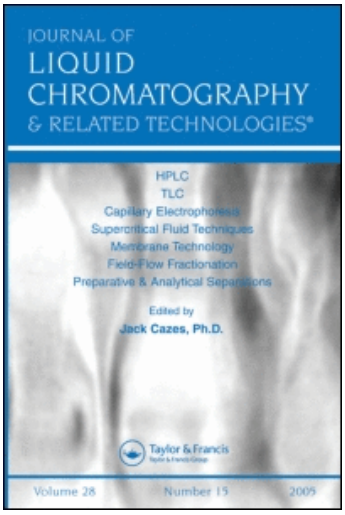

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Quantitation of Serotonin in Human Plasma, Serum and Cerebrospinal Fluid Samples by HPLC-EC Using 6-Hydroxytryptamine as an Internal Standard

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QUANTITATION OF SEROTONIN IN HUMAN PLASMA, SERUM AND CEREBROSPINAL FLUID SAMPLES BY HPLC-EC USING 6-HYDROXYTRYPTAMINE AS AN INTERNAL STANDARD

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ABSTRACT

A simple method for sample clean up and concentration of serotonin (5-HT) in biological samples, such as human cerebrospinal fluid and serum, is described. To the sample 6-hydroxytryptamine (6-HT) is added as an internal standard and it is then absorbed either on C_{18} SEP-PAK cartridge or Biorex-70 short column. 5-HT and 6-HT are then eluted from the column with methanolic formic acid. After evaporation, the residue is dissolved in the mobile phase and an aliquot is used for LC-EC quantitation.

INTRODUCTION

In previous communication, the use of 5-hydroxyindole as an internal standard for the quantitation of 5-hydroxytryptophan, 5-hydroxytryptamine (serotonin, 5-HT), 5-hydroxyindoleacetic acid, homovanillic acid was described (1). In this instance, samples such as cerebrospinal fluid (CSF), blood platelets and tissue homogenates were directly injected into the LC-EC system with the internal standard (5-HI) added to the sample. However, the other biological samples samples such as serum or plasma, some tissue homogenates need extensive sample cleanup before quantitation by

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LC-EC. Extraction procedures or ion exchange columns where acidic and basic components are separated have been used for the clean up (2,3). Various types of compounds were used as internal standards by some investigators while others have used no internal standard at all (2,3,4). In our earlier work with a catechola- mine metabolite by GC and GC/MS successfully used isomeric compounds as internal standards (5). I now report the use of 7-hydroxytryptamine (6-HT) as an internal standard for the quantitation of 5-HT in biological samples.

MATERIALS AND METHODS

Serotonin hydrochloride and 6-hydroxytryptamine creatinine sulfate were obtained from Sigma Chemical Company (St. Louis, MO). All solvents were HPLC grade from Burdick and Jackson or Baker Chemical Company. C_{18} SEP-PAK cartridges were obtained from Waters Associates (Milford, MA). HPLC-EC system is from Bioanalytical Systems (BAS, West Lafayette, IN) with LC-4 detector. C_{18} reverse phase columns, 5 um 15 x 0.46 cm, 3 um 10 x 0.46 cm from Rainin, 5 um 10 x 0.46 cm from Biorad, 5 and 3 um 15 x 0.46 cm from Supelco were used. Two mobile phases (1) 0.05 M Triethylamine phosphate (pH 3.0) with acetonitrile 3.5% and (2) 0.035 M ammonium acetate buffer (pH 4.6) containing 5% acetonitrile were used. All solvents were filtered through millipore filters and degassed before use. The ionization was set at 0.65 V.

GC/MS Conditions:

A Finnigan 4000 GC/MS 6110 Data System was used for GC/MS analysis. A 3% OV-17 on Gas Chrom Q column (2m x 4mm i.d.) was used for GC at an oven temperature 180°C isothermal. All quantitative GC/MS analyses were carried out at ionization potential 70ev, separator temperature 250°C, ion source temperature 260°C

and ionization current 0.45 A. The standard method (6) using d_4-5-HT as internal standard and tri-trifluoracetyl (TFA) derivative was used for validating LC-EC results. Ions m/z 351 and 354 were monitored for 5-HT and internal standard.

Procedure for Sample Cleanup:
Plasma SEP-PAK Procedure:

To one milliliter of plasma, 200 ng of the internal standard (6-HT) was added, the sample alkalinized with 0.5 ml 10% sodium carbonate, and the mixture passed through a C_{18} SEP-PAK cartridge activated prior to use (7). The plasma was followed by 1 ml of water wash, 5-HT and internal standard were eluted by passing 4 ml of methanolic formic acid (90 ml methanol + 10 ml formic acid) through the C_{18} cartridge. The eluate was collected, evaporated under nitrogen and the residue redissolved in 0.2 ml of the mobile phase and 50 ul used for LC-EC.

A standard calibration curve was obtained by using 50, 100, 150 and 200 ng of 5-HT and 200 ng of 6-HT (internal standard and plotting peak height ratio vs. concentration of 5-HT.

The procedure was repeated on duplicate samples of plasma with 200 ng of d_4-5-HT added as internal standard. The eluate after evaporation under nitrogen was heated with trifluoroacetic anhydride for 15 min. in a reactive vial at 60°C. The reagent was removed under nitrogen, residue dissolved in 20 ul and 2 ul injected into GC/MS system. From a standard calibration curve obtained under same conditions 5-HT in plasma sample is calculated.

Plasma Ion Exchange Procedure:

A 2.5 cm long 0.5 cm diameter column of Biorex 70 (H^+ form) was prepared by pouring slurry of the resin into small chromatographic columns (Biorad) with sintered discs. The column was washed with deionized glass distilled water prior to use. To 1 ml

of plasma sample in a test tube 200 ng of 6-HT (internal standard) was added in the plasma and 1 ml of water wash pushed through the column. This was followed by 2 ml of distilled water wash and 1 ml of methanol wash. 5-HT and 6-HT were then eluted with 2 ml of methanolic formic acid (90 ml methanol and 10 ml 10% formic acid). The eluate was collected in a 5 ml centrifuge tube, evaporated under nitrogen, residue dissolved, in 200 ul of mobile phase and 40 ul of this injected into HPLC system.

A standard calibration curve was obtained with 1 ml each of water solutions containing 50, 100, 150, 200 ng of 5-HT and 200 ng of 6-HT as internal standard following the same procedure. Peak height ratios of 5-HT and 6-HT were plotted against 5-HT concentrations.

Platelet Samples:

Blood samples were collected in sodium citrate tubes, the samples centrifuged at low speed to obtain platelet rich plasma. After obtaining the platelet count (range 500,000 to 600,000 ul) 0.5 ml of the sample was diluted with an equal volume of mobile phase, 500 ng of internal standard added. The mixture was then sonicated for 30 sec and 25 ul injected directly into HPLC. The remaining sample was then divided into two equal parts and one part processed through SEP-PAK after adding 0.5 ml of carbonate buffer. The second part is adjusted to pH 6.5 with carbonate buffer (4 drops) and processed through Biorex-70 column as described above.

Cerebrospinal Fluid Samples:

To 2 ml of CSF sample 5 ng of 6-HT is added and the sample is processed through either SEP-PAK cartridge or Biorex-70. In the former case, the sample is made alkaline with 0.5 ml of carbonate buffer. The SEP-PAK cartridge is washed with 1 ml of hexane and

then eluted with 2 ml of methanolic formic acid. The ion exchange column is washed with 2 ml of water, 1 ml of methanol and then eluted with 2 ml of methanolic formic acid. The eluates are evaporated to dryness under nitrogen at 40°C, the residue is dissolved in 100 ul of mobile phase and 40 ul used for HPLC. A calibration curve is obtained using 1, 2, 3 and 4 ng of 5-HT standards in water following the same procedure.

RESULTS

The separation of 5-HT and 6-HT standards and in a platelets sample by direct injection is shown in Figure 1. The retention data on different columns and mobile phases are shown in Table I. Samples run without the internal standard indicated no interference from any endogenous compound. Further, the SEP-PAK and Ion exchange procedures were standardized by using d_4-5-HT as internal standard and quantitating 5-HT by GC/MS method (1,6). The reproducibility by both methods was good (c.v. 1.7%, n=4). In four experiments using 1 ml aliquots of the same 5-HT/6-HT mixture, the ratio of peak heights was very reproducible; mean $\pm$ SD $\pm$ 0.03 by SEP-PAK method and 1.35 $\pm$ 0.03 by Biorex-70 method. A standard calibration curve with 4, 5, 6, and 8 ng of 5-HT and 10 ng 6-HT gave a value of 0.992. The values for 5-HT on some plasma serum and platelet rich plasma samples by both methods are shown in Table II. The results are in close agreement. Further, in platelet rich plasma samples the results are also comparable to the values obtained by direct injection of platelet samples (Table II). In all, we analyzed 30 control platelet rich plasma samples using either 6-HT or 5-hydroxyindole as internal standard, by direct injection method. The values by either of these methods range between 700 and 1300 ng/10^9 platelets with a mean 851 $\pm$ 120 ng/10^9 platelets in agreement with the value reported by

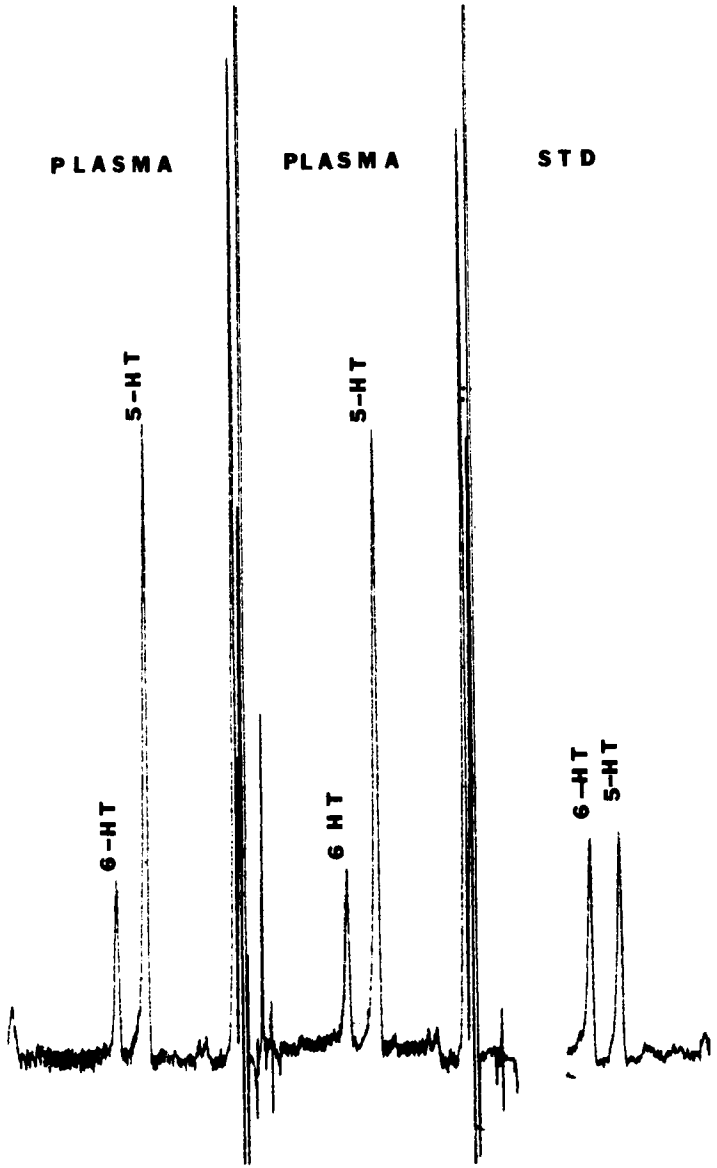

FIGURE 1A. Chromatograms of standard mixture of 5-HT and 6-HT (5 ng each) and plasma sample of phenelzine-treated subject using Biorex-70.

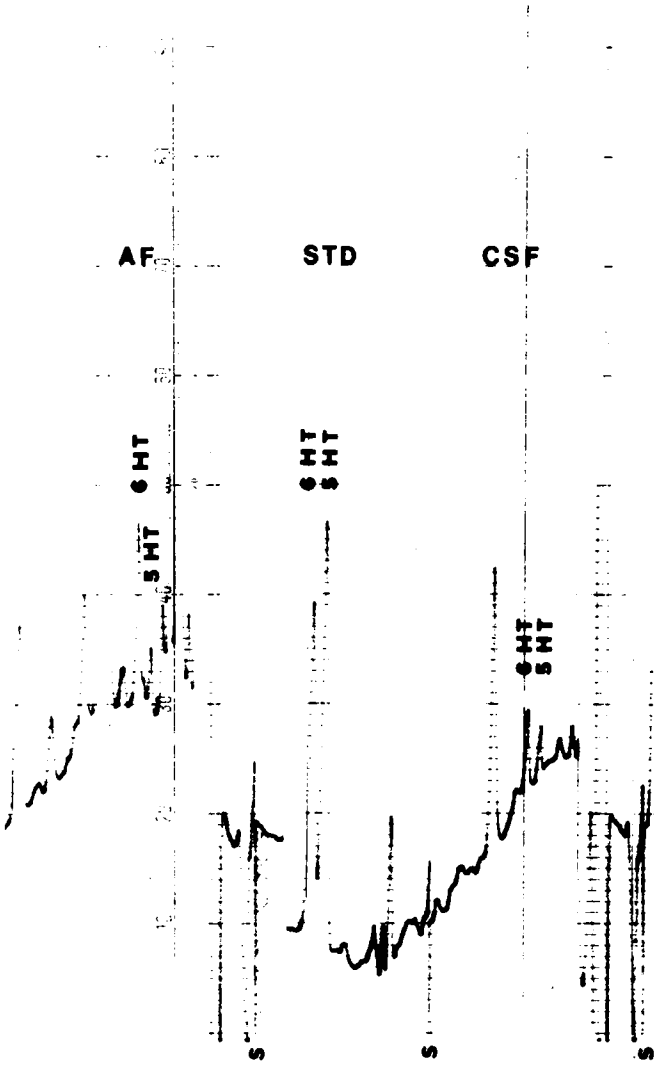

FIGURE 1B. Chromatgrams of cerebrospinal fluid (CSF) and amniotic fluid (AF) samples, 2 ml sample was used in each case with 10 ng of 6-HT for CSF and 25 ng for AF.

TABLE 1

Retention Data for 5-HT and 6-HT

Column	MOBILE PHASE Flow Rate	5-HT	6-HT
		Minutes	
Rainin C_{18} 5u 15 x 4.6	A.0.8 ml.	3.75	4.50
Rainin C_{18} 3u 100 x 4 mm	C.0.8 ml.	1.25	1.5
Same	B.1.0 ml.	4.33	5.66
Biorad C_{18} 5u 15 x 4.6	Same	2.8	3.44
Same	B.1 ml.	3.18	4.18
Supleco C_{18} 3u	B.0.8 ml.	1.63	2.13
Same	C.1.0 ml.		
Supelco C_{18} 5u	C.1 ml.		

A. pH 4.65 5% CH_3CN.
B. pH 3.0 3.5% CH_3CN.
C. pH 4.65 7.5% CH_3CN.

Markey et al. using a GC/MS method (8). We have found a similar correlation using a different GC/MS method (1,6).

The 5-HT levels in a few CSF samples run on SEP-PAK or Biorex-70 columns are shown in Table III. The limit of detectable level in our present method is 200 pg/ml. The values we have obtained are in the range reported in the literature using radioenzymeassay procedure or radioimmunoassay procedure (9). The reproducibility of the assay is established by replicate analysis (Table III). In three human amniotic fluid samples run on Biorex-70 column, 5-HT levels were 3.5, 2.8 and 3.2 ng/ml. The

TABLE 2

Serotonin Levels in Plasma, Serum and Platelet-rich Plasma Samples. ng/ml

Sample	Biorex-70	SEP-PAK	Direct Injection
Plasma (MAOI)	728	730	
Plasma (MAOI)	550	500	
Serum pooled (N=4)	110±13		
PRP	930	960	970
PRP	510	525	510
PRP	750	728	760
PRP	920		900
PRP	650		630

TABLE 3

Serotonin Levels in Human CSF

Sample	5HT(ng/ml)	Method
1	0.81	SEP-PAK C_{18}
2	1.25	SEP-PAK C_{18}
3	0.33	Biorex-70
4	0.5	Biorex-70
Pooled CSF (N=4)	1.25±0.15	SEP-PAK

Standard Calibration Curve $r = 0.992$

chromatograms of plasma, CSF and amniotic fluid are shown in Figure 1.

DISCUSSION

Several methods are reported in the literature for the quantitation of 5-HT and the relative merits of the methods have been discussed (10). Of these, the most specific method is the GC/MS method using d_4-5-HT as internal standard (6). More recently, the HPLC method with amperometric detection has been used for 5-HT determination in biological fluids (1-4). However, samples need cleanup, especially those with very low levels. Solvent extraction methods give very poor extraction efficiency (8). Methods to improve 5-HT recovery have been reported (8). Earlier methods using a combination of ion exchange procedure for sample cleanup and HPLC-EC for quantitation did not use an internal standard (2,3). We have not found any interference by any endogenous compound under the HPLC conditions used in this study. The method has been successfully applied to a variety of biological samples such as serum, platelets, cerebrospinal fluid and the results validated by GC/MS method. We have recently reported an elegant method for extraction of trace amines using C_{18} SEP-PAK cartridges and have now extended it for the extraction of serotonin from plasma and CSF samples with the isomeric 6-HT as internal standard. This method is particularly useful for CSF samples where the levels are extremely low (1 ng/ml) and any organic solvent extraction will only yield extremely poor recoveries. This is the first report of the use of isomeric non-biological compound in LC-EC analysis. We have also demonstrated an alternate ion exchange procedure which gives comparable results and is less expensive. This is an improvement over the RIA method. We have validated the LC-EC results with platelets, or platelet-rich plasma using either SEP-PAK or ion exchange column procedure by comparing the results of direct

injection with 6-HT as internal standard and GC/MS procedure. This method is now in routine use in our laboratory for the assay of 5-HT in serum and CSF samples of different diagnostic groups of patients. In an earlier study, we suggested that measurement of platelet or plasma or serum serotonin would be a better indicator of treatment response with MAO inhibitors (tranylcypromine or phenelzine) (10). The HPLC-EC method described here which is readily accessible and less expensive could be used in clinical studies with MAO inhibitors.

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