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ON THE ACCURATE DETERMINATION OF SEROTONIN IN HUMAN PLASMA

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Serotonin (5-hydroxytryptamine, 5-HT) in blood is stored in platelets and has vascular and platelet stimulating effects when released into plasma. Accurate measurements of 5-HT in plasma are complicated by inadvertent platelet activation causing sampling artifacts and by analytical problems when determining trace levels. We developed an assay for plasma 5-HT based on solid-phase extraction (Sep-Pak C_{18}), aqueous acetylation, pentafluoropropionylation, and negative ion chemical ionization gas chromatography-mass spectrometry. The method was able to recover 5-HT from plasma by >90% and to quantitate with a precision of 7.5% at a level of 0.5 nmol/l. It was used to define blood sampling and sample handling procedures giving low and consistent values for 5-HT. A good blood sampling technique, adequate platelet stabilization in the test tube, and rapid high speed centrifugation of the blood resulted in low plasma levels of both 5-HT and ß-thromboglobulin (a platelet release product). Using these procedures plasma 5-HT levels in healthy volunteers were found to be 0.77±0.38 (mean±S.D.; range 0.27-1.49) nmol/l (n=18), which is 4-100-fold lower than previously reported values.

Serotonin (5-hydroxytryptamine; 5-HT) is stored in blood platelets and has vascular and platelet stimulating effects of considerable interest (1). In healthy humans, 5-HT acts mainly as an arterial vasodilator, and a vasoconstrictor on the venous side (2). 5-HT appears to be important in cardiovascular pathology, as it causes endothelium dependent vasodilatation in normal human coronary arteries, but vasoconstriction in atherosclerotic coronary arteries (3). Animal studies have shown an important role for 5-HT in the thrombogenesis caused by vascular injury (1,4).

Several investigators have measured 5-HT in plasma, but results have been discouragingly variable (5-17). Generally, precautions to minimize artifactual release of 5-HT from platelets during sampling and sample handling have not been taken. Most studies report mean plasma 5-HT levels above 10 nmol/l (≥ 1.8 ng/ml). We have noted problems with sampling artifacts when using plasma levels of the platelet specific protein ß-thromboglobulin (ßTG) as a marker of platelet activation *in vivo* (18,19). Since platelets contain large amounts of 5-HT artifactual release of 5-HT from platelets during sampling and sample handling might explain the marked discrepancies in reported plasma 5-HT levels. Accurate measurements of plasma 5-HT may be difficult also because of analytical problems, if the levels are low. The present investigation was thus undertaken to define better procedures for sampling, sample handling and analysis of plasma 5-HT.

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MATERIALS AND METHODS

Chemicals. 5-HT creatinine sulphate monohydrate and EDTA were from E. Merck AG (Darmstadt, Germany); pentafluoropropionic anhydride (PFPA) from Supelco Inc. (Bellefonte, PA, USA); theophylline from Fluka Chemie AG (Buchs, Switzerland); prostaglandin E_1 (PGE_1) from Sigma Chemical Co. (St. Louis, MO, USA); Iloprost from Schering AG (Berlin, Germany). Other chemicals were analytical grade and from various sources. The internal standard, tetra-deuterated 5-HT picrate (5-HT-$^2H_4$), was prepared as described previously (20).

Volunteers. Healthy male and female volunteers, aged 28-50 years, who had not taken any platelet active medication (including aspirin) during the preceding two weeks, gave informed consent to participate in the studies, which were approved by the local Ethics Committee.

Collection and handling of blood samples. All volunteers had easily accessible forearm veins, to ensure good flow upon venepuncture. Blood was sampled in the reclining position by puncture of an antecubital vein, after local anesthesia with lidocaine creme (EMLA, Astra AB, Södertälje, Sweden). Different veins were punctured when comparing sampling techniques. Unless otherwise stated, we used a platelet stabilizing/anticoagulating solution yielding (final conc.) 9.0 mmol/l EDTA, 1.0 mmol/l theophylline and 1.4 μmol/l prostaglandin E_1 (PGE_1) (18,19).

Three sampling techniques were compared: venepuncture by 18G Wasserman needles (Sterijekt, TSK Laboratory., Japan) or by the Vacutainer technique (21G needles; Becton & Dickinson, Meylan Cedex, France), or sampling via an indwelling venous catheter (20G Venflon, Viggo AB, Helsingborg, Sweden). When using Wasserman needles the first two ml of blood were discarded and eight ml were then allowed to drip freely into ice-cooled plastic test tubes containing 800 μl of the platelet stabilizing solution. Sampling through venous catheters followed the same procedure, but plastic syringes (Terumo, Leuven, Belgium) were used. Vacutainer tubes were prepared with 1/10th volume of the stabilizing solution immediately before use. After blood collection the test tubes were gently inverted and placed on ice. Blood from the glass Vacutainer tubes were decanted into plastic tubes after mixing. The blood samples were centrifuged at 15 000 x g for 30 min (+4°C), within five min if not specified. Plasma from the mid-portion was then carefully removed, aliquoted into separate tubes for βTG and 5-HT measurements and stored at -80°C until analyzed.

The importance of rapid centrifugation was studied by preparing parallel samples of plasma from blood taken by the Wasserman procedure; one aliquot of blood was centrifuged rapidly (within 5 min) and the other after 45 min on ice. In a separate experiment (Wasserman sampling procedure) PGE_1 and the stable prostacyclin derivative Iloprost (final conc. 0.2 μg/ml) were compared as additives in the platelet stabilizing solution. For comparison, we also sampled blood into Vacutainer tubes containing only the conventional concentration of EDTA (1 μmol/l).

Preparation of plasma for 5-HT measurements. Plasma (1.0 ml) was pipetted into a plastic test tube containing internal standard (11.1 pmoles 5-HT-$^2H_4$ in water). After addition of 1 ml sodium borate buffer (0.1 mol/l, pH 10) the mixture was applied (flow rate ≈2 ml/min) onto a solid-phase extraction cartridge (C_{18} Sep-Pak, Waters Assoc., Milford, MA, USA). The cartridge had been activated with 3 ml of methanol and 3 ml of water prior to use. Following sample application, the cartridge was washed with 5 ml of 10 mmol/l borate buffer (pH 10), and dried by sucking air through it during 1-2 min. Finally, the analyte was eluted with 2 ml of methanol and collected in a glass test tube. The eluate was evaporated under nitrogen at 35°C until 0.1-0.2 ml remained.

After the addition of 1.0 ml of ice-cold water and 0.3-0.4 g $NaHCO_3$, 0.1 ml of acetic anhydride was added. The sample was repeatedly mixed and kept on ice for 20-30 min and then allowed to attain room temperature (20-22°C) with occasional mixing. When the reaction had ceased (no more CO_2 bubbles emerging) the sample was extracted with 3.0 ml of diethyl ether. The organic layer was transferred to a new glass test tube and evaporated to dryness under nitrogen. The residue was treated with 70 μl of PFPA for 30 min at 60°C. Excess reagent was evaporated under nitrogen and the residue was dissolved in 15 μl ethyl acetate.

GC-MS analysis. The prepared extracts were analysed on a gas chromatographic mass spectrometric (GC-MS) system consisting of an HP 5890 II gas chromatograph (Hewlett-Packard Co., Palo Alto, CA, USA) connected via a heated transfer line to a Finnigan MAT Incos 50 quadropole mass spectrometer (Finnigan MAT Co., San Jose, CA, USA).

An aliquot (1-2 μl) of each sample was introduced via a "moving needle" injector (250°C) onto a 30 m DB-5 fused silica capillary column (i.d. 0.25 mm, film thickness 0.25 μm, J & W Scientific, Folsom, CA, USA). Helium was used as carrier gas. The column temperature was kept at 200°C and increased to 275°C at a rate of 15°C/min immediately after injection. The capillary column was inserted directly into the ion source via a heated transfer line (275°C).

The mass spectrometer was operated in the negative ion chemical ionization (NCI) mode with methane as reagent gas. Mass numbers m/z 348 (5-HT) and 351 (internal standard) were recorded

in the selected ion monitoring (SIM) mode. Mass spectrometric conditions were: ion source temperature 120°C; electron energy 110 eV.

Quantitation. Calibration curves were prepared by analysing aqueous solutions of 5-HT creatinine sulphate reference substance (0-8.5 nmol/l) with the same procedure as for plasma samples. Quantitation was achieved by comparing peak height ratios m/z 348/351 of unknowns with reference to the calibration curve. The peak height ratio (m/z 348/351) was linearly related to the amount of 5-HT in a sample, with an intercept close to the origin.

Plasma ßTG measurements. Commercially available radioimmunoassay kits (IM-88, Amersham International, Amersham, Bucks, UK) were used with modifications described previously (21).

Statistics. Non-parametric statistical evaluation was performed by Wilcoxon's test for paired observations. Mean (S.D.) or median (range) values are given.

RESULTS

The present analytical procedure was able to reliably determine 5-HT in plasma from different individuals. The GC-MS analysis of extracts demonstrated good chromatographic qualities of the derivative, and lack of interferences in the plasma extract (Fig. 1). The NCI mass spectra showed base peaks at m/z 348 for 5-HT and 351 for 5-HT-$^2H_4$ (Fig.2). These ions were formed by loss of HF and ^{2}HF, respectively, and were used in the selected ion monitoring.

The limit of detection (signal to noise ratio of 3:1) was ≈ 0.03 nmol/l. The recovery of 5-HT over the solid-phase extraction, aqueous acetylation and dietyl ether extraction steps was >90%. The coefficient of variation (CV) in the quantitation of 5-HT was 7.5% within-day (at 0.50±0.04 nmol/l, n=6), and 10% between-day (at 0.72±0.07 nmol/l, n=6). The mean recovery of varying amounts of 5-HT (1.69, 3.38, 6.75 nmol/l) added to a plasma pool was 90 (86-95) %.

The analytical method was used to establish blood sampling and sample handling procedures. In one experiment the necessity of rapid centrifugation of blood was demonstrated. A delay of about 40 min at +4°C resulted in a 332% increase in the plasma 5-HT, from 0.88 (0.33-1.49) to 2.92 (1.26-8.53) nmol/l (n=7, p=0.018), and a 41% increase in plasma ßTG, from 23.9 (18.9-67.0) to 33.7 (19.0-144) ng/ml (n=7, p=0.063). Samples with the greatest increases in ßTG also had the greatest increases in 5-HT, but the latter variable was more sensitive in this respect (Fig.3).

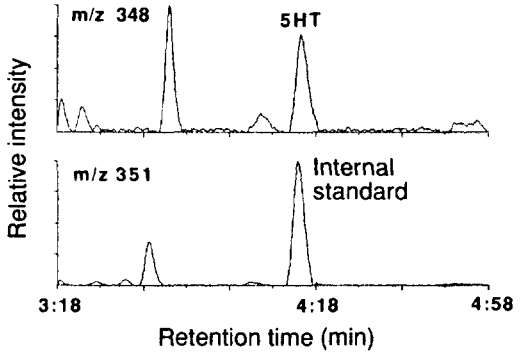

Figure 1. A representative ion trace chromatogram obtained from the analysis of 5-HT in a human plasma sample by gas chromatography-mass spectrometry.

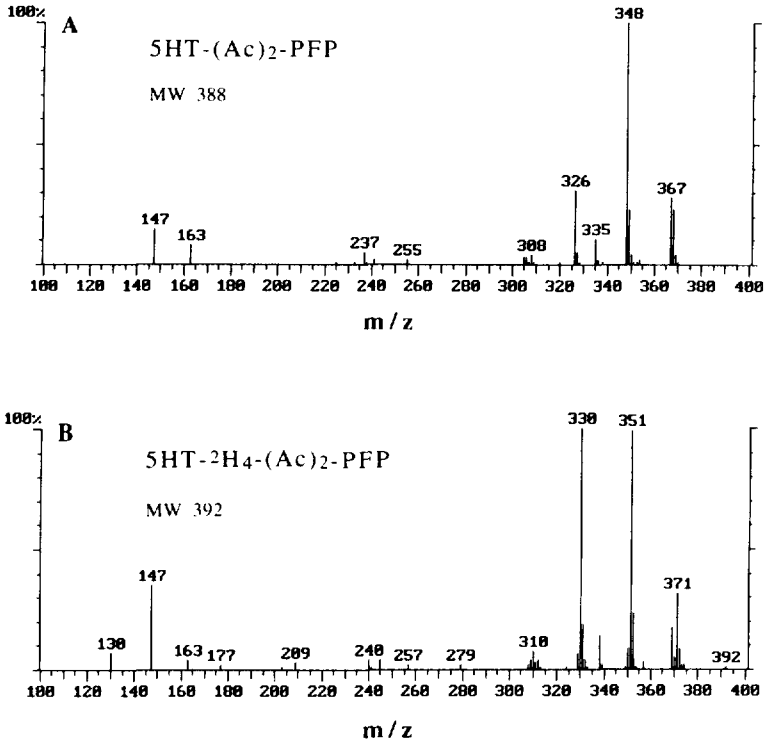

Figure 2. Negative ion chemical ionization (methane) mass spectra of the diacetyl-pentafluoro-propionyl derivatives of 5-HT and 5-HT-$^2H_4$. The molecular ions are seen on m/z 388 and 392, respectively.

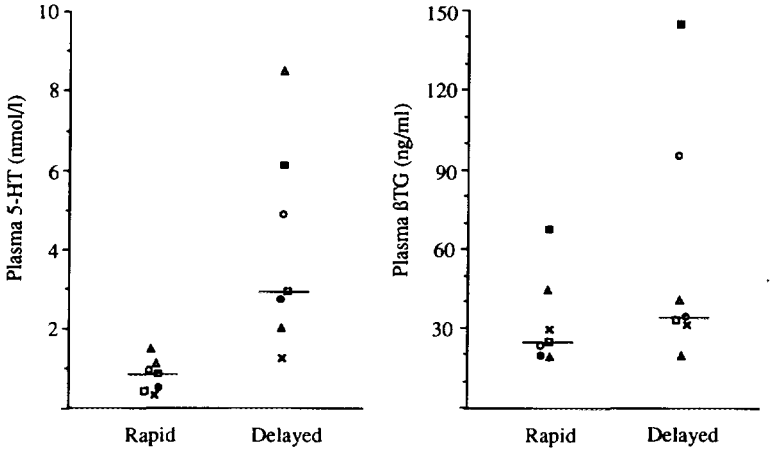

Figure 3. Influence of rapid (≤5 min) vs. delayed (45 min) centrifugation of blood after sampling on 5-HT and βTG levels in plasma. The blood was sampled with the standard procedure involving 18G Wasserman needles and platelet stabilization with EDTA, theophylline and PGE_1.

Different aspects of the blood collection procedure were evaluated. Sampling with wide-bore Wasserman needles, which in our hands has given low plasma βTG levels and few sampling artifacts (18,19), yielded lower and less variable values for 5-HT and βTG than did sampling via an indwelling teflon catheter. Median 5-HT levels were 0.85 (0.52-1.26) vs. 1.40 (0.85-3.01) nmol/l (p=0.075) and βTG levels 20.0 (19.2-43.0) vs. 47.2 (36.5-94.2) ng/ml (p=0.028). Prolongation of sampling (Wasserman needles) resulted in high values in some samples; when >25 ml of blood was collected 5-HT levels increased to 1.26 (0.59-5.94) nmol/l and βTG levels to 26.6 (17.6-50.8) ng/ml. The Wasserman procedure was also compared to Vacutainer sampling with EDTA, theophylline and PGE_1. The subjects were not subjected to standardized rest in this experiment. Compared to the Wasserman technique, Vacutainer sampling tended to result in even lower levels of 5-HT in plasma (0.66±0.08 vs. 1.27±0.23 nmol/l; p=0.088), whereas βTG levels differed less (15.6±1.0 vs. 17.1±1.1 ng/ml; p=0.625). Rapid flow during sampling is thus important.

Iloprost and PGE_1 were compared as platelet stabilizing additives (with EDTA and theophylline) in eight subjects. These samples were taken without preceding rest and were centrifuged after ≈20 min, i.e. under suboptimal conditions for low levels of 5-HT and βTG in plasma. 5-HT levels were 1.54±0.79 nM with PGE_1 and 1.69±0.81 nM with Iloprost (p=0.438), and βTG levels were 24.0±6.7 and 26.3±6.1 ng/ml, respectively (p=0.036). Sampling into standard Vacutainer EDTA tubes gave higher values than the Wasserman technique described above; 2.43±0.95 vs. 0.90±0.28 nmol/l (p=0.028) for 5-HT and 40.6±8.2 vs. 23.8±9.4 ng/ml (p=0.028) for βTG.

Using the Wasserman sampling procedure, platelet stabilization (PGE_1, EDTA and theophylline) and high speed centrifugation (within 5 min), the mean value for 5-HT in plasma was estimated to be 0.77±0.38 (S.D.; range 0.27-1.49) nmol/l (data from 18 resting healthy volunteers).

DISCUSSION

A prerequisite for accurate measurements of plasma 5-HT is a sensitive and specific analytical technique. Previous reports have been based on liquid chromatographic methods. The GC-MS procedure described here was based on work by Markey et al (22), showing that the spirocyclic derivative of 5-HT formed by aqueous acetylation and subsequent pentafluoropropionylation gives excellent sensitivity and selectivity when using negative ion chemical ionization GC-MS. Aqueous acylation has advantages when analyzing biogenic amines (23,24), and appeared attractive also for 5-HT because of its polar character which complicates its isolation from aqueous media by solvent extraction. Problems encountered when applying it to plasma samples were losses of 5-HT during protein precipitation with perchloric acid and interfering peaks in the mass spectrometric analysis. These problems were solved by including the initial isolation step by solid-phase extraction. Some difficulty was experienced with the chromatographic performance of the 5-HT derivative, in disagreement with a report showing excellent GC properties on packed columns (22). Broad and unsymmetrical peaks were obtained on certain brands of fused silica capillary columns. This was, however, not experienced with any DB-5 column.

The present study shows that appropriate sampling and sample handling are important in order to reduce the risk of obtaining artifactual release of 5-HT in the sample. This has usually been

overlooked in previous reports on plasma 5-HT. Precautions to minimize sampling artifacts and the analytical method based on GC-MS, resulted in basal mean levels of 5-HT in plasma of about 0.8 nmol/l (140 pg/ml). This is way below previously reported levels (5-17). However, Anderson and coworkers (8) reported values only 4-fold higher than ours.

Adequate platelet stabilization in the test tube is obviously important. The combination of EDTA, theophylline and PGE_1 has been found by us to result in low plasma ßTG values and few samples with signs of artifactual release of ßTG (18,19) and was found to give low levels of 5-HT as well. The more stable prostaglandin analogue Iloprost yielded almost the same results, whereas anti-coagulation by EDTA only resulted in higher values. Rapid high speed centrifugation (to eliminate all platelets from plasma) is another precaution which is recommended.

The blood sampling technique is also important for good results. Rapid blood collection using wide-bore Wasserman needles or the Vacutainer technique, which is more convenient, gave good results. Sampling through an indwelling catheter or allowing the blood to flow through the needle during a longer period of time resulted in elevated 5-HT levels in plasma. Our experience indicates that subjects with easily punctured veins should be selected for studies of this nature, as rapid flow through the sampling cannula reduces artifactual platelet activation.

Interestingly, 5-HT was even more sensitive to sampling artifacts than ßTG. This may be related to the storage of the two substances in different platelet granules; 5-HT is stored in dense granules and ßTG in α-granules (25). There is little evidence for differential control of secretion from these granules, but the former may be more easily activated (26). Our data support that contention and show that 5-HT is a sensitive marker for artifactual platelet activation.

In conclusion, we demonstrate low (subnanomolar) levels of 5-HT in human plasma when assayed by a sensitive and specific analytical method and when artifactual release of 5-HT from the platelets during sampling and sample handling is kept low. The latter aspect has previously not received appropriate attention, but appears to be most important. Rapid blood flow during sampling (wide bore cannulae or Vacutainer sampling), effective platelet stabilization in the test tube (EDTA, theophylline and PGE_1 or Iloprost) and rapid high speed centrifugation to remove all platelets from plasma resulted in low plasma 5-HT levels (≈ 0.8 nmol/l or 140 pg/ml). It cannot be excluded that methodological developments may reduce these levels even further.

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