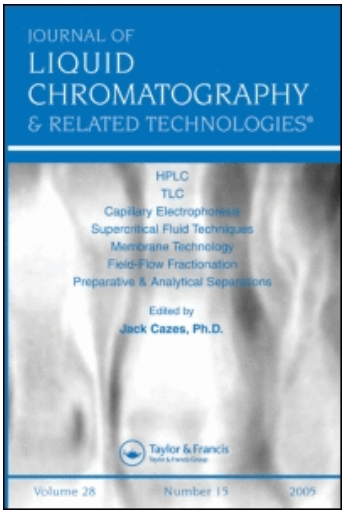

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An Extraction Method to Quantitate Serotonin in Human Serum, Amniotic Fluid and Urine Samples by HPLC Using 6-Hydroxy Tryptamine as Internal Standard

N. Narasimhachari[a]; B. Landa[a]
[a] Department of Psychiatry, Medical College of Virginia, Richmond, Virginia

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AN EXTRACTION METHOD TO QUANTITATE SEROTONIN IN HUMAN SERUM, AMNIOTIC FLUID AND URINE SAMPLES BY HPLC USING 6-HYDROXY TRYPTAMINE AS INTERNAL STANDARD

N. Narasimhachari and B. Landa
Department of Psychiatry
Medical College of Virginia
Richmond, Virginia 23298

ABSTRACT

A method for extracting serotonin (5-HT) from human serum, amniotic fluid and urine samples is described. A mixture of sample, 6-hydroxy tryptamine (6-HT) as internal standard and sodium carbonate is extracted with ethyl acetate. The ethyl acetate layer is mixed with 200 ul of triethylamine phosphate buffer (pH 3.0) used for mobile phase and centrifuged. An aliquot of the aqueous layer was used for HPLC quantitation with amperometric detection. With this method 30 samples can be assayed in 6 hours.

INTRODUCTION

Among the various quantitative methods used for the quantitation of serotonin in biological fluids, the gas chromatographic mass spectrometric method with d4 5-HT as internal standard is the most specific method (1). However, in view of expensive and sophisticated instrumentation, this method is not suitable for routine clinical laboratory setup. Liquid chromatography with electrochemical detection (LC-EC) is now the most widely used method for quantitation of 5-HT in biological fluids and tissue samples (2,5). We have recently reported on cross validation of LC-EC method by GC-MS (5). In many

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instances it is necessary to adopt sample cleanup procedures to selectively extract serotonin from biological samples such as blood, plasma, serum, or urine samples. For this purpose ion exchange columns or Sep-Pak cartridges have been used with 6-HT as internal standard (6). In our work on serum or plasma levels of 5-HT in psychiatric patients where baseline levels and levels after MAO inhibitor or lithium treatment were routinely determined, we needed a rapid and simple method for its quantitation. We report here a simple extraction procedure for 5-HT from biological fluids with 6-HT as internal standard and subsequent quantitation by LC-EC.

MATERIALS AND METHODS

Ethyl acetate was from Burdick and Jackson HPLC grade. 6-Hydroxytryptamine creatinine sulfate and serotonin hydrochloride were from Sigma Chemical Co. (St. Louis, MO).

Standard solutions:

Standard solutions of 5-HT and 6-HT were prepared in deioinized distilled water to contain 1 mg/ml of the respective bases. From the stock solution working standards were individually prepared to contain 2, 5, and 10 ng of 5-HT and 10 ng 6-HT. A mixture of 5-HT and 6-HT containing 2 ng/ul was also prepared for use as a standard in LC-EC.

HPLC: The HPLC system consisted of a Waters M-45 pump with a Rheodyne valve and LC-4B (BAS) amperometric detector. In the present study we have used both C_8 and C_{18} short columns and mobile phases of three different pH values with or without acetonitrile. These are listed in Table 1. The mobile phase was prepared by dissolving in 4 liters of deionized distilled water (1) 7 ml of phosphoric acid and 14 ml of triethylamine (pH 3.0) or (2) 6 ml of phosphoric acid and 14 ml of triethylamine (pH 3.5) or (3) 20 ml of distilled acetic acid and 12 ml of triethylamine (pH 4.0). To each of these solutions 50 mg/L of EDTA disodium salt and 50 mg/L of sodium heptane sulfonate were added.

Table 1. Retention Data For 5-HT And 6-HT For Different Columns and Mobile Phases.

Column	Mobile Phase	Flow rate ml/min	Retention Time (min) 5-HT	6-HT
SGE, 10 cm, 3 um, C_8	MP-1 + 1.5% CH_3CN	1.2	6.96	8.88
Beckman, 7.5 cm, 3 um, C_{18}	MP-1	1.2	4.56	6.24
	MP-3	1.2	4.08	5.28
Rainin, 5 cm, 3 um, C_8	MP-1	1.4	3.36	3.84
PE, 3 cm, 3 um, C_8 (cartridge)	MP-1	1.0	2.16	2.64
PE, 3 cm, 3 um, C_{18} (cartridge)	MP-1	1.0	4.0	5.6
Same	MP-2 + 2% CH_3CN	1.4	3.2	4.2

SGE = Scientific Glass Engineering; PE = Perkin Elmer

MP-1 = TEA PO_4 pH 3.0; MP-2 = TEA PO_4 pH 3.5; MP-3 = TEA-Acetate pH 4.0.

All solutions were microfiltered and degassed before use. The oxidation potential was set at 0.65 volts.

Blood samples:

Blood samples from psychiatric patients and normal controls were collected in venoject tubes without any anticoagulant (red top) or in plain propylene tubes by venopuncture. After leaving in the refrigerator for one hour serum samples were obtained by centrifugation and the samples stored frozen at -40^0. In addition serum samples were also obtained from the clinical toxicology department, samples received for alcohol content or lithium levels. Plasma samples were prepared as platelet rich (PRP) or platelet poor (PPP) from sodium citrate tubes first by slow speed centrifugation followed by regular centrifugation. Amniotic fluid samples were obtained from clinical toxicology and Ob/Gyn from samples received for billirubin and oc-fetal protein determinations.

Calibration Curve:

Platelet-poor plasma samples from the blood bank were used for the preparation of standards for calibration. The samples were assayed for 5-HT content and found to contain generally less than 20 ng/ml (range 5-20 ng/ml). Standard solutions of plasma containing 0, 100, 200, 300, 400, and 500 ng/ml of 5-HT were prepared and stored frozen. Similarly water solutions of standards were also prepared for use as standards for calibration curve. A second set of standards containing 20, 40, 60, 80, 100 and 200 ng/ml in water was also prepared. In routine analysis the use of three water standards, 50, 100 and 200 ng/ml was found to be adequate to cover the range of biological fluids.

Extraction procedure:

A mixture of 0.25 ml of serum, PRP, or PPP or urine sample, 10 ul of 6-HT (100 ng) and 0.1 ml of 2N sodium carbonate was extracted with 4 ml of ethyl acetate by vortexing for 10 seconds. After centrifuging for 5 minutes at 250C rpm, the ethyl acetate layer was transferred into a 5 ml culture tube

containing 200 ul of TEA PO_4 buffer (pH 3.0) used for the mobile phase in HPLC. After vortexing for 10 secs, the tubes were centrifuged and 40 ul aliquots drawn from the bottom aqueous layer were used for HPLC. From the peak height ratios of 5-HT/6-HT of the samples, 5-HT in the sample is directly calculated from the calibration curves. For the assay of amniotic fluid, 1 ml of the sample was used. Calibration standards were run under identical conditions as the samples.

GC-MS validation:

A few of the serum samples (N=6) in both low and high range values were assayed by gc-ms selected ion monitoring using d_4 5-HT as internal standard as described earlier (5).

Comparison of extraction and column methods:

Equal aliquots of the same serum samples (0.25 ml) containing the same amount of 6-HT as internal standard (100 ng) were assayed by the extraction procedure and Biorad-70 column procedure (6) and the peak height ratios by the two methods were compared.

RESULTS

The separation of 5-HT and 6-HT on a variety of C_{18} columns was reported in an earlier paper (6). The retention data on short 3 um C_{18} and C_8 columns and cartridges are presented in Table 1. The chromatograms in Fig. 1 illustrate the separation in standards and different serum samples on a 3 cm, 3 um, C_8 cartridge. The same kind of separation is also achieved on several other short columns with a run time of 5 minutes per sample. The chromatograms of plasma and serum samples show no other peaks indicating selective extraction of 5-HT and 6-HT possibly due to relatively higher concentrations compared to other biogenic amines. It is also noticed that extraction efficiency of endogenous serotonin is considerably increased by the addition of 6-HT as internal standard. Extraction efficiency on the basis of the recovery of internal standard was 57 $\pm$ 3.5% (n=50). Calibration curves

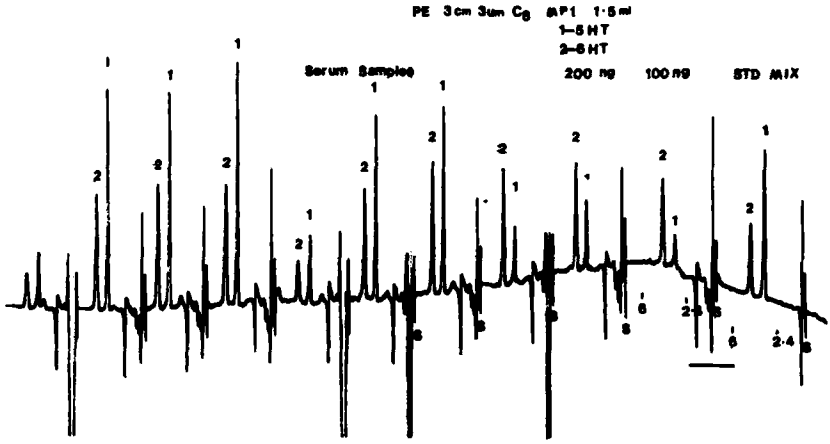

Figure 1. From right to left: Neat standards, calibration standards 100 and 200 ng/ml, serum samples from a subject on phenelzine. Time course of MAO inhibition based on rise in serum 5-HT.

Table 2. Comparison of Serum 5-HT Using Plasma and Water Standards.

Sample	Plasma*	H_2O*
1	219.5	202.3
2	59.2	55.7
3	186.5	172.6
4	49.3	46.8
r value for standard curve.	0.994	0.999

Table 3. Replication Analysis Of 5-HT in Serum Samples.

	Pool A	Pool B
1	164.4 ng/ml	140.9 ng/ml
2	162.7	136.8
3	172.2	148.9
4	174.3	147.5
5	169.7	
mean	169.7 $\pm$ 4.45	143.5 $\pm$ 5.68

are linear from 0 to 500 ng/ml and the r value was reproducible from day to day (r=0.996 ± 0.003, n=6). Similarly the peak height ratios of 5-HT/6-HT by column and extraction procedures were consistently almost identical (r=0.99, n=6). In our experience plasma and water standards gave similar values for unknown samples (Table 2) and the slopes and r values were very similar except that the plasma curve did not pass through zero. The reproducibility of the procedure is shown by replicate analysis of two pooled serum samples (Table 3). The results on select serum and urine samples from different clinical categories are given in Table 4. The normal levels found in serum vary as reported in the literature (2,3). Plasma samples obtained from volunteers, using heparinized tubes (green top) showed wide variation in 5-HT levels possibly due to varying amounts of platelets. However, if these plasma samples were recentrifuged and only platelet-poor plasma was taken, the levels were consistent and less than 20 ng/ml. Platelet rich plasma samples gave identical results by the present extraction method and the direct method described earlier (6).

Amniotic fluid samples were assayed both by column and extraction procedures; and the levels ranged between 5 ng and 40 ng/ml. No attempt was made to ascertain the stage of pregnancy or any other clinical picture of these subjects as this was only intended to develop an analytical method. Urine samples also give a relatively clean chromatogram by this extraction procedure and LC-EC (Fig. 2); and the levels were in the range of 50-180 ng/ml in the four samples of normal volunteers and much higher in phenelzine-treated patients (Table 4).

DISCUSSION

We had recently reported a column procedure for the extraction of low levels of serotonin from cerebrospinal fluid, serum, plasma, amniotic fluid and urine samples using either SEP-PAK C_{18} cartridge or Biorad-70 ion exchange

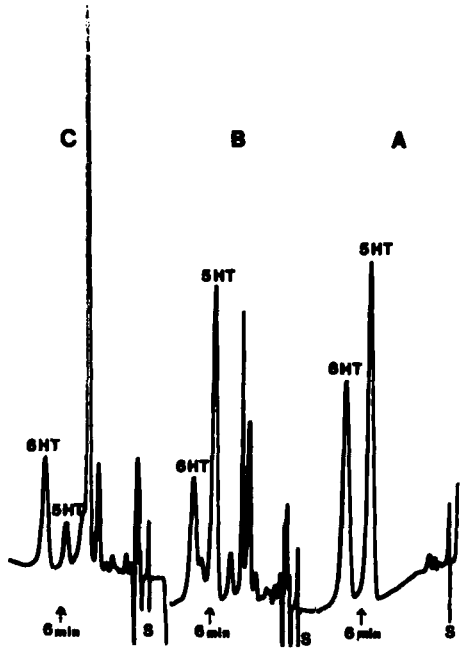

Figure 2. Chromatograms showing extracts of (A) water standards (B) urine sample from phenelzine treated subject and (C) baseline urine sample from the same subject.

columns (6). We have now extended this study using a simple extraction procedure and the same 6-HT as an internal standard and validated the method by comparing the results with column procedure. Serum, plasma, and urine samples contain adequate levels of 5-HT (20 ng/ml) making extraction with ethyl acetate feasible. The back extraction into the mobile phase at pH 3.0 is quantitative and reduces the sample handling time. Thus, for example, in platelet-poor plasma samples and amniotic fluid we were able to quantitate levels as low as 5 ng/ml using this procedure. Further, in serum or plasma samples, with the mobile phases we used and the oxidation potential of 0.65 V, the chromotagrams were very clean showing in most cases only two peaks of 5-HT and 6-HT without interfering shoulders or overlapping peaks. With urine

Table 4. Serum 5-HT Levels In Different Clinical Samples.

Nature of Sample	5-HT ng/ml
Serum (normal)	111
	95
	107
	140
Serum (depressed patients)	73
	67
	94
	82
Serum (MAOI)	260
	391
	289
	419
Platelet-rich Plasma	600
	800
	720
	650
Urine (normal)	53
	141
	180
	162
Urine (MAOI)	543
	2962
	1440
	3000
Amniotic Fluid	32
	20
	5
	39

samples especially from subjects on MAO inhibitor, we observed more peaks in the chromatograms which we could identify as tyramine, metanephrine, and 3-methoxy-tyramine. The accuracy of the extraction method was checked by cross validation of the assay by column procedure or LGC-MS procedure (6). The correlation between different methods (r=0.97) was more than adequate for assay of biological samples. The two-step extraction method is much simpler than the butanol-HCL method where it is not selective and other indole compounds could interfere , especially in fluorometric detection. In our

experience the direct injection of serum samples deproteinized with perchloric acid did not yield reproducible results due to interfering substances. The chromatograms with only measurable peaks of 5-HT and internal standard 6-HT enhance the specificity of the entire procedure. In our earlier paper we have used ethyl acetate extraction for blood platelet samples where 3H 5-HT was used as an internal standard for monitoring the extraction efficiency which was around 35% (7). In our present work using 6-HT as an internal standard the monitoring efficiency is 57%, making it possible to determine low levels of 5-HT with only 0.25 ml of the sample. Markey, et al. (8) have used an acetylation technique to increase the extraction of very low vels of 5-HT; and we used the ion exchange column procedure (6).

The pH 3.0 buffer extracts were found to be stable for 48 hours when stored in the refrigerator. When the extracts of serum samples were analyzed after storage for 24 hours and 48 hours no significant difference was found between assay values. One cautionary note has to be emphasized with regard to the stability of the internal standard 6-HT. Frozen standard containing 1 mg/ml or 100 ng/ml was found to be stable for over a year. However, in dilute working standards 10 ng/ul, used as internal standard, and 2 ng/ul used in working standard mixture, we noticed that 6-HT samples showed a second peak (Fig. 3) after 2 or 3 weeks storage; and when this happened the deterioration was subsequently very fast, the percentage of second peak increasing relative to 6-HT. It is therefore necessary to prepare fresh working standards every week and change the standards when this contamination is noticed. Dilute 5-HT standards were stable when stored in the refrigerator for 4 weeks.

In conclusion we have to state that we have described a simple and specific method for 5-HT determination using an isomeric non-biological compound as internal standard and as many as 30 samples can be assayed in 6 hours. Since abnormal 5-HT metabolism is reportedly associated with a number of psychiatric and other clinical conditions (9), this method should prove useful in such clinical studies. The results of our own clinical studies

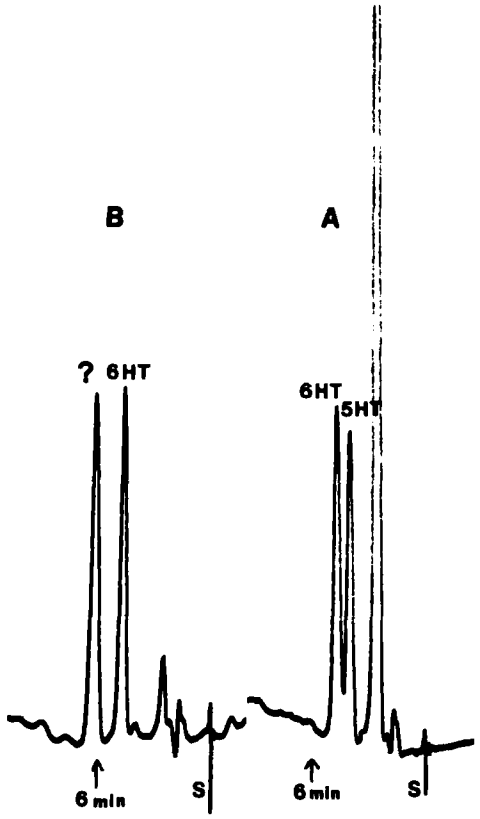

Figure 3. Chromatogram of (A) mixture of 5-HT and 6-HT and (B) 6-HT and its decomposition product.

using this analytical methodology on the effects of lithium and of phenelzine, an MAO inhibitor, on serum 5-HT have recently been presented (10,11).

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