the standard. If the suspected compound and the sample are the same, their voltammetric curves will be identical at any potential. Hydrodynamic voltammograms for serotonin and the serotonin peak of a urinary Amberlite eluent are shown in Fig. 7. In this example, a slight amount of distortion does occur, but the agreement is evident. For every other possibility, the voltammograms coincide even more closely, except for the special case of 5-HIAA in urine, as will be explained below.

The hydrodynamic voltammograms point out not only the identity of the compound but also the specificity of the method for each compound. Inter-

ferences are virtually eliminated by the three criteria they must satisfy. First of all, an interfering molecule must behave in the same manner as the compound of interest on the isolation columns, then must co-chromatograph with that compound, and finally must be oxidized at the electrode potential used. The latter point can be of great advantage. For instance, the catecholamines behave most like serotonin and thus are eluted off the Amberlite, and may chromatograph close to serotonin, but they require a higher oxidation potential than 500 mV and thus are not detected. The accurate determination of serotonin in urine (and in other matrices, for that matter) is greatly improved by operating at 500 mV vs. e.g. 900 mV, as shown in Fig. 8.

Another way the detector potential offers selectivity is illustrated by the chromatograms of the Sephadex elution from a urine sample (Fig. 9). In the chromatograms, a large interference appears just after 5-HIAA which would completely mask the latter if it weren't for the fact that the interference doesn't begin to respond until about +550 mV. Thus, restricting the potential to 500 mV permits determination of 5-HIAA without the need to eliminate or resolve the interference.

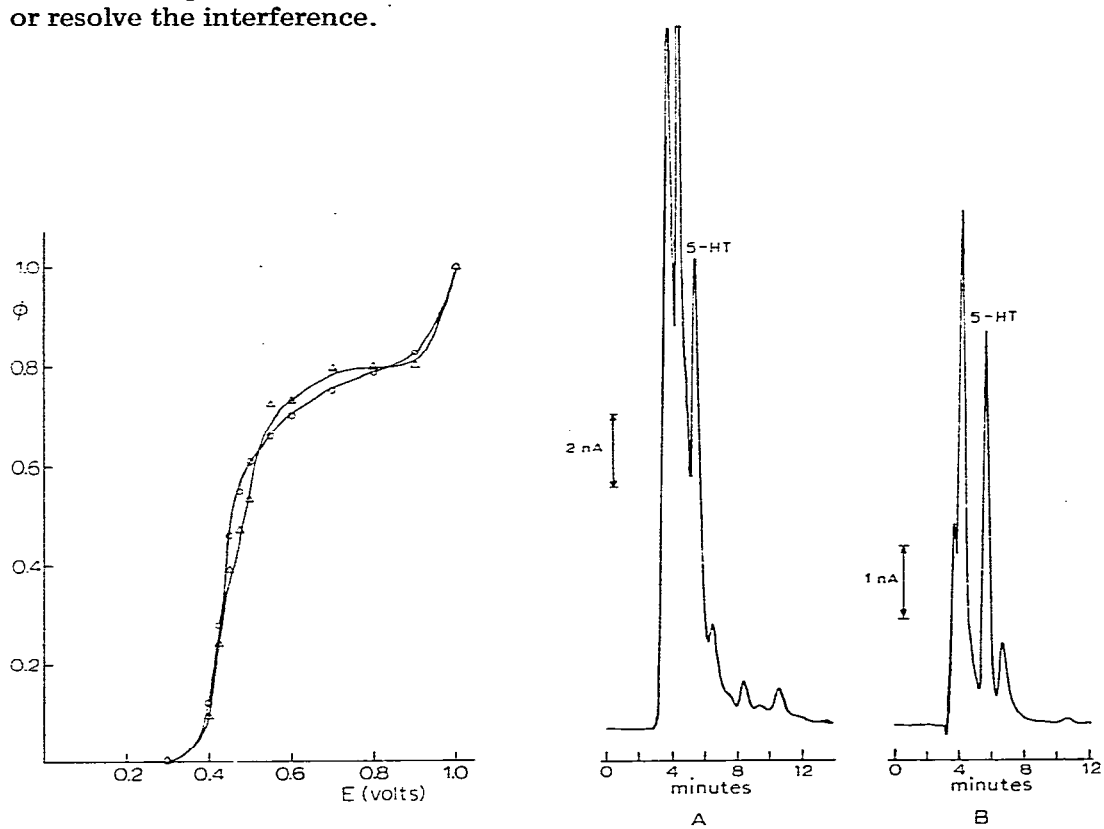

Fig. 7. Hydrodynamic voltammograms of seroronin standard (○) and the Amberlite eluate from a urine specimen (△).

Fig. 8. Selectivity based on the oxidation potential. Chromatograms of the same urine Amberlite eluate at two different potentials vs. the Ag/AgCl reference. (A) 900 mV; (B) 500 mV.

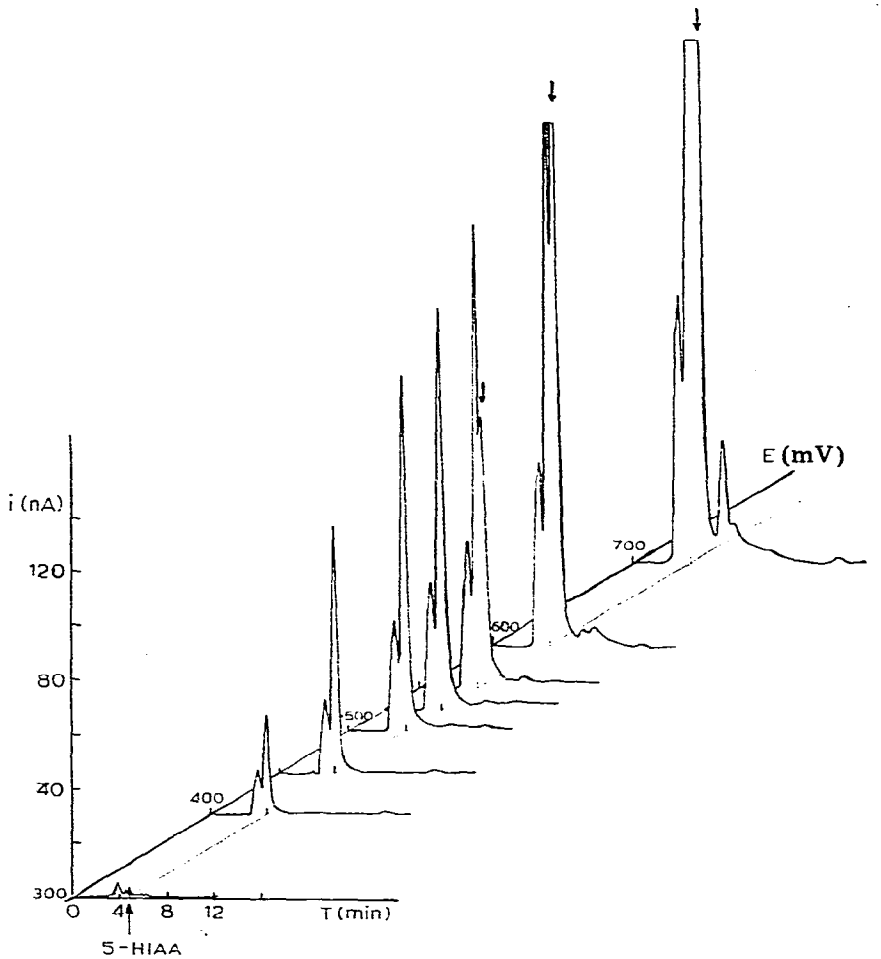

Fig. 9. Chromatograms of urinary 5-HIAA at different detector potentials, illustrating that judicious choice of the potential can eliminate interferences. Repeated injections of the sample were made at the following potentials vs. the Ag/AgCl reference: (mV) +700, +600, +550, +525, +500, +450, +400, +300. Each chromatogram is plotted vs. potential. Arrows indicate growth of the interfering peak.

DISCUSSION

At this point, the method has been used to determine tryptophan, serotonin, and 5-hydroxyindole-3-acetic acid levels in 25 rat brains, in 20 human plasma and serum samples, and in numerous urines from healthy humans and patients with disease states such as carcinoid tumor, melanoma, and neuroblastoma. The method also has been very successful when extended to other applications such as cerebrospinal fluid, free tryptophan in plasma, pineal gland, and other rat brain regions.

As the procedure has been described for quantitating all three compounds, it is possible for a single technician to complete 16 samples per day from

frozen specimens. However, the method is versatile and can be used when only one or two of the compounds are studied, increasing the sample rate considerably. For instance, 48 serotonin determinations per day are possible. If only 5-HT and 5-HIAA are desired, the Dowex step in the procedure may be omitted and the Amberlite eluates applied directly to the Sephadex. It is also possible to handle tryptophan alone, using only the Dowex columns. However, when performing the 5-HIAA individually, a slight addition to the procedure is helpful which cleans up every Sephadex chromatogram. This extra step is a wash with 5.0 ml of 0.02 *M* acetic acid which should follow the water wash. A large number of interferences are then eliminated, while 5-HIAA recovery is unchanged.

A study of the influence of the volume of sample applied to the isolation columns showed that 500 μl can be successfully used instead of 2 ml. This can be advantageous when the amount of sample is at a premium or the volume is low, e.g. homogenates of small brain regions. The elution volume also may be varied to suit individual purposes. As has been mentioned, 3.0 ml of 0.1 *M* NH_4OH is generally used to elute tryptophan, but when the amount of compound is high it is wise to increase the elution volume to enhance both the recovery and the linearity of the determination. Examples where this is most important include the determinations of tryptophan in urine, total tryptophan in plasma, and 5-HIAA in urine, since the unspiked concentrations are already large. Alternatively, and perhaps more satisfactorily, this problem is eliminated by replacing the TL-3 detector cell with one where the auxiliary electrode is positioned directly opposite the working electrode [39] (Bioanalytical Systems, Model TL-5A), which extends the linearity substantially. At the other extreme of elution volume, small amounts applied to the isolation columns demand elutions of minimal volume such as 500 μl. The first 500 μl contain little or none of the molecule desired, thus it is discarded in favor of collecting the second or third 500-μl fraction.

Another variable is the nature of the eluent. Different eluents can be employed to desorb the compounds which improve recoveries; however, other factors make them less desirable than those currently employed. For example, 3 *M* HCl elutes serotonin from the Amberlite with an 80% recovery, but the void volume response is much greater than that for 3 *M* ammonium acetate, which matches the mobile phase more closely. Improvement in the recoveries also results when more resin is packed into the columns, though at the expense of speed since the flow-rates will decrease.

The need for a fast, reliable procedure for tryptophan and its metabolites is apparent in view of the vast number of studies and the difficulties in interpreting results from the many different laboratories investigating similar problems. The LCEC method described here has several principal advantages which should make it useful in meeting this need. The three-way selectivity of liquid—solid extraction, liquid chromatography, and amperometric detection combine to provide very good specificity. The high sensitivity, simplicity, and good reproducibility with inexpensive reagents help to make it ideal for routine use. The versatility of the method, allowing analysis of one, two, or all three molecules, and applicability to every physiological sample commonly encountered are other desirable traits.

ACKNOWLEDGEMENTS

We thank Craig Bruntlett, Greg Davis, and Ron Shoup for helpful discussion and encouragement and Leon Thacker, John Schultz, and Rich Harner for valuable assistance. This investigation was supported by the National Institute for General Medical Sciences and the National Science Foundation.

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