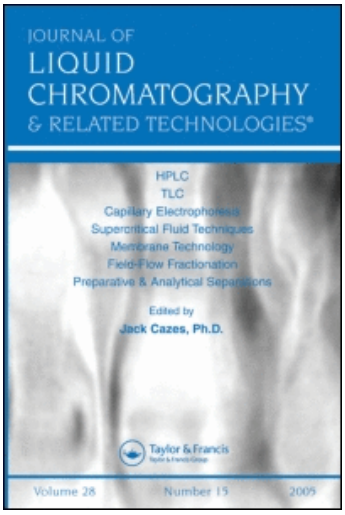

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Tryptophan Determination in Tissue Homogenates and Biological Fluids Using HPLC-EC

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TRYPTOPHAN DETERMINATION IN TISSUE HOMOGENATES AND BIOLOGICAL FLUIDS USING HPLC-EC

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ABSTRACT

A method for quantitating tryptophan in tissue homogenates and biological fluids using 6-hydroxy-tryptamine as internal standard is described. Tryptophan in CSF and free tryptophan in serum or plasma can be quantitated by directly injecting CSF or ultrafiltrate into the HPLC system with the internal standard. Serum, plasma, amniotic fluid, and saliva are deproteinized by addition of equal volume of acetonitrile. Urine samples are processed through Biorex-70 columns. Cross validation was done by comparing the values of column procedures with direct injection method.

INTRODUCTION

Determination of tryptophan in human serum and cerebrospinal fluid has great relevance in the studies of psychiatric disorders where the role of serotonin in depression and schizophrenia has been well recognized (1,2,3,). Tryptophan is the precursor amino acid for serotonin synthesis and tryptophan levels in the brain may determine the effective synthesis of serotonin in the CNS. Though a large volume of literature exists on the levels of

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5-hydroxy-indole-acetic acid in human CSF in a variety of psychiactive disorders, the literature on tryptophan levels is rather sparse and this aspect is not studied as intensely. Recently, with the development of HPLC systems and sensitive detection such as fluorescence detection and electro-chemical detection, reliable methods are now available for the determination of tryptophan in biological fluids (4,5,6). One of the earlier methods described by Koch and Kissinger used an elaborate clean-up procedure for serum samples using ion exchange columns to separate fractions containing 5-HT, tryptophan, and 5-HIAA (7). The recent methods of Beck et al. (4) uses trichloroacetic acid for deproteination of tryptophan in serum, and cerebrospinal fluid samples. We now report a simple and rapid HPLC-EC method for tryptophan in human serum and CSF using 6-hydroxy-tryptamine as an internal standard.

MATERIALS AND METHODS

L-tryptophan and 6-hydroxy tryptamine creatinine sulfate were obtained from Sigma (St. Louis, MO); acetonitrile and ethyl acetate were HPLC grade from Burdick and Jackson. A Rainin Rabbit HPLC pump with Rheodyne valve interfaced with a BAS 4B electrochemical detector and glassy electrode was used. Triethylamine and Phosphoric acide were analytical grade.

HPLC Conditions

Mobile Phase: 0.25M Triethylamine phosphate buffer pH 3.0 $\pm$ 0.05, flow rate 1.0 ml/min.. Column: Rainin C_8 Supershort-one 3 um, 4.6mm ID x 5 cm L., Supelco C_{18} 15 cm with Mobile Phase, pH 3.0 $\pm$ 2.5% CH_3CN. Waters m-45 pump, BAS LC-4B electrochemical detector, oxidation potential 0.80 volt.

Serum or Plasma Tryptophan Determination

The following three methods were extensively studied and evaluated before selecting the routine method.

Method I:

A mixture of 0.25 ml of serum, 0.25 ml of mobile phase and 1 ug of 6-hydroxy tryptamine (internal standard) was vortexed for 30 seconds and filtered through a 0.45 uM filter ((Amicon sterile disposable filters). Ten ul of the clear filtrate was directly injected into HPLC system

Method II:

0.25 ml of serum sample containing 500 ng of 6-HT was processed through a short Biorex-70 column according to the procedures described earlier (8) for serotonin. The eluate from the column with methanolic formic acid which contained both serotonin and tryptophan was evaporated under nitrogen and the residue redissolved in 0.25 ml of mobile phase for HPLC analysis. The complete absorption and elution were monitored by analyzing effluent and fractions from the column. For determination of tryptophan levels only, the column was eluted with 1 ml of mobile phase only and 50 ul of the eluate is injected into HPLC system .

Method III:

To 0.25 of serum containing 500 ng 6-HT (5 ul 100 ng/ml) in 5 ml centrifuge tubes or 5 ml culture tubes, 0.5 ml of acetonitrile was added and after vortexing for 30 seconds the mixture was centrifuged for 10 minutes, supernatant was drawn with a pasteur pipette, transferred to another centrifuge tube or culture tube. The clear supernatant was evaporated to dryness under nitrogen in a 50°C. waterbath. The residue was dissolved in 250 ul mobile phase pH 3.0 and extracted with 2 ml ethyl acetate in the same tube. The tubes were centrifuged for 5 minutes and 10 ul aliquot of the aqueous layer was injected into HPLC system. 0.25 ml of whole blood or 0.25 ml of red blood cells was hemolized with 0.25 ml of deionized distilled water and processed exactly as the serum sample.

Calibration curves with water standards containing 2 ug/ml of 6-HT and 5, 7.5, 10, and 12 ug of tryptophan were run under the same conditions for each method. A similar standard curve was obtained with 0,2,4,6, and 8 ug/ml tryptophan added to a plasma sample.

Method IV; CSF Samples:

20 ul of CSF sample and 5 ul (10 ng) 6-HT were directly injected into HPLC system. From the peak height ratios of Tryptopham/6-HT of the sample and the standard, tryptophan levels in the sample were calculated.

Free Tryptophan in Serum or Plasma:

0.5 ml or 1 ml aliquots of serum or plasma samples were transferred to Amicon Centrifree filters (Amicon Corp., Danvers, MA) and centrifuged at 3000 rpm for 1 hour. The ultrafiltrate volume was measured and 5 ul of the filtrate directly injected along with 5 ul of internal standard 6-HT (10 ng). From the peak height ratio of Tryptophan/6-HT for samples and the standard, tryptophan level in 5 ul of ultrafiltrate is calculated. From the known volume of ultrafiltrate, free tryptophan per ml of serum or plasma is calculated from the equation:

Tryptophan in 5 ul of UF x $V_{UF}/0.005$ x $1/V_S$

where V_{UF}=volume of ultrafiltrate

V_S=volume of sample taken for filtration

RESULTS

The separation of 5-HT, 6-HT, and tryptophan on a C_{8} 3u short column 5 cm is shown in Fig. 1. The retention times for 5-HT, 6-HT, and tryptophan are 2.88, 3.36, and 4.68 minutes respectively. In the extraction procedure used for 5-HT (8) tryptophan is not extracted into ethyl acetate. In the deproteinization using acetonitrile, tryptophan is quantitatively

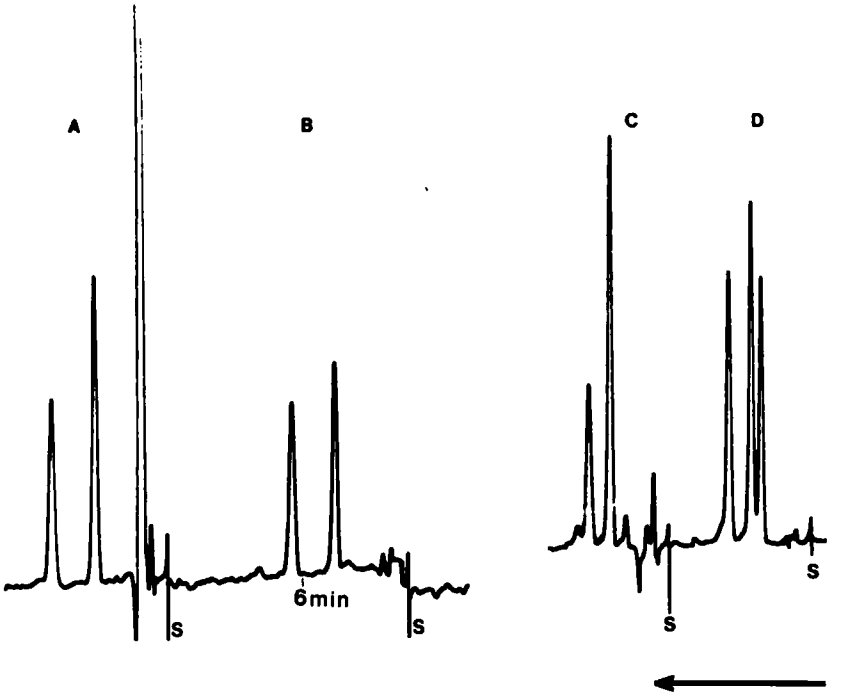

FIGURE 1. Chromatograms of Standards. B. 6-HT and Tryptophan. D. 5-HT, 6-HT, and Tryptophan. Serum Samples A by Method I, C by Method II.

recovered and 6-HT as internal standard compensates for volume changes and losses in manipulation. At oxidation potential used here (0.80 V) it provided medium sensitivity more than adequate for the levels found in serum and CSF. The chromatograms obtained by the use of the three methods described are shown in Fig. 1 and 2. The standard curve with added tryptophan to a plasma sample is shown in Fig. 3 (r=0.998). The tryptophan level in plasma from the curve was 5.38 ug/ml and by direct calculation of peak height ratio of 5-HT/6-HT was 5.20 ug/ml. The quantitative data obtained by the three methods are comparable. However, reproducibility is better in Methods I and III. The main disadvantage in the direct

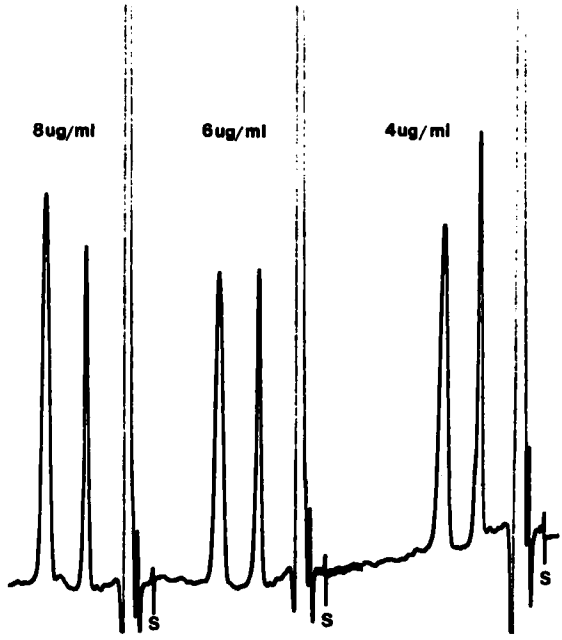

FIGURE 2: Chromatogram of Part of a Calibration Curve with Added Tryptophan to Serum Samples, Method III.

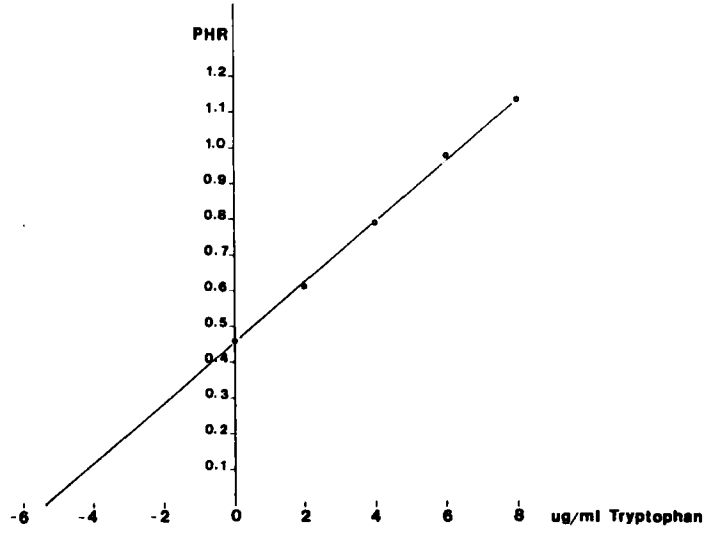

FIGURE 3: Standard Curve of a Plasma Sample with Added Tryptophan (r=0.998)

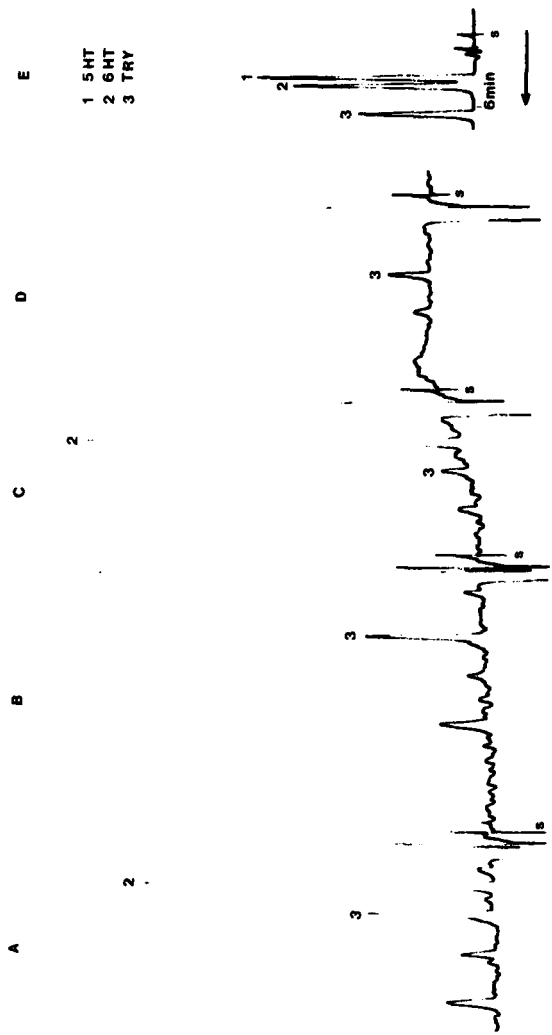

FIGURE 4: Chromatogram of Direct Injections of 20 ul of CSF with and Without Internal Standard 10 ng 6-HT. A=CSF PM + 6-HT; B=CSF PM no 6-HT; C=CSF JJ + 6-HT; D=CSF JJ no 6-HT; E=standard.

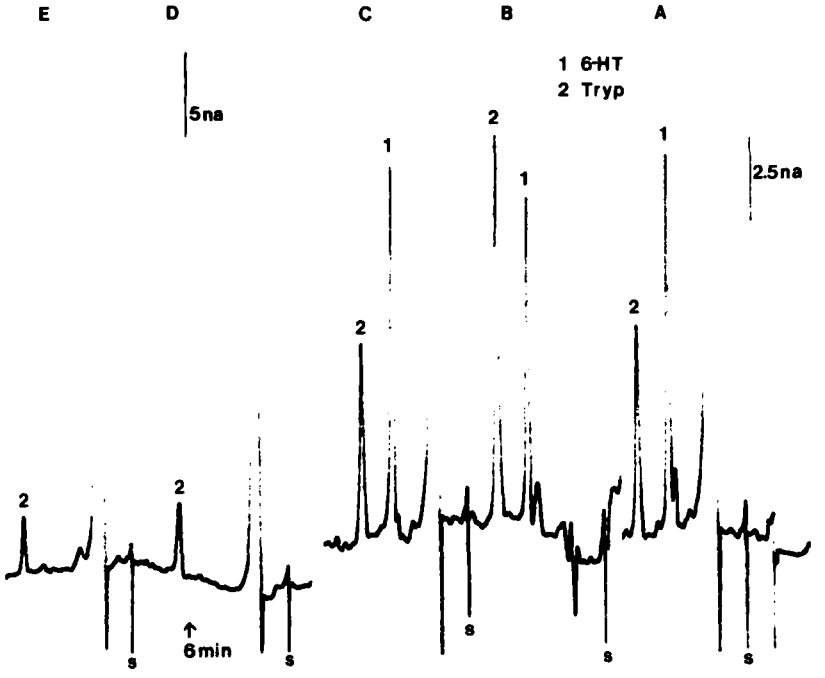

FIGURE 5 Chromatograph of Ultrafiltrates from Plasma With and Without Internal Standard 10 ng 6-HT. A,B,C=UF + 6-HT; D,E=UF no 6-HT.

injection is the fast deterioration of the guard column and build up of back pressure. Since deproteination with acetonitrile and evaporation is not a time-consuming procedure, we have adopted this for our routine laboratory use.

Very low volumes (20 ul) of CSF used for tryptophan determinations give very clean chromatograms, e.g., very often with only a tryptophan peak in that region. 6-HT serves as a useful internal standard to compensate for any variations in detector sensitivity, particularly because it elutes close to tryptophan. the chromatograms with and without internal standard for a low and a high level of tryptophan in two CSF samples and 4 ultrafiltrates

from serum or plasma are shown in Fig. 4, 5. The general applicability of this method is ilustrated by the analysis of amniotic fluid, saliva, human brain, and urine samples (Fig. 6, 7). Only in the case of urine, the column procedure was used. Amniotic fluid and saliva were treated by the acetonitrile procedure (Method III), the human brain homogenate (1:4)(pH 7.0, 0.1M phosphate buffer) was centrifugred and 20 ul of supernatant was directly injected. We have analyzed a variety of clinical samples using this method from different diagnostic categories such as depression, alcoholism, head injury, depressed patients on Lithium or MAO inhibitor (phenelzine) and also normal controls. The data will be published in appropriate studies along with clinical correlates. A few illustrative values are given in Table 1 and Table 2.

DISCUSSION

Several methods have appeared recently on the quantitation of tryptophan in serum and CSF using HPLC and both fluorescence and electro-chemical detection (4,5,6). Of these, the method of Beck et al. uses 1 and 2 ml of serum and deproteinization with TCA. The method of Laakso et al. (5) for CSF uses a very high sensitivity and a phenyl column for separation. The peak heights for tryptophan are small and they recommend using 200 ul of CSF to get all the other metabolites, too. In this present study, we have demonstrated direct injection using small volumes (5 to 10 ul for serum, 20 ul for CSF) and a common internal standard for 5-HT as well as tryptophan. A second procedure for 5-HT and tryptophan is described where acetonitrile is used as a deproteinizing reagent. In this an extra step of solvent extraction removes neutral and acidic fraction, reducing the HPLC run time and also minimizing interfering peaks. The general applicability of this method is illustrated by the analysis of other biological fluids such as amniotic fluid, saliva, brain homogenate, and urine samples. The method is well tested by reproducibility studies, comparison of data by two methods, and increment analysis. We have studied a

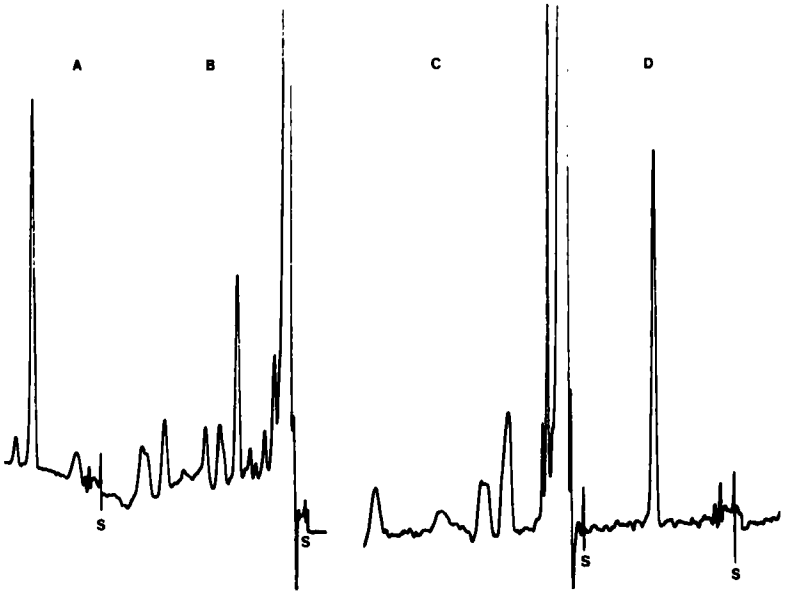

FIGURE 6. Chromatograph of Amniotic Fluid and Human Saliva, No Internal Standard. A. Standard: B. Amniotic Fluid; C. Saliva; D. Standard.

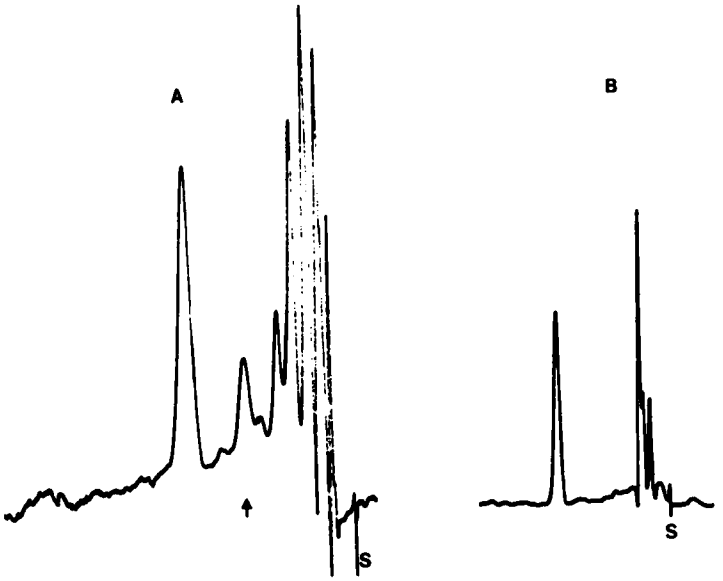

FIGURE 7. Chromatograph of Urine and Brain Homogenate. A. Urine; B. Brain (20 ul Centrifuged Homogenate is Directly Injected).

TABLE 1

Illustrative Tryptophan Values in Serum, Plasma and Ultrafiltrates

Serum Plasma	
Normal serum	7.35 ug/ml tryptophan
	5.55
	4.07
	3.09
Normal plasma	8.76
	7.22
	8.12
	8.07
Alcohol serum	10.18
	13.15
	12.87
	6.20
Lithium Serum	14.17
	9.07
	11.85
	12.78
Ultrafiltrates	
Serum	238 ug/ml tryptophan
	419
	103
	593
	453
	610
	557

TABLE 2

Illustrative Tryptophan Values in Amniotic Fluid and CSF

Amniotic Fluid	326 ng/ml tryptophan
	229
	697
CSF	
Head Injury	2008
	1373
	1988
	969
	809
	775
	494
Depression	106
	41
	422
	65
	57

number of clinical samples and in these studies our findings are in agreement with those of others in the literature (5,7). We therefore consider this as an useful addition in the methodology for tryptophan determinations.

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