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Note

Determination of serotonin in plasma by liquid chromatography with electrochemical detection

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Serotonin (5-hydroxytryptamine; 5-HT) is located primarily in the enterochromaffin cells of the intestine, the serotonergic neurons of the brain and the platelets of the blood. Serotonin has been established both as a neurotransmitter in the central nervous system and as a vasoconstrictor, and is implicated in some physiological functions such as sleep regulation [1] and pathological phenomena such as depression [2], carcinoid syndrome [3] and essential hypertension [4].

Various methods have been applied for the determination of serotonin in biological fluids and nervous tissues, including fluorimetric techniques [5—10], gas chromatography—mass spectrometry (GC—MS) [11] and radioimmunological [12, 13] and radioenzymatic methods [14]. However, they suffer from a lack of sensitivity and specificity (fluorimetric techniques) or are expensive (GC—MS). Recently, high-performance liquid chromatography (HPLC) with electrochemical detection (ED) has proved to be a sensitive and inexpensive method for measuring catecholamines, indoleamines and their metabolites in tissues and body fluids [15—20].

This paper describes a method with an internal standard for the measurement of serotonin in platelet-poor plasma (PPP) and in platelet-rich plasma (PRP), using reversed-phase HPLC—ED. The method requires two successive liquid extractions to concentrate and purify the samples. We carried out this study on healthy young adults in order to determine normal values for extra-platelet serotonin (from PPP) and intra-platelet serotonin (from PRP).

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EXPERIMENTAL

Chemicals and reagents

Serotonin creatinine sulphate and N-methyl-5-hydroxytryptamine oxalate (internal standard, I.S.) were obtained from EGA-Chimie (Strasbourg, France). The other chemicals were of analytical-reagent grade: chloroform, 1-pentanol, sodium chloride, glycine, disodium hydrogen phosphate dihydrate, citric acid, the disodium salt of ethylenediaminetetraacetic acid (Merck, Nogent, France), methanol and sodium hydroxide (Prolabo, Paris, France).

Standard solutions

Stock solution of serotonin was prepared by dissolving 22.8 mg of serotonin creatinine sulphate in 100 ml of distilled water to give 100 μg/ml free base. A stock solution of the internal standard was prepared by dissolving 14.7 mg in 100 ml of distilled water to give a concentration of 100 μg/ml. These solutions remain stable for one month if stored at 4°C.

Apparatus

The chromatographic system was composed of a Waters 6000A pump (Waters Assoc., Paris, France), and a Rheodyne injection valve (Touzart et Matignon, Vitry sur Seine, France) fitted with a sample loop (20 μl). A guard column filled with μBondapak C_{18} was placed before the C_{18} reversed-phase analytical column (250 × 4.6 mm I.D., particle size 5 μm) (Altex). The column effluent was monitored by using an electrochemical detector (Eldec 102) (Chromatofield, Chateauneuf les Martigues, France). The output of the detector was connected to a Model S recorder (Servogor, St. Etienne, France) with a chart speed of 120 mm/min. The mobile phase was pumped at a flow-rate of 1 ml/min (147 bars).

Operating conditions

The HPLC mobile phase consisted of 0.1 *M* citric acid buffered with 0.1 *M* $Na_2HPO_4 \cdot 2H_2O$ (pH 3.8) containing 12% of methanol and 0.1% of 0.1 *M* EDTA. To minimize the background noise and improve the detection sensitivity, it is important to filter the mobile phase under vacuum through a 0.22-μm Millipore filter and degas it. Chromatography was carried out at room temperature. The potential of the working electrode (glass carbon) was set at +0.6 V versus the Ag—AgCl reference electrode. The applied cell potential was determined by hydrodynamic voltammetry of serotonin and the internal standard in the mobile phase. The sensitivity of the detector was 1 and 20 nA for PPP and PRP, respectively.

Patients

Serotonin levels in PPP and PRP were determined in twenty healthy adults (ten females and ten males) with a mean age of 30 ± 11 years and a mean weight of 57 ± 12 kg.

Blood collection and processing

Volumes of 10 ml of venous blood were collected from individuals in the fasting state at 8.00 a.m. in siliconized glass tubes containing 0.1 ml of EDTA solution (15%, w/v). The samples were mixed and placed in ice—water and the blood was rapidly centrifuged at 100 *g* for 10 min at 4°C to obtain PRP. A platelet count on PRP was obtained using a Model S Plus Coulter Counter (Coultronics, Margency, France). A 1-mg amount of sodium disulphite, used as antioxidant, was added and the PRP stored at −70°C until taken for assay.

A 1.5-ml aliquot of PRP was centrifuged at 6000 *g* for 15 min at 4°C to produce PPP. Subsequently, the supernatant was carefully decanted and transferred into a clean tube containing 1 mg of sodium disulphite and stored.

Sample preparation

Platelet-poor plasma samples. A 1-ml volume of PPP was introduced in a 20-ml glass tube, then 1 ml of buffer (pH 11) containing glycine, 0.1 *M* NaOH and 0.1 *M* NaCl was added. The plasma was adjusted to pH 11 with 0.1 *M* NaOH and 7 ml of chloroform—1-pentanol (60:20, v/v) saturated with water were added and the mixture was shaken mechanically for 10 min. After centrifugation for 10 min at 2000 *g*, the supernatant organic phase (5 ml) was transferred into a 10-ml tube containing 400 μl of 0.1 *M* hydrochloric acid and the mixture was then shaken for 10 min. After centrifugation for 10 min at 2000 *g*, 20 μl of the aqueous phase were injected into the HPLC system.

Calibration graphs were generated by spiking blank PPP (950 μl) with 50 μl of serotonin solution at concentrations of 3.12, 6.25, 12.5, 25, 50, 100 and 200 ng/ml. All the PPP and the standards were previously supplemented with internal standard (50 μl of N-methyl-5-hydroxytryptamine solution at a concentration of 100 ng/ml). The standard solutions were processed in the same manner as the samples.

Platelet-rich plasma samples. The extraction procedure used for assaying PRP was similar to that described above. Only 500 μl of PRP were taken; the calibration graphs were prepared by supplementing blank plasma (450 μl) with 50 μl of serotonin solution at concentrations of 62.5, 125, 250, 500, 1000, 2000 and 4000 ng/ml. All the PRP samples were supplemented with the internal standard (50 μl of solution at a concentration of 2000 ng/ml).

RESULTS AND DISCUSSION

Effects of centrifugation

Plasma serotonin is mostly intra-platelet, a small proportion being "free" extra-platelet. We had to determine the optimal centrifugal speed to obtain good sedimentation of platelets without damaging them. We centrifuged PRP for 15 min at increasing speeds of 1000, 6000, 13 000, 23 000, 33 000, 52 000 and 70 000 *g*. Then we determined the serotonin concentration of the supernatant (Fig. 1). The proportion of extra-platelet serotonin was lowest at 6000 *g*. When a high centrifugal speed was applied the concentration of serotonin in the PPP obtained increased; the platelets were damaged during high-speed centrifugation and serotonin was released. We therefore chose 6000 *g* at 15 min for the routine preparation of PPP.

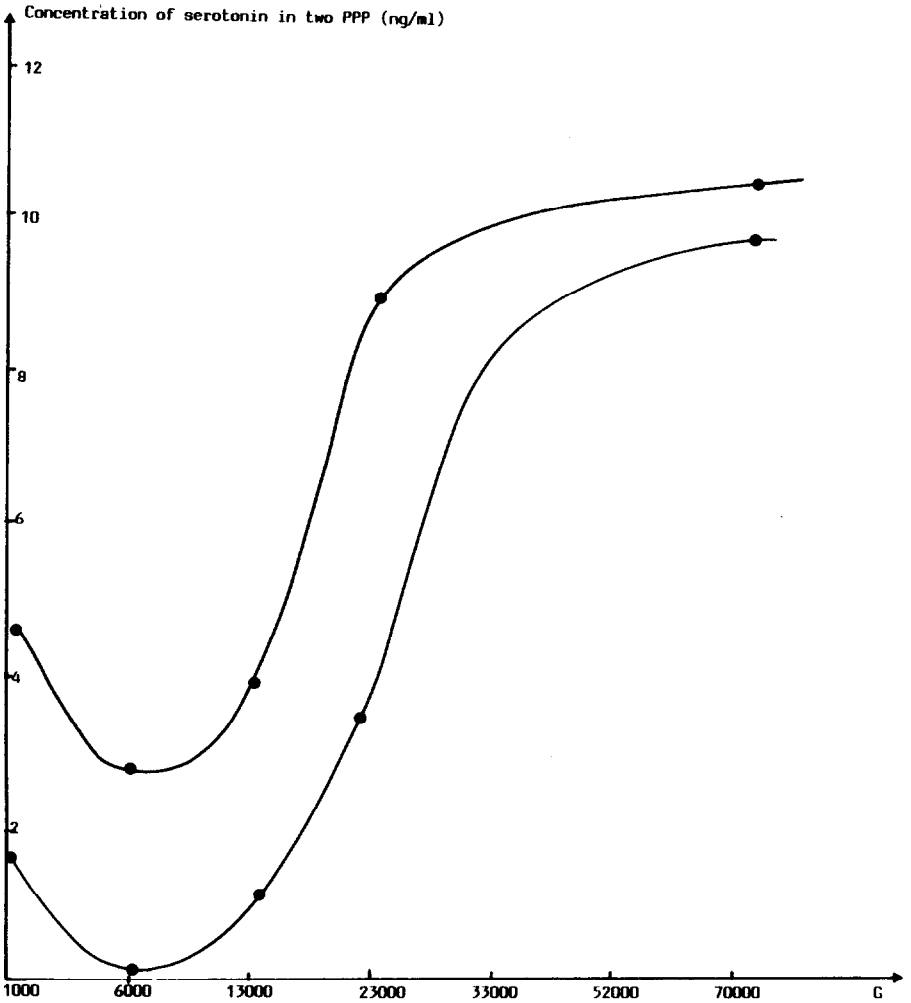

Fig. 1. Effects of centrifugation on the concentration of serotonin in the PPP.

Extraction

Different organic solvents were tried for the extraction of serotonin from biological fluids and the results are presented in Table I. The highest recovery was obtained with chloroform—1-pentanol. Neither the kind nor the molarity of the acid used for the back-extraction had any influence on the recovery.

TABLE I

VARIATION OF RECOVERY WITH THE ORGANIC SOLVENT USED FOR EXTRACTION

Organic solvent	Recovery (%)
Chloroform—1-pentanol	90
Ethylacetate	0
Hexane	5
Chloroform	10

Quantification

Calibration graphs of the serotonin-to-I.S. peak-height ratio versus serotonin concentration were plotted and that for serotonin in plasma was linear throughout the concentration range studied. The correlation coefficient of the linear calibration was 0.9994 for PPP, the equation being $y = 0.3745x - 0.0079$, where y represents the serotonin-to-I.S. peak-height ratio and x the concentration of serotonin. For PRP the correlation coefficient was 0.9995 and the equation was $y = 0.0217x - 0.0042$.

Reproducibility and repeatability

Good intra-day repeatability was obtained; the coefficients of variation for ten assays were 4.9% at 1 ng/ml and 5.3% at 5 ng/ml. The inter-day precision evaluated by analysis over several days ($n = 10$) was 5% at a concentration of 5 ng/ml.

Chromatographic conditions

The best separation of the two chromatographic peaks corresponding to the internal standard and serotonin was obtained with a mobile phase containing 12% of methanol. Under these conditions the retention times were 9 and 11 min, respectively. For a signal-to-noise ratio of 3 the lower limit of quantitation was 200 pg/ml with a sensitivity of 0.2 nA. In each assay, an endogenous peak with the same retention time as the internal standard was carefully screened by means of a blank. In the same way, we checked that other molecules with an indolic nucleus (tryptophan, 5-hydroxytryptophan, 5-hydroxyindolacetic acid, melatonin, tryptamin) did not interfere with serotonin and the internal standard.

Normal values

For PPP the concentrations of plasma serotonin in healthy adults were of 2.71 ± 0.62 ng/ml for men and 1.83 ± 0.90 ng/ml for women. For PRP the concentrations of intra-platelet serotonin were obtained by subtraction of the extra-platelet serotonin concentration. The results, expressed in terms of 10^9 platelets (10^9 pl) were 626 ± 207 ng per 10^9 pl for men and 625 ± 86 ng per 10^9 pl for women.

TABLE II

INTRA-PLATELET AND EXTRA-PLATELET CONCENTRATIONS OF SEROTONIN DETERMINED BY VARIOUS WORKERS

Reference	Techniques	Serum (ng/ml)	PPP (ng/ml)	PRP (ng per 10^9 pl)
6	Fluorimetry			670
7	Fluorimetry		6—19	
8	Fluorimetry		5—12	295—659
9	Fluorimetry		80	150—450
10	Fluorimetry			716—755
12	Radioimmunoassay		6—15	300—380
13	Radioimmunoassay	66—158	0.8—2.4	
14	Radioenzymatic		3.9	
17	HPLC—ED		9.1	
18	HPLC—ED	67—77	3—3.6	

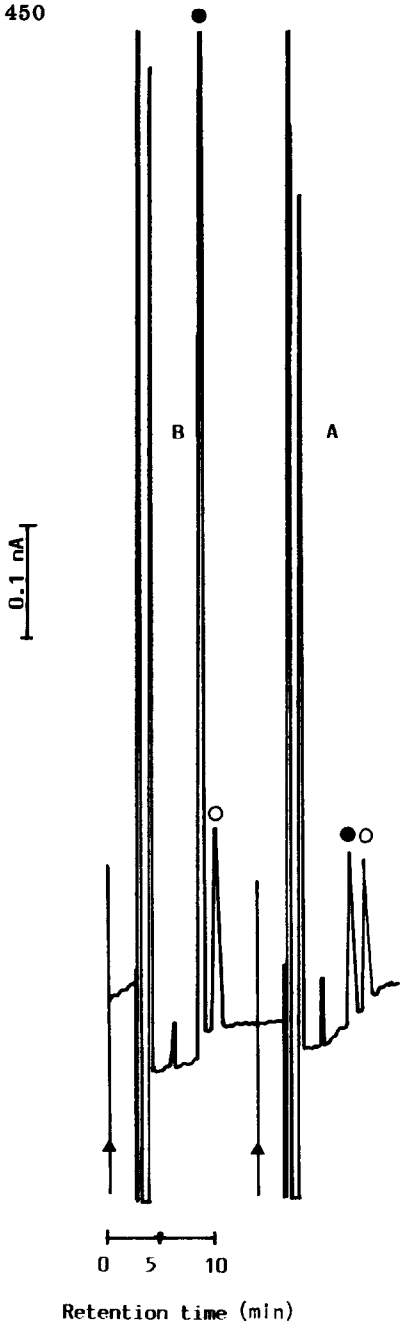

Fig. 2. Chromatograms of (A) normal and (B) pathological plasma samples (PPP). PPP serotonin concentrations: (A) 1.5 ng/ml; (B) 35 ng/ml. Chromatographic conditions: column, μBondapak C_{18}, 250 × 4.6 mm I.D.; mobile phase, 0.1 *M* citric acid buffered with 0.1 *M* $Na_2HPO_4 \cdot 2H_2O$, pH 3.8, containing 12% of methanol and 0.1% of 0.1 *M* EDTA; electrode potential, +0.6 V. ●, Serotonin; ○, internal standard.

It seemed of interest to compare the serotonin levels in PRP and PPP as assessed by different workers (Table II). The results differed not only with the mode of detection used, but even when the same mode was used by different

workers. These variations were probably due to the difference in the specificities of the techniques and to the lack of standardization of sample preparation, PPP, PRP, serum, centrifugal speed [21], intra-platelet serotonin level expressed as ng/ml or ng per 10^9 pl and the choice of anticoagulant.

The serotonin levels in PPP obtained in this work are in accord with those of other workers [13, 14, 17, 18] using different techniques, but the values are lower than all the other results but one [13]. The values in PRP are too difficult to compare owing to the lack of standardized methods of preparation, as mentioned above. We can nevertheless make a comparison with the closely related techniques described by Koch and Kissinger [18] and Tagari et al. [17]. What is interesting in Koch and Kissinger's technique is enrichment on a pre-column, which increases the sensitivity and consequently permits smaller test samples to be used. However, it requires very expensive and heavy equipment, and is therefore difficult to use. Moreover, the use of an internal standard confers greater reliability and better reproducibility.

CONCLUSION

The technique described for the assay of intra-platelet and extra-platelet serotonin is highly sensitive (detection limit 200 pg/ml), reproducible (intra-day coefficients of variation 4.9% and 5.4%; inter-day coefficient of variation 5%) and specific. The assay of extra-platelet serotonin seems to be an important tool for the clinical diagnosis of carcinoid tumours (Fig. 2); moreover, it allows a biological approach to depression and the effects of some anti-depressants.

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