HPLC Determination of Serotonin and Its Metabolites From Human Platelet-Rich Plasma; Shift to 5-Hydroxytryptophol Formation Following Alcohol Consumption

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Abstract

A sensitive, simple, and reliable high-performance liquid chromatographic method with electrochemical detection is developed for the measurement of four natural products, the serotonin-related indols from human platelet-rich plasma (PRP) using *N*-methylserotonin as internal standard. Separation of serotonin (5HT), 5-hydroxytryptophan (5HTP), 5-hydroxytryptophol (5HTOL), and 5-hydroxyindole-acetic acid (5HIAA) is carried out on Supelcosil LC-18DB stationary phase. A mixture of 48mM citric acid, 28mM sodium phosphate dibasic, 0.027mM Na_2EDTA, and 3% methanol (pH 3.18) serves as the mobile phase. Measurements are carried out at 25°C at E_{ox} = 0.65 V. The calibration curves are linear through the range of 10–200 pg/mL. Method validation is performed according to internationally accepted criteria. Blood is collected from healthy controls and schizophrenic subjects. Significantly higher PRP serotonin is measured in schizophrenics; patients with recent alcohol consumption could be characterized with significantly elevated 5HTOL/5HIAA ratio.

Introduction

Serotonin (5HT; 3-(β-aminoethyl)-5-hydroxyindole, 5-hydroxytrypamine) is an ubiquitous compound widely distributed in the animal and plant kingdoms. It can be found in plants (including fruits and vegetables), mushrooms, gastropods, insects, annelids, vertebrate animals, as well as in humans. Among plants walnut and hickory contain 5HT in the highest concentration (25–400 µg/g), while pineapple, banana, kiwifruit, tomato, and plums (1) are also rich in 5HT (3–30 µg/g). 5HT (together with the related tryptamines) is a constituent of toad venoms and in different insects it functions as a neurotransmitter or a neuromodulator or neurohormone (2). In vertebrates and humans 5HT acts as a monoamine neurotransmitter and is localized to three key systems in the body: the gastrointestinal tract (GI; ~90% of the whole 5HT content of the body), platelets (~8%), and the brain (central nervous system = CNS; ~1%). 5HT is synthesized in the enterochromaffin cells of the gastrointestinal mucosa and in the raphe nuclei in the brain. However, platelets containing approximately 99% of the blood 5HT do not synthesize it, but accumulate from the blood circulation (3,4,5) by two different transport systems and store it in dense granules.

Serotonin has widespread peripheral and CNS functions. Platelet serotonin has a key role in promoting blood coagulation (hemostasis) and thrombus formation in connection of atherosclerosis, and it is involved in such vascular diseases as Raynaud's disorder and coronary vasospasm. The "classical" response of blood vessels to serotonin is contraction, particularly in the case of splanchnic, bronchial, renal, and cerebral vessels. Serotonin has positive inotropic and chronotropic effect on the heart, but also produces extreme bradycardia and hypotension.

In addition to its release from the serotonergic nerve terminals in the CNS, serotonin is also released from nonsynaptic varicosities. Thus, it acts both as a neurotransmitter and a neuromodulator (5) and has a pivotal role in aggression, body temperature, sleep, mood, sexuality, appetite, and vomiting. Low levels of serotonin and/or dysfunctions of CNS serotonin metabolism are associated with several psychiatric (depression, schizophrenia, anxiety disorders) and neurologic (migraine, epilepsy, chronic pain) diseases. In different types of headaches, serotonin dysfunction is particularly important (6,7). Several groups of therapeutic drugs are available with primary effect on the serotonergic system (8).

Taken orally serotonin does not pass into the serotonergic pathways. It is synthesized both in the periphery and the CNS from the essential amino acid L-tryptophan by a two-step pathway. The rate-limiting enzyme in the biosynthesis is L-tryptophan-5-monooxygenase (EC. 1.14.16.4, tryptophan

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hydroxylase), which converts tryptophan to 5-hydroxytryptophan (5HTP). This compound also occurs as a natural amino acid in turkey muscles and cheese, and it is used in over-the-counter therapeutic supplements (9). Two different isoenzymes (TH1 and TH2) are known; TH1 is found in the gut and the pineal gland, and TH2 is selectively expressed in the brain (11). In humans two different TH genes exist, located on chromosomes 11 and 12, respectively (12). The other enzyme involved in the synthesis of serotonin is the aromatic L-amino acid decarboxylase (L-AADC, EC 4.1.1.28) known also as DOPA-decarboxylase.

L-AADC is not saturated by 5HTP under physiological conditions; therefore, it is possible to raise serotonin levels by either increasing the amount of tryptophan or 5HTP in the dietary intake. The increase in the rate of 5HT synthesis results in part from alterations in the kinetic properties of THs by post-translational calcium-dependent phosphorylation (short term demands) or by the synthesis of more TH protein (13).

The principal metabolism route of serotonin is a two-step process involving monoamine oxidase (MAO; EC 1.4.3.4) existing in two isoforms (MAO-A and MAO-B) that were cloned (14) and can be distinguished initially on the basis of substrate and inhibitor specificities. Serotonin is the preferential/selective substrate for MAO-A both in vertebrates and humans. Besides the central nervous system, the intestines, kidney, liver, and lungs are the main organs where MAO-A metabolizes serotonin to the intermediate 5-hydroxyindole-3-acetaldehyde (5HIAL). Depending on the tissue NAD/NADH ratio, 5HIAL is further metabolized to the reduced metabolite 5-hydroxytryptophol (5HTOL) by alcohol dehydrogenase (ADH, EC 1.1.1.1.) or the oxidized metabolite 5-hydroxyindoleacetic acid (5HIAA) by aldehyde dehydrogenase (ALDH, EC 1.2.1.3.). Acetaldehyde is in competition for ALDH with 5HTOL (Figure 1).

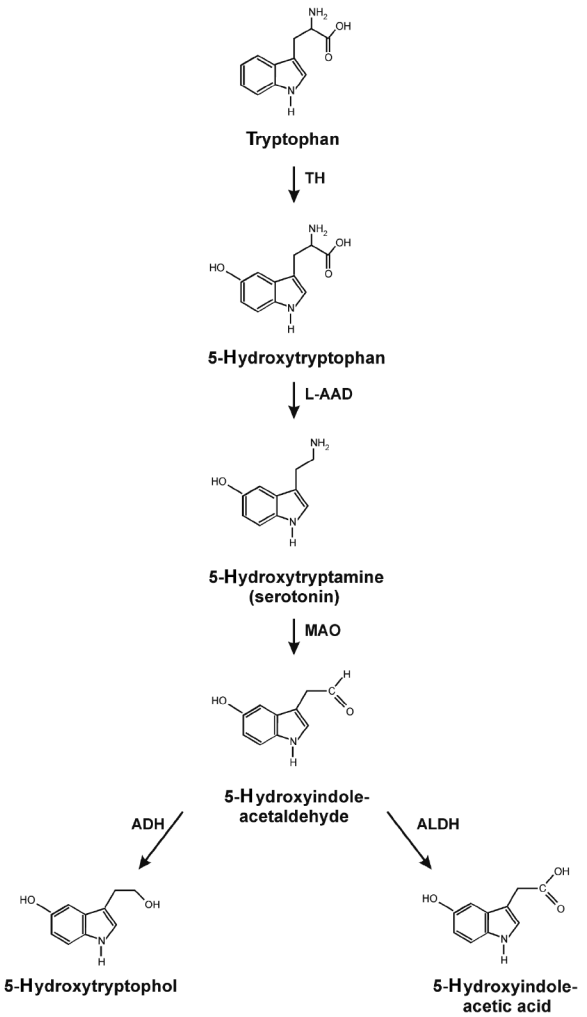

Figure 1. Major steps in the synthesis and metabolism of the serotonin. TH = tryptophan hydroxylase; L-AAD = aromatic L-amino acid decarboxylase; MAO = monoamino oxidase; ADH = alcohol dehydrogenase; ALDH = aldehyde dehydrogenase.

Several methods have been reported for the quantitative determination of serotonin and related indols in biological samples (15–19). In humans, mainly high-performance liquid chromatography with electrochemical detection (HPLC–EC) has been used both for urinary excretion of 5HT, 5HIAA, and 5HTOL (20) and blood/platelet serotonin (21,22). This method has the advantage of many approaches to sample preparation and is sensitive, accurate, and reliable, and provides comparable data between different investigators.

In the present study, we report on a simple and sensitive HPLC–EC method suitable to determine five serotonin-related indols from human platelet rich plasma to obtain information about the synthesis and metabolism of serotonin in one run with high precision and sensitivity.

Methods

Chemicals

Chemicals were purchased from commercial sources in the best available quality: 5HTP, serotonin creatinine sulfate, 5HTOL, 5-hydoxyindole-acetic acid, *N*-methylserotonin oxalate, sodium phosphate dibasic dodecahydrate ($Na_2HPO_4 \times 12\ H_2O$), citric acid monohydrate, ethylenediamino tetraacetic acid disodium salt dihydrate (Na_2EDTA), phosphoric acid, and methanol were from Sigma-Aldrich (St Louis, MO): perchloric acid 70% (PCA) was from Fluka (Buchs, Switzerland). The water was double distilled and deionized and of HPLC grade.

Subjects and sample preparation

Healthy volunteers (N = 29, mean age: 37 ± 11 years, 16 males and 13 females) not having been treated with any drugs and not taking alcohol in the last 72 h served as controls. Patients who fulfilled the criteria for schizophrenia (N = 36, mean age: 42 ± 15 years, 27 males, 9 females) were treated in hospital (4th Department of Psychiatry, Jahn Ferenc Hospital, Budapest and Department of Psychiatry and Psychotherapy, Semmelweis University, Budapest). Patients gave informed consent. The schizophrenic group was further divided according to recent alcohol consumption (N = 21) and alcohol-free (N = 15). Venous blood samples were withdrawn between 8 and 10 h AM into 2-mL vacutainers containing 0.1M K_3-

EDTA as anticoagulant, and after a 2 h storage at 4°C, the supernatant (platelet–rich plasma, PRP) was transferred to Eppendorf tubes. Platelet counts were determined from appropriately diluted PRP using a CellDyn900 type counter (Abbott Park, Illinois) immediately after sedimentation. Aliquots (50 µL) of PRP were treated by equal volume of 0.8M perchloric acid containing *N*-methylserotonin (NMS) as internal standard (5 ng/20 µL) and centrifuged by 10.000 × g for 10 min at 4°C in an Eppendorf centrifuge (A. Hettich, Tuttlingen, Germany). The gained supernatant was used for HPLC analysis. Samples were stored at –80°C before analysis, except for stability studies. Data are given in ng/10^9 platelet ± SD or pg/10^9 platelet ± SD, as indicated.

Chromatography

The supernatants were injected on the HPLC column (Supelcosil LC-18DB, 15 cm × 4.0 mm, 3 µm and SSI 25-148 post-column filter 0.2 µm). The instrument consisted of an ISCO 2350 pump, VALCO C6W injector and PAR 400 detector (Teledyne-ISCO Instruments, Lincoln, NE). A mixture of 48mM citric acid, 28mM sodium phosphate dibasic, 0.027mM Na_2EDTA and 3 v/v % methanol was used as the mobile phase at 1 mL/min; its pH was adjusted to 3.18 with 85% H_3PO_4.

Calibration curves were established using seven dilutions from a 10 µg/mL stock solutions prepared with 0.8M PCA in the range of 5–200 ng/mL, in triplicate. The linearity was evaluated by least-squares regression analysis.

Statistical analysis

Data were analyzed using SSPS version 6.1 (StatSoft Inc.) software by two-tailed *t*-test followed by Mann-Whitney U test. Probability levels of $p < 0.05$ were considered as statistically significant.

Results

As shown in Figure 2, the calibration curves for 5HTP, serotonin, *N*-methylserotonin (NMS), 5-HTOL, and 5-HIAA were linear in the range of 10–200 ng/mL, and the limits of quantitation (LOQ) were 2.3, 3.7, 3.8, 2.8, and 4.6 pg/mL, respectively. Regression analyses yielded the following equations:

5HTP: $y = 3.02x + 3.14$ ($R = 0.9996$)
Serotonin: $y = 2.5x + 2.69$ ($R = 0.9998$)
NMS: $y = 3.60x + 5.31$ ($R = 0.9992$)
5-HTOL: $y = 5.17x + 1.72$ ($R = 0.9998$)
5-HIAA: $y = 4.49x + 1.52$ ($R = 0.9997$)

Optimal potential measurements were carried out at E_{ox} 0.40–0.80 V, and the optimal potential was found as $E_{ox} = 0.65$ V. At lower potentials, the peak-to-noise ratios were un-satisfactory (less than 3:1), while at higher potentials 5HTOL and 5HIAA could not be adequately measured. It was found to be very important to use a standard column temperature: all measurements were performed at 25 ± 0.15°C. After comparing several analytical columns for the optimal separation of the five indols, the deactivated basic Supelcosil LC-18DB column was found the best, with 8 min retention time and narrower peak width ($w_h = 0.238$ min) for serotonin. This was accompanied by a significant increase in sensitivity and selectivity.

Based on literature data (23), *N*-methylserotonin was used as the internal standard. In case of the schizophrenic patients, some, but not all (8 of 36) samples contained a small, unidentified peak at the retention time of 9.6 min; however, it did not disturb the serial measurements (Figure 3A).

For intraday reproducibility determination the samples were injected nine times a day and the calculated relative standard

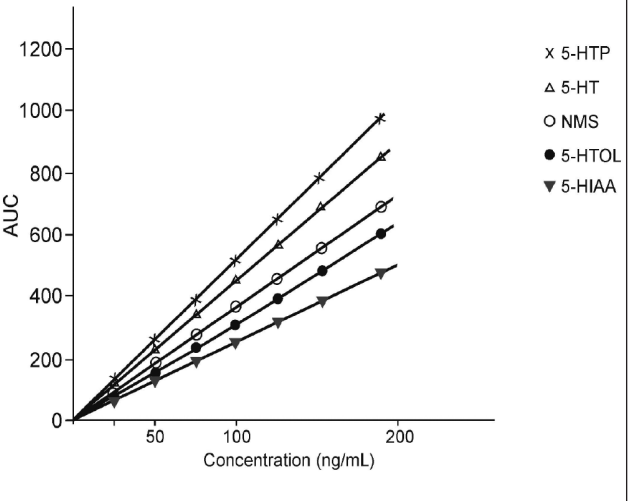

Figure 2. Calibration lines for the determination of indolamines involved ion serotonin synthesis and metabolism. 5-hydroxytryptophan = 5HTP; serotonin = 5HT; *N*-methylserotonin = NMS; 5-hydroxytryptophol = 5HTOL; 5-hydroxyindole-acetic acid = 5HIAA; AUC = area under the chromatographic peak/curve.

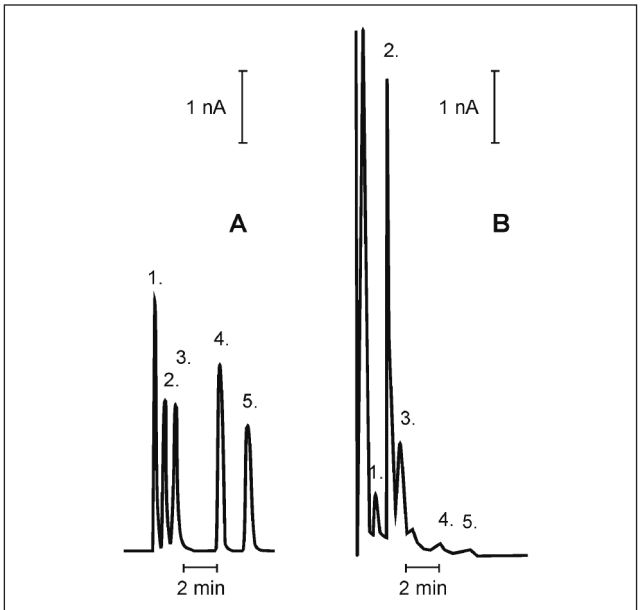

Figure 3. HPLC determination of serotonin (2); serotonin precursor [5HTP (1)]; and some metabolites [NMS (3); 5HTOL (4); 5HIAA (5)] from a standard mixture (A) and from platelet rich plasma of a control patient (B).

deviation (RSD) value was 0.89 (0.72%–2.25%). For interday reproducibility determination the samples were measured on seven consecutive days. It was found that the 5-HIAA and serotonin contents were the most sensitive to degradation. If the samples were stored even at –20°C, the first day was critical causing approximately 70% loss both in the serotonin and 5HIAA contents of the samples; therefore, for serial determinations the samples were stored at –80°C and were thawed just before injection on the column. Accuracy, precision, and the limit of detection were also calculated according to the requirements. A representative chromatogram of a control subject is shown in Figure 3B, and the calculated data are summarized in Table I.

5HTP content of PRP was found to be similar in both groups of subjects. The recent alcohol consumption of schizophrenics was also without effect on the plasma 5HTP level. However a significant elevation in the serotonin content of PRP was observed in both groups of schizophrenic patients, independently from their alcohol consumption. When the concentrations of serotonin metabolites were calculated, we have found that schizophrenics with recent alcohol consumption showed very high plasma 5HTOL levels. In schizophrenics, the 5HIAA content was slightly elevated as compared to controls, but it did not reach a significant level. Calculating the 5HTOL/5HIAA ratios it was found that for schizophrenics with recent alcohol consumption the ratio was one order of magnitude higher than in controls or in alcohol-free schizophrenics.

Discussion

Several techniques including HPLC have been reported to determine the concentrations of serotonin, its precursor 5HTP and the two metabolites 5HTOL and 5-HIAA in different biological samples. In clinical practice, HPLC–EC detection seems to be preferred as a robust, versatile separation technique. Using the presented simple and sensitive method, the five serotonin-related indols can be determined with a high precision and sensitivity in one run from human platelet-rich plasma in both control and schizophrenic patients. The method can also be used for other biological samples.

The main findings of the present study are that in platelet-rich plasma (PRP) of schizophrenics significantly higher serotonin concentration was observed than in age-matched controls, and there was a shift to 5HTOL formation in schizophrenics with recent alcohol consumption. There may be several explanations for the significantly elevated PRP serotonin level in schizophrenics. The serotonin content of the platelet may be regulated by synthesis (mainly peripheral in the enterochromaffin cells), uptake, storage, metabolism, and release. As patients were treated with different types of neuroleptic drugs, one may hypothesise that high PRP serotonin content is a consequence of drug treatment. However it was shown (24) that neuroleptics in vivo had no effect on the 5HT content of the platelet and on 5HT uptake by platelets. As we have found, no difference between the controls and schizophrenics in the 5HTP (precursor of serotonin) content of their PRP, it can be assumed that decreased degradation by monoamine oxidase (25) and/or its release may contribute to the experienced high level.

It is known that aldehyde dehydrogenase catalyses the irreversible oxidation of 5-hydroxyindole-acetaldehyde into 5-HIAA. As no significant change was found in the 5HIAA level in the PRP of schizophrenics (in spite of the high 5HT levels) we assume that this may reflect elevated urinary excretion of this metabolite. Human studies have demonstrated that urinary output of 5HIAA is markedly increased after ingestion of food with high 5HT content (26). The other metabolite of serotonin, 5HTOL, is formed from the same intermediate as 5HIAA in a reaction catalyzed by alcohol dehydrogenase. It is known that there is a competition among the two endogenous aldehydes, alcohol-derived acetaldehyde and 5-hydroxyindole-

Table I. Indoleamines in the human platelet-rich plasma*

Subjects	Human platelet-rich plasma indoleamines				
	5HTP pg/10^9 platelet ± SD	5HT ng/10^9 platelet ± SD	5HTOL pg/10^9 platelet ± SD	5HIAA pg/10^9 platelet ± SD	5HTOL/5HIAA ± SD
Control patients (*N* = 29)	133.2 ± 67.1	916.2 ± 78.3	57.3 ± 16.8	8.69 ± 1.31	0.006 ± 0.0008
Schizophrenic patients (*N* = 36)	145.3 ± 88.2	1798.3 ± 863.1†	342.6 ± 110.2	10.7 ± 4.1	0.03 ± 0.008†
Schizophrenic patients with recent alcohol consumption (*N* = 21)	161.7 ± 76.8	1688.3 ± 582.2†	583.2 ± 106.2	9.8 ± 3.0	0.059 ± 0.0009†
Schizophrenic patients, alcohol-free (*N* = 15)	153.8 ± 71.2	1908.8 ± 616.8†	65.6 ± 12.9	12.9 ± 2.6	0.005 ± 0.0007

* Abbreviations are as follows: serotonin (5HT); 5-hydroxytryptophan (5HTP); 5-hydroxytryptophol (5HTOL); 5-hydroxyindole-acetic acid (5HIAA).
† where $p < 0.05$.

acetaldehyde, for this enzyme. Our observation that recent alcohol consumption by schizophrenics results in significant increase in the 5HTOL–5HIAA ratio is in line with the finding of others (27) measuring urinary excretion of serotonin metabolites. The shift to 5HTOL formation from serotonin in schizophrenics measured in PRP seems to be less pronounced than found in urine (15).

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