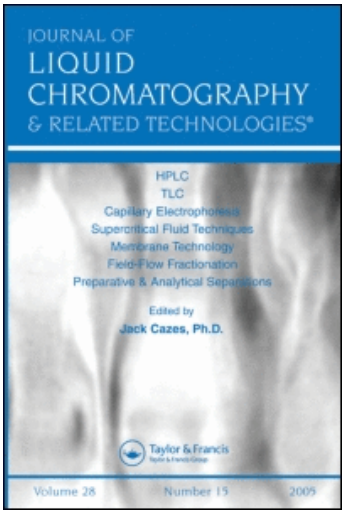

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Narasimhachari, N.(1986) 'A Micro Method for the Quantitation of Tryptophan in Biological Fluids by HPLC-EC', Journal of Liquid Chromatography & Related Technologies, 9: 10, 2223 — 2235
To link to this Article: DOI: 10.1080/01483918608074144
URL: http://dx.doi.org/10.1080/01483918608074144

JOURNAL OF LIQUID CHROMATOGRAPHY, 9(10), 2223-2235 (1986)

A MICRO METHOD FOR THE QUANTITATION OF TRYPTOPHAN IN BIOLOGICAL FLUIDS BY HPLC-EC

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ABSTRACT

A micromethod for the quantitation of tryptophan in biological fluids, such as whole blood, RBC, serum, plasma or tissue homogenates using HPLC and electrochemical detection is described. -Methyl tryptophan is used as an internal standard. Ten microliters of the sample are added to a mixture of 50 ul of acetonitrite and 10 ul of internal standard (20 ng/ul). This mixture is then diluted with 180 ul of triethylamine phosphate-buffer (pH 3.0). After centrifugation 25 ul of the sample are injected into HPLC system. Standards containing 100 ng and 200 ng of tryptophan are similarly prepared and 25 ul aliquot injected into HPLC.

INTRODUCTION

In a recent communication we described a method for the quantitation of tryptophan in biological fluids using

 0148-3919/86/0910-2223$3.50/0

6-hydroxytryptamine (6-HT) as an internal standard (1). In this method we established the high sensitivity of the electrochemical detector makes it possible to use small volumes of the sample for quantitation. Further we showed that prior sample clean up is not necessary and therefore we could use the basic compound 6-HT as an internal standard which made it possible to quantitate serotonin and tryptophan in the same sample. 6-HT is not easily available from commerical sources and therefore we have investigated other closely related nonbiological compounds as internal standards. We chose 1-methyltryptophan and α-methyltryptophan for this purpose. Further, in view of the relatively high concentration of tryptophan compared to other amines and their metabolites we found that micro quantities (10 ul) of the samples are adequate for rapid determination of this important amino acid in various types of biological materials, such as blood, serum, tissue homogenates etc. Such a method which has been in routine use in our laboratory for the past few months is described in this short communication.

Materials and Methods:

1-Methyl tryptophan and α-methyl tryptophan were purchased from Sigma Chemical Company (St. Louis, MO.). All solvents were hplc grade and the chemicals analytical grade. Blood samples were collected from normal healthy volunteers,

psychiatric patients on antidepressant medication, in green top venoject tubes (heparin), or red top tubes for serum. In addition 10 microliters of whole blood samples were obtained from volunteers by the needle puncture technique using an autolet instrument. Amniotic fluid samples were from the toxicology laboratory and cerebrospinal fluid samples were those received for catecholamine metabolite levels. Human brain autopsy samples were received for antidepressant levels from over dose suicide cases and the homogenates prepared for drug assays (2) were used for tryptophan assay. Rat and rabbit blood and CSF samples were obtained from experimental animals from ongoing studies on drug metabolism (3). All tissues were homogenized with 4 volumes of phosphate buffer (pH 7.0, 0.1 M.) with a polytron homogenizer and centrifuged at 2500 rpm for 10 minutes and the supernatant used for tryptophan assay.

Ultrafiltrates:

Ultrafiltrates from plasma, serum, CSF and amniotic fluid were obtained by three different methods, using Amicon centrifree, Amicon Cones or filter discs (0.25 microns).

HPLC Conditions:

The HPLC system consisted of M-45 Waters pump, Rheodyne valve with 200 ul loop and Bio Analytical Systems (BAS)

electrochemical detector (LC-4B) with a glassy electrode. The oxidation potential was set either at 0.85 or 0.90 V. A wide variety of columns were used to evaluate the resolution characteristics and also for cross validation of results and are listed in Table I.

Mobile Phase:

Triethylamine phosphate buffer (pH 3.0) was prepared by adding 7 ml of phosphoric acid to 4 liters of deionized distilled water followed by 14 ml of triethylamine. The same buffer of pH 3.5 was prepared by mixing 6 ml of phophoric acid and 14 ml of triethylamine. To each of the buffer solutions 50 mg/L of EDTA disodium salt and sodium heptane sulfonate were added. In addition, ammonium acetate buffer (pH 4.5) was prepared by mixing 40 ml of distilled glacial acetic acid and 20 ml of conc ammonium hydroxide solution in 4 L of deionized water. Acetonitrile was mixed with the buffer solutions ranging from 1%-7.5% depending on the column used for analysis (Table I). All solutions were microfiltered and degassed before use.

Sample Preparation:

Ten microliters of the sample of whole blood, plasma, serum, RBC or tissue homogenate are added to 50 ul of

TABLE 1. Retention Data for Tryptophan and α-Methyltryptohan

Column	Mobile Phase + % CH_3CN	Flow Rate ml/min	Retention Time (mins) TRP	α-MeTRP
PE Cartridge 3 cm 3 um C_8	MP-1 + 2	1.2	3.36	5.76
	MP-1 + 4	1.2	2.64	4.8
Ranin 5 cm 3um C_8	MP-1 + 4	1.2	2.88	4.32
SGE 10 cm 3 um C_8	MP-1 + 4	1.4	4.08	7.2
	MP-1 + 6	1.2	4.0	6.0
PE 3 cm 3um C_{18}	MP-1 + 4	1.0	1.92	3.36
	MP-2 + 3	1.0	3.36	4.8
Beckman 7.5 cm 3 um C_{18}	MP-1 + 6	1.5	3.6	5.5
Vydac 25 cm 10 um C_{18}	MP-1 + 7.5	1.4	5.28	7.44
	MP-1 + 5	1.4	7.2	11.3

acetonitrile in a 5 ml culture tube. In the case of amniotic fluid 50 ul of the sample was used. To this mixture are added 200 ng of α-methyltryptophan (10 ul of 20 ng/ul) as internal standard and 180 ul of TEA PO_4 buffer pH 3.0. The mixture was vortexed for 10 secs and centrifuged for 5 minutes and 25 ul of the clear supernatant injected into hplc system. Five microliters of CSF sample or ultra filtrate sample and 5 ul of internal standard (2 ng/ul) were directly injected into hplc system.

Standard Solutions:

Ten microliters of standard tryptophan solutions containing 10 ng/ul and 20 ng/ul are added separately to two

tubes S_1 and S_2 containing a mixture of 50 ul acetonitrile, 10 ul of internal standard (200 ng) and 180 ul of TEA PO_4 buffer pH 3.0. These two standards of 100 ng (S_1) and 200 ng (S_2) are prepared fresh daily and used as standards at the beginning and the end of the sample runs. 25 ul of the standards are injected into hplc system.

All tryptophan levels are calculated from peak height ratios of tryptophan/α-methyltryptophan of the samples and standards.

RESULTS

The retention times for tryptophan and α-methyltryptophan for different columns and column conditions are given in Table I. 1-Methyltryptophan elutes after α-methyltryptophan. However, we found tryptophan as a contaminant (1%) in the sample of 1-methyltryptophan and therefore did not use it as an internal standard. The applicability of this micro method for a variety of biological samples is clearly illustrated in Fig 1 and 2. The clean chromatograms with well separated peaks for tryptophan and the internal standard without any interference from other endogenous compounds render identification and quantitation much simpler. The peak height ratios of Trp/I.S. for standard mixtures under different column conditions are

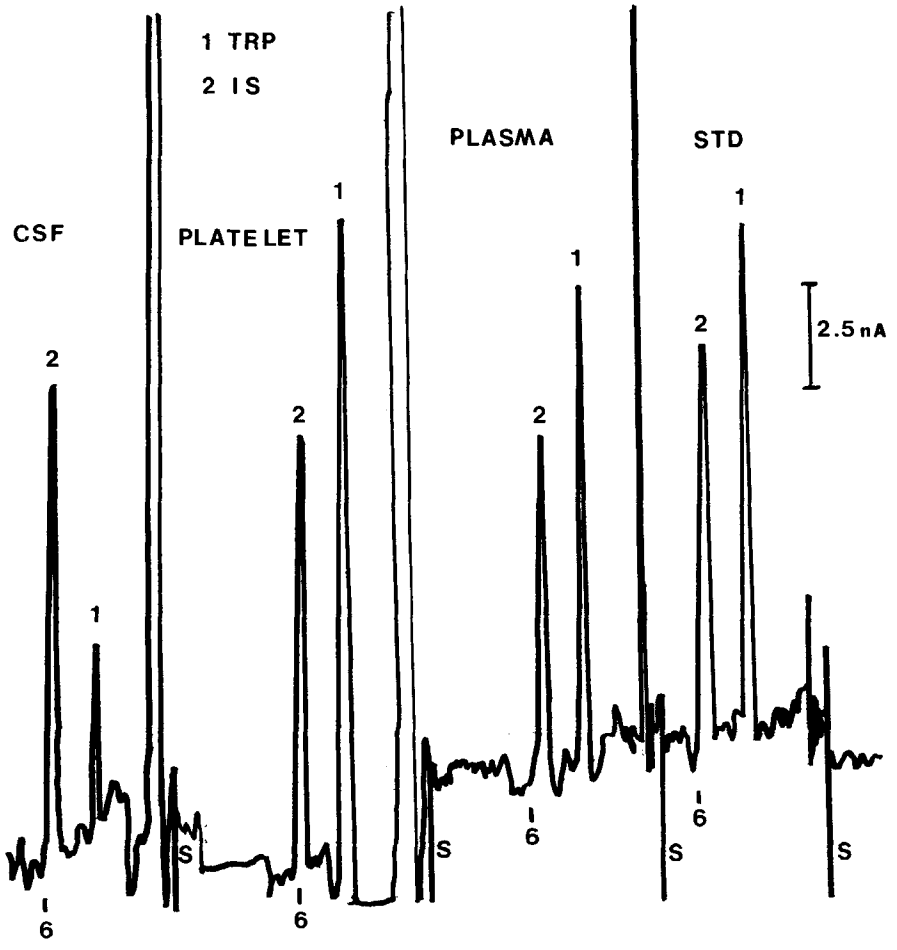

Fig. 1. Chromatogram of standard and samples. Platelet sample was from a volunteer 1 1/2 hours after 0.5 g oral tryptophan and had 2.8 ug/10^9 platelets.

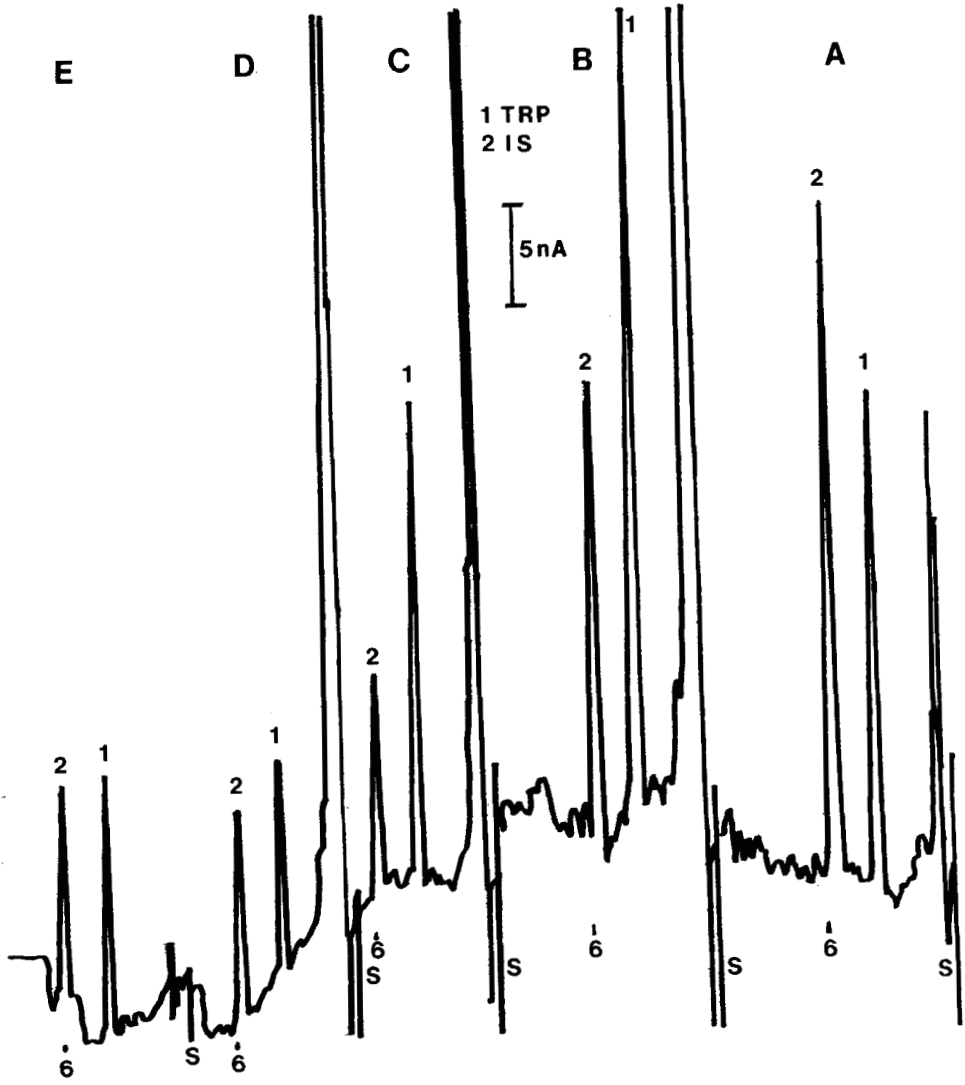

Fig. 2. Chromatogram of (A) standard, (B,C) serum (duplicate), (D,E) amniotic fluid (duplicate).

shown in Table 2. Replicate analysis of two samples is also shown in Table 2. Samples analyzed by the earlier method (1) were reanalyzed by this micro method and the results compared very well (r=0.99). Serial samples drawn from the same subject at different time intervals in a day and on different days by the pin prick method were analyzed by this procedure. The results are shown in Tables 3 and 4. Similarly whole blood samples were obtained by the same procedure from volunteers receiving a single oral dose of 0.5g or 1.0g of tryptophan. A dose dependent rise was observed between 1 1/2 and 2 hours and returned to base line levels at 8 hours (Table 5). Tryptophan levels in different blood components using this micromethod are presented in Table 6.

DISCUSSION

The micro method described here has several advantages over the earlier methods. Since the concentration of tryptophan/ml either in serum or whole blood is usually in microgram level (8-10 ug/ml), 10 ul of the sample is adequate because of the high sensitivity of LC-EC method. Acetonitrile deproteinization does not produce any changes in the pH value and dilution with the mobile phase provides smooth LC run. In most cases the quantity injected is 10 to 20% of the final

Table 2. Peak Height Ratios (PHR), Trp/-MeTrp Of Standards and Samples In Replicate Analyses.

PHR				
Standards			Samples	
1.51 ± 0.01	1.80 ± 0.01	1.1 ± 0.008	1.30 ± 0.02	2.15 ± 0.03
(N=5)	(N=6)	(N=5)	(N=4)	(N=5)

Table 3. Mean Tryptophan Levels in Normal Volunteers of Six Samples Drawn in 24 hour period

Number	Sample	Tryptophan ug/ml (Mean + SD)
1.	Plasma	10.87 ± 0.4
2.	Whole blood	7.47 ± 0.77
3.	Whole blood	10.97 ± 1.63
4.	Serum	10.04 ± 0.45
5.	Serum	12.83 ± 1.17
6.	Serum	12.42 ± 1.18
7.	Serum	9.01 ± 0.57
8.	Serum	9.94 ± 1.6

Table 4. Serum Tryptophan Levels From Normal Volunteers on Samples Drawn on Different Days

	Tryptophan ug/ml		
Subject No.	6/12/85	6/18/85	6/19/85
1	13.6	15.1	12.9
2	12.0	12.6	10.3
3	9.8	7.8	5.7
4	12.5	10.7	15.2
5	9.2	8.8	8.3
6	9.0	8.8	9.0

Table 5. Whole Blood Tryptophan Levels After Single Oral Dose

Time (Hours)	Dose 0.5g Whole Blood Tryptophan ug/ml	Dose 1.0g
0	10.0	10.0
0.5	28.0	55.0
1.0	40.0	73.2
2.0	31.2	54.6
3.0	n.d.	36.2
4.0	18.5	40.5
6.0	12.0	19.1
8.0	10.2	12.0
24.0	9.8	9.2

Table 6. Tryptophan levels in blood components from normal volunteers

Subject	Whole blood	RBC	Plasma	Serum
1	7.7	3.3	10.2	12
2	8.4	3.8	10.24	11.6
3	7.5	3.7	10.3	
4	9.6	3.2	11.7	
5	4.2	3.1	4.95	5.3

volume, equivalent to 1 or 2 ul of the blood or serum. In the case of CSF and ultrafiltrate samples 5 ul of the sample is found to be adequate to give measurable peak height at the oxidation potential of 0.9 V. We found a small volume (0.2 ml) and a short centrifugation time of 10 minutes adequate for obtaining ultrafiltrate samples. We observed differences

between tryptophan values on whole CSF or amniotic fluid samples and their ultrafiltrates indicating some protein binding in these instances also. We found significant difference between platelet rich plasma and the platelet poor plasma which prompted us to look for tryptophan in pure platelet preparations. We could identify and measure tryptophan in homogenates of platelet pellets (fig. 1). RBC samples also contain high levels of tryptophan both free and bound. In the present study we found that 6-HT could not be recovered quantitatively when added to RBC or whole blood and therefore was not suitable as an internal standard.

Tryptophan is finding increasing use as a chemotherapeutic agent either alone or in combination with other drugs and there is a need to assess the tryptophan deficiency and also to follow the pharmacokinetics after oral administration or intravenous perfusion (4). This simple micro method using microquantities of whole blood samples obtained by needle puncture will be a fast and less invasive technique for getting multiple samples from the same subject. The time for obtaining tryptophan level from blood drawing can be as short as 15 minutes and 40 samples can be assayed in a 6 hour run.

We have used this method in recent studies on depressed patients on MAO inhibitor, phenelzine or lithium (5,6) and also for tryptophan determination in discrete regions of the rat brain. Full details will be published elsewhere.

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