Radioimmunoassay of Serotonin (5-Hydroxytryptamine) in Cerebrospinal Fluid, Plasma, and Serum

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We describe a direct radioimmunoassay for serotonin (5-hydroxytryptamine) in cerebrospinal fluid, platelet-poor plasma, and serum. We raised antisera in rabbits against serotonin diazotized to a conjugate of bovine albumin and D,L-p-aminophenylalanine. Polyethylene glycol, alone or in combination with anti-rabbit immunoglobulins, is used to separate bound and unbound tritiated serotonin. The minimum concentration of serotonin detectable is 2 nmol/L in a 200-μL sample. Within-day precision (CV) is 4.3%, between-day precision 7.7%. Analytical recoveries of serotonin are 109% and 101% for cerebrospinal fluid and plasma, respectively. Tryptophan, 5-hydroxytryptophan, 5-hydroxyindoleacetic acid, and 5-hydroxytryptophol do not interfere with the assay. However, 5-methoxytryptamine and tryptamine cross-react. Of samples of cerebrospinal fluid from patients with disc herniations (n = 21) or low-pressure hydrocephalus (n = 10), one-third had concentrations of 2–4 nmol/L and two-thirds were below the minimum detectable concentration. The observed range for the concentration of serotonin in plasma of 14 normal subjects was 5–14 nmol/L (mean ± SD, 9 ± 3 nmol/L). The observed ranges for serotonin in serum were: for 10 women 520–900 (mean ± SD: 695 ± 110) nmol/L and for 10 men 380–680 (520 ± 94) nmol/L.

Additional Keyphrases: reference interval · sex-related effect · use of inhibitors to assure stability of analyte · sample handling

Serotonin (5-HT)3 is located primarily in the enterochromaffin cells of the intestine, serotonergic neurons of the brain, and platelets of the blood. Because it is also a vasoconstrictor and a neurotransmitter, it has been implicated as a causative agent in cerebrovascular and affective disorders (1, 2).

Classical methods for its determination are based on fluorescence measurements (3, 4). Recently, more specific and sensitive methods—radioimmunossay (5), liquid chromatography with electrochemical detection (6), and radioenzymatic assay (7, 8)—have been described.

We have raised antisera to 5-HT (5) and developed a direct radioimmunossay for it, which is very sensitive and specific. Inhibitors of monoamine oxidase (EC 1.4.3.4) and of platelet uptake of 5-HT are added to all specimens just after sampling, to ensure the conservation of 5-HT. We have measured the concentrations of 5-HT in cerebrospinal fluid (CSF), platelet-poor plasma, and serum with this method.

Materials and Methods

Reagents

5-[1,2-3H(N)]-Hydroxytryptamine (creatinine sulfate complex; 25–32 kCi/mol) was provided by New England Nuclear, Boston, MA 02118. Bovine albumin (RIA grade), bovine gamma-globulin (Cohn Fraction II), activated charcoal, paraglyne, theophylline, 5-HT (creatinine sulfate complex), and the 5-HT analogs were from Sigma Chemical Co., St. Louis, MO 63178. Polyethylene glycol 6000 (PEG) was from Merck, Darmstadt, F.R.G. Rabbit gamma-globulin was from Calbiochem-Behring Corp., La Jolla, CA 92037. Swine immunoglobulins to rabbit immunoglobulins were from DAKO Immunoglobulins a/s, Copenhagen, Denmark. Chlorimipramine (Afanfranil®) was a gift from Ciba-Geigy, Copenhagen, Denmark; and clorgyline was a gift from Pharma-Rhodia, Birkerød, Denmark (a representative of May & Baker Ltd.). Analytical grade chemicals and glass-distilled water were used throughout.

Anticoagulant: per liter, 618 mmol of dipotassium, ethylenediaminetetraacetate (EDTA), 470 mmol of KOH, and 20 mmol of theophylline. Inhibitor solution: per liter, 250 μmol of chlorimipramine, 250 μmol of clorgyline, 250 μmol of paraglyne, and 150 mmol of NaCl. Phosphate buffer: per liter, 38.5 mmol of Na2HPO4, 61.5 mmol of NaHPO4·2H2O, and 1 mmol of Na2H2EDTA·2H2O, pH 6.9.

Borate buffer: per liter, 200 mmol of Na2BO3, 54 mmol of Na2B4O7·10H2O, and 1 mmol of Na2H2EDTA·2H2O, pH 8.4.

IgG solution A: 20 g of bovine gamma-globulin and 125 μmol of D,L-3,4-dihydroxyphenylalanine in 1 L of borate buffer. Store in 5-mL aliquots at −20 °C until use.

IgG solution B: 200 mg of rabbit gamma-globulin and 125 μmol of D,L-3,4-dihydroxyphenylalanine in 1 L of borate buffer. Store in 5-mL aliquots at −20 °C until use.

PEG solution A: 222 g of PEG in 1 L of borate buffer; store at 4 °C until use.

PEG solution B: 90 g of PEG in 1 L of borate buffer; store at 4 °C until use.

Isotope solution: 250 nCi of tritiated 5-HT per milliliter of IgG solution A or B. Prepare freshly each day.

Anti-rabbit IgG solution: Mix 7.5 mL of borate buffer and 2.5 mL of swine immunoglobulins to rabbit immunoglobulins. Prepare freshly each day.

Binder solution A: 7.5 μL of antiserum to 5-HT per milliliter of borate buffer.

Binder solution B: 7.5 μL of antiserum to 5-HT per milliliter of anti-rabbit IgG solution. Prepare freshly each day.

Preparation of Immunogen; Immunization

The immunogen used consisted of 5-HT diazotized to a conjugate of bovine albumin and D,L-p-aminophenylalanine as described (5). Dialyze the final reaction mixture against...
phosphate buffer and remove insoluble reaction products by centrifugation. The immunogen concentration was about 150 μmol of protein-bound 5-HT per liter, as estimated by the absorbance at 480 nm. Store the immunogen in 1-mL aliquots at −20 °C in the dark. It is stable for one year.

Immunize rabbits with a mixture of equal volumes of immunogen and Freund’s incomplete adjuvant (9). Dialyze the antisera against phosphate buffer containing activated charcoal, 5 g/L, to eliminate interference by endogenous 5-HT.

Sample Collection and Preparation

CSF. Collect 2.5 mL of CSF into a polystyrene tube containing 100 μL of inhibitor solution kept at 0 °C. Invert the tube to mix. Centrifuge at 1500 × g for 30 min at 4 °C. Transfer the upper two-thirds of the fluid to a fresh tube. Store at −20 °C until the day of the assay. After thawing, analyze by method A (see Procedure).

Plasma. Collect 2.5 mL of blood into a polystyrene tube containing 100 μL of anticoagulant solution kept at 0 °C. Without delay, pour the stabilized blood into another polystyrene tube containing 100 μL of inhibitor solution kept at 0 °C. Centrifuge at 1500 × g for 30 min at 4 °C. Transfer the upper two-thirds of the platelet-poor plasma to a fresh polystyrene tube (10). Store at −20 °C until the day of the assay. After thawing, dilute fourfold in borate buffer and analyze by method B (see Procedure).

Serum. Collect 2.5 mL of blood into a glass tube containing 100 μL of inhibitor solution, and mix by inversion. After 1 h at room temperature, centrifuge as above. Transfer the upper two-thirds of the serum to a fresh polystyrene tube and store at −20 °C until the day of the assay. After thawing, dilute 20-fold in borate buffer and analyze by method A, or dilute 15-fold in borate buffer and analyze by method B.

Radioimmunoassay Procedure

To a polystyrene tube add 200 μL of sample or diluted sample, 100 μL of isotope solution, and 200 μL of binder solution. Incubate at 37 °C for 15 min. Transfer the tube to a 4 °C bath. After 5 min add 1 mL of PEG solution and mix. Incubate for a further 15 min at 4 °C and centrifuge (1500 × g, 10 min, 4 °C). Invert the tube and wipe the inside of it with a swab. Dissolve the pellet in 1 mL of water. Transfer an 800-μL aliquot of the redissolved pellet to a counting vial, add 10 mL of a water-miscible liquid scintillation cocktail, and count the radioactivity. In each series include standards of 5-HT in borate buffer and controls, to determine the total amount of radioactivity and the nonspecific binding.

Method A: Use IgG solution A for the isotope solution, and use binder solution A and PEG solution A.

Method B: Use IgG solution B for the isotope solution, and use binder solution B and PEG solution B.

Results

Immunization

Eight rabbits were immunized. During the process of immunization we estimated the binding capacities and the equilibrium constants for the antisera (11). Concentrations of free and free plus bound radioactive ligand were measured by equilibrium dialysis. Useable antisera were obtained from four rabbits after five months of immunization. The antisera with the greatest equilibrium constant was chosen for the experiments described in this paper.

Analytical Variables

pH optimum. We examined the binding of tritiated 5-HT to the antisera in various buffers over a pH range of 6.4 to 9.7. Binding was optimum in the borate buffer at pH 8.4.

Concentrations of tritiated 5-HT and antisem. We included D,L-3,4-dihydroxyphenylalanine in the IgG solutions in order to decrease the nonspecific binding in the absence of CSF, plasma, or serum to the amount found in the presence of these components. At most, only 10 μL of serum or plasma can be used in method A without increasing the nonspecific binding. Therefore, we developed method B, the second-antibody–PEG method. By using method B, 50 μL of serum or plasma can be used without increasing the nonspecific binding and without impairing the recovery of added 5-HT.

For method A, the concentrations of tritiated 5-HT and antisem were adjusted such that the amount of tritiated 5-HT bound at zero dose was 28% (SD 2%) of the total amount of tritiated 5-HT (n = 27), and B/B0 was about 0.5 in the presence of 200 μL of 43 nmol/L 5-HT. The nonspecific binding was 4% of the total amount of radioactivity.

For method B, we used the same concentrations of tritiated 5-HT and antisem. In this case, the amount of tritiated 5-HT bound at zero dose was 39% (SD 1%) of the total amount of tritiated 5-HT (n = 7), and the nonspecific binding was 0.9% of the total amount of radioactivity.

Standard curve. A typical standard curve obtained with method A is shown in Figure 1. The standard curve is linear, when plotted as log B/B0 vs log dose, over the range 5–200 nmol/L. The standard curve for method B is very similar (not shown).

Minimum detectable concentration. The within-day CV of the zero-dose standard is 1.8%. Therefore, the minimum detectable concentration is the concentration at which B/B0 is equal to 0.964 (1 – 2 CV), or 2 nmol/L.

Specificity. Tryptophan (1–50 μmol/L), 5-hydroxytryptophan (2–10 μmol/L), 5-hydroxyindoleacetic acid (0.1–1 μmol/L), and 5-hydroxytryptophol (10–50 nmol/L) did not significantly displace tritiated 5-HT from the antisem. However, tryptamine and 5-methoxytryptamine did cross react (Figure 2).

Precision. Serum diluted 40-fold in borate buffer was used to estimate within-day precision. Some diluted serum was stored in aliquots at −20 °C and used to estimate the between-day precision. The within-day CV was 4.3% (n = 12) at 21 nmol/L. The between-day CV was 7.7% (n = 14) at 20 nmol/L.

Recovery. A pool of CSF was enriched with 5-HT at concentrations ranging from 5 to 25 nmol/L. Analytical recovery was 109% (SD 3%).

Fig. 1. A typical standard curve for the 5-HT radioimmunoassay, calculated from the experimental data by log-logit transformation and linear-regression analysis. The abscissa represents the concentrations of 5-HT in the standards.
An aliquot of plasma was enriched with 5-HT at concentrations ranging from 10 to 200 nmol/L. Analytical recovery was 101% (SD 3%).

Conditions for preparation of serum. We studied the effects of inhibitors of platelet uptake (12) and inhibitors of monoamine oxidase A and B (13) on the concentration of 5-HT in serum by adding the inhibitors chlorimipramine, clorgyline, and pargyline, separately or together, to blood in vitro before coagulation. Table 1 shows the results of two experiments with blood from a healthy volunteer. Furthermore, in vitro addition of the three inhibitors to blood from five healthy volunteers before coagulation increased the yield of 5-HT by 8% (paired Student’s t-test, t = 2.14, p < 0.10). Evidently, the presence of all three inhibitors is needed for maximum recovery of 5-HT in serum.

We also examined the effects of inducers of the platelet-release reaction (14) on the concentration of 5-HT measured in serum (Table 2). Addition of ADP, epinephrine, or thrombin did not increase the yield of 5-HT in serum at either 25 or 37 °C.

Furthermore, addition of reserpine (15) did not increase the yield of 5-HT in serum incubated at 37 °C.

Reference Intervals

CSF. Lumbar CSF were sampled from 21 patients with disc herniations. These patients were being subjected to myelographic examination, and the samples were collected during this procedure.

Ventricular CSF was sampled from 10 patients with low-pressure hydrocephalus. These patients were being subjected to measurement of intraventricular pressure, and the samples were collected during this procedure.

In two-thirds of these samples (Table 3) the concentrations of 5-HT were below the minimal detectable concentration, 2 nmol/L. The upper limit of the observed range was 4 nmol/L.

Plasma. Blood samples were drawn from 14 blood donors, seven men and seven women, and the concentrations of 5-HT, beta-thromboglobulin, and platelet factor 4 were determined in the plasma. Concentrations of beta-thromboglobulin and platelet factor 4 were within the normal range. The results of the 5-HT determinations are shown in Table 4. The concentration of 5-HT is only 2% of that in serum.

Serum. Blood was sampled from 20 blood donors after donation of 500 mL of blood. The 5-HT concentrations were measured in the sera (Table 4). The concentration of 5-HT in serum from women is about 1.3-fold that from men. This difference is statistically significant (unpaired Student’s t-test, t = 3.83, df = 18, p < 0.0025).

Intra-individual variation. Blood was collected once a week for eight weeks from a healthy volunteer, and the concentration of 5-HT in serum measured in quintuplicate. It averaged 870 nmol/L, with a CV of 5%.

Discussion

We determine 5-HT by radioimmunoassay, because this method is more sensitive and specific than are previously applied methods. The minimal detectable concentration is 2 nmol/L in a 200-μL sample. Tryptophan (16, 17), 5-hydroxytryptophan (18, 19, 20, and I. Magnussen and M. H. Van Woert, personal communication), 5-hydroxyindoleacetic acid (19, 20), and 5-hydroxytryptophol (21) at concentrations found in vivo do not interfere with the assay. However, 5-methoxytryptamine (22, 23), and tryptamine (24, 25) do cross react with 5-HT, and these compounds may therefore contribute to the measured concentrations of 5-HT in CSF and plasma.

Two monoamine oxidase inhibitors (13) and an inhibitor of the platelet uptake of 5-HT (12) are added to all specimens just after sampling, to ensure the conservation of 5-HT. In fact, addition of these inhibitors increases the yield of 5-HT in serum, and optimum yield is only achieved when all three are present. Furthermore, CSF and stabilized blood are kept at 0 °C during handling, to slow the enzymic processes.

For ethical reasons, the samples of CSF were from patients from whom CSF was being collected during a diagnostic procedure. Samples of lumbar CSF were collected from patients with disc herniations during myelography. This examination demonstrated unhindered passage through the subarachnoid space. The samples of ventricular CSF were collected from patients with chronic low-pressure hydrocephalus. Thus, none of these patients had recently had increased intracranial pressure or intracranial bleeding, which might influence the concentration of 5-HT in CSF. We therefore believe that these samples of CSF resemble CSF from normal persons, at least with respect to the concentration of 5-HT.

Previously, the concentration of 5-HT in CSF has been reported to be 17–900 nmol/L (26) or 0–75 nmol/L (27) as measured by fluorometric methods. However, with the more specific and sensitive radioimmunoassay we find the upper range to be 4 nmol/L. Because higher concentrations would have been detected if present, 4 nmol/L must be the upper limit for the 5-HT that is actually present in CSF.

**Table 1. Effects of Inhibitor of Platelet Uptake and Inhibitors of Monoamine Oxidase A and B on Concentration of 5-HT in Serum**

<table>
<thead>
<tr>
<th>5-HT concn, nmol/L,</th>
<th>after Incubation for</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
</tr>
<tr>
<td>No inhibitor</td>
<td>755</td>
</tr>
<tr>
<td>Chlorimipramine</td>
<td>860</td>
</tr>
<tr>
<td>Clorgyline</td>
<td>730</td>
</tr>
<tr>
<td>Pargyline</td>
<td>780</td>
</tr>
<tr>
<td>Chlorimipramine + clorgyline + pargyline</td>
<td>860</td>
</tr>
</tbody>
</table>

From a single venipuncture, sequential blood samples were drawn into glass tubes containing 40 μL of 250 μmol/L inhibitor per milliliter of blood and incubated at 37 °C before centrifugation.

**Fig. 2. Displacement of tritiated 5-HT from the antiserum by 5-HT (●), 5-methoxytryptamine (○), and tryptamine (△)**
Table 2. Effects of Inducers of the Platelet Release Reaction on Concentrations of 5-HT in Serum

<table>
<thead>
<tr>
<th>5-HT concn, nmol/L, after incubation at</th>
<th>37 °C, 30 min</th>
<th>20 °C, 90 min</th>
<th>37 °C, 30 min</th>
<th>37 °C, 30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>No inducer</td>
<td>940</td>
<td>850</td>
<td>890</td>
<td>830</td>
</tr>
<tr>
<td>ADP, 20 μmol/L</td>
<td>920</td>
<td>900</td>
<td>740</td>
<td>—</td>
</tr>
<tr>
<td>Epinephrine, 20 μmol/L</td>
<td>980</td>
<td>880</td>
<td>960</td>
<td>—</td>
</tr>
<tr>
<td>Thrombin, 1000 NIH units/L</td>
<td>955</td>
<td>850</td>
<td>880</td>
<td>—</td>
</tr>
<tr>
<td>Reserpine, 2 μmol/L</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>870</td>
</tr>
</tbody>
</table>

Sequential blood samples were drawn into glass tubes containing 10 μL of the appropriate inducer and 40 μL of inhibitor solution per milliliter of blood. *Final concentration.

A ventriculo/lumbar gradient for 5-hydroxyindoleacetic acid has been reported (28). To see if a similar gradient exists for 5-HT, we analyzed samples of both ventricular CSF and lumbar CSF for 5-HT. In both cases, the concentrations of 5-HT in two-thirds of the samples were below the minimum detectable concentration of our assay. Thus, we could not demonstrate a ventriculo/lumbar gradient. A more sensitive method for the detailed examination of the concentrations of 5-HT in CSF is evidently needed.

Meaningful measurements of 5-HT in platelet-poor plasma require that the 5-HT be neither taken up nor released from the platelets during the preparation of plasma. The concentration of 5-HT in platelet-poor plasma, which was prepared from platelet-rich plasma, has been measured by radioenzymic assay and found to be 25–50 nmol/L (7, 8). The conditions we use for blood sampling are designed to reduce the platelet release reaction in vitro (10). Furthermore, an inhibitor of the 5-HT uptake is added immediately after the blood sampling. With these precautions, we find the concentration of 5-HT in platelet-poor plasma to be 5–14 nmol/L. Because we find lower concentrations of 5-HT in platelet-poor plasma, we believe that platelets must be better preserved during our isolation procedure.

We find that the concentration of 5-HT in plasma is only on the average 2% of that in serum.

The major compartment for 5-HT in the blood is the platelets, from which it is released by the release reaction during blood coagulation (14). The concentration of 5-HT in serum will be a measure of the 5-HT content of the platelets if 5-HT is released completely and if 5-HT is not metabolized in coagulated blood or serum. Because we have found that inducers of the release reaction do not increase the yield of 5-HT in serum, all releasable 5-HT must be released during blood coagulation in vitro. Fluorometrically, the 5-HT content of platelets has been found to be 670–710 ng/10⁹ platelets (28, 30). These results lead one to expect a concentration of 5-HT in serum of 1700 nmol/L. However, only one-third (this study) or one-half (6) of this concentration is measured in the serum. Therefore, not all platelet 5-HT may be releasable (31, 32), or the fluorescence methods used in the previous studies may not be specific for 5-HT.

We find that women have 1.3-fold higher concentrations of 5-HT in serum than men have. A similar sex-related difference has also been observed for 5-HT extracted from whole blood (33).

Because the intra-individual variation of the concentration of 5-HT in serum is small (34), the measurement of this concentration might be useful in monitoring the effects of diet or medication on 5-HT metabolism.

We have used the described method to measure the concentrations of 5-HT in ventricular CSF from patients with ruptured intracranial aneurysm, to study the relationship between the concentration of 5-HT in CSF and cerebral vasospasm (35).

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References