Rapid and Specific Enzyme Immunoassay of Serotonin

J. Chauveau, V. Fert, A. M. Morel, and M. A. Delaage

A new, highly sensitive enzyme immunoassay (EIA) of serotonin (5HT) is described. The assay is based on the competition between N-succinyl-glycinamide-serotonin (N-SGA-5HT, obtained by acylation of the 5HT in the sample to be assayed) and an enzymic tracer, N-succinyl-5HT-acetylcholinesterase, for binding to rabbit polyclonal antibody coated onto the wells of microtiter plates. The antibody is directed against an immunogen obtained by coupling N-succinyl-5HT to glycy-bovine serum albumin. The EIA permits the accurate measurement of as little serotonin as 0.5 nmol/L (1.8 pg per well) in blood, plasma, serum, cerebrospinal fluid, urine, platelets, and other tissues, with no significant cross-reactivity with other compounds. The results obtained correlate well with those obtained by HPLC after extraction. The assay has the advantage of permitting the measurement of 5HT in up to 500 samples in as little as 3 h.

Additional Keyphrases: acetylcholinesterase • cerebrospinal fluid • platelets • urine

The vasoconstrictor effect of serotonin (5-hydroxytryptamine; 5HT) has been known for about a century.\(^1\) The compound, characterized as an indolealkylamine (1), was chemically synthesized almost 40 years ago (2). 5HT occurs in large amounts in the gastrointestinal tract, platelets, spleen, and brain. Within the cell, tryptophan is first hydroxylated to 5-hydroxytryptophan and then decarboxylated to 5HT. Mammalian platelets take up 5HT from their environment. The main catabolic pathway is by oxidative deamination of 5HT, catalyzed by monoamine oxidase, to form 5-hydroxyindoleacetic acid.

5HT is implicated in several pathological situations, including carcinoid tumors (3, 4), hypertension (5, 6), migraine (7–10), depression (11–13), cirrhosis of the liver (14), premenstrual tension (15), Alzheimer’s (16, 17) and Parkinson’s diseases (18), and the Kleine–Levin syndrome (19). Moreover, 5HT has been implicated in neuroendocrine, temperature, and blood pressure regulation and also plays a role in pain perception, sleep, and behavior (20). The availability of a reliable, sensitive, and convenient assay for 5HT in biological fluids is evidently a prerequisite for its use as a biochemical indicator.

Currently methods for measuring 5HT include radioenzymatic assay (21–24), radioimmunoassay (25–29), fluorometric assay (30–32), and HPLC with electrochemical detection (33–35).

The radioenzymatic assay is a multi-step procedure with the final step being extraction of the titrated product. The RIA involves use of a gamma-labeled tracer and, unlike HPLC or fluorometric methods, does not require prior extraction of the biological sample; however, iodinated derivatives of serotonin are not sufficiently stable for large-scale routine use. HPLC has the advantage that 5HT and its metabolites can be measured in the same run, but only a few samples can be assayed per day.

The small size of 5HT and its susceptibility to oxidation are two major difficulties in the design of an immunoassay. To obviate the risk of 5HT oxidation during iodination, we have used an enzymic tracer instead of a radiolabel. Acetylcholinesterase (AChE; EC 3.1.1.7) (36) is suitable as a nonisotopic tracer because of its high turnover number per mole (1.8 × 10⁶ mol of acetylthiocholine hydrolyzed per hour). The hydrolyzed product can be detected at very low concentrations: 3.5 × 10⁻¹⁸ mol (absorbance increase of 0.01 during 30 min, sample volume = 0.2 mL, light path = 0.44 cm) compared with 1.5 × 10⁻¹⁷ mol for ¹²⁵I (detection limit, 50 counts/min; specific radioactivity, 74 TBq/mol; counting efficiency, 75%).

The EIA of 5HT takes advantage of an efficient chemical derivatization of small amines, previously used successfully in the RIA of histamine (37). We designed the immunoassay so that the serotonin derivative closely resembled the immunogen. Serotonin was first converted to N-succinylated serotonin and then conjugated to glycyl-bovine serum albumin (BSA) as immunogen. The N-succinylated 5HT was coupled directly to AChE as the enzymatic tracer. We used the reagent N-hydroxysuccinimide ester-succinyl glycaminid (NHS-SGA; patented by Immunotech, Marseille, France) to convert 5HT in standards and biological samples into an SGA derivative. The structures of the compounds used in the serotonin EIA are shown in Figure 1.

Materials and Methods

Apparatus

The solid-phase EIA was performed by using standard microtitration equipment, including an automated plate washer (ADIL, Strasburg, France) and a spectrophotometer (SLT, Salzburg, Austria), and monitored with ELIOT™ (Immunotech) software, which involves the use

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1 Immunotech, Case 915, 13288 Marseille Cédex 09, France.

1 Nonstandard abbreviations: AChE, acetylcholinesterase (EC 3.1.1.7); BSA, bovine serum albumin; CEP, cerebrospinal fluid; DMF, dimethylformamide; EIA, enzyme immunoassay; 5HT, serotonin; MES, 2-(N-morpholino)ethanesulfonic acid; NHS-SGA, N-hydroxysuccinimide ester succinyl glycaminid; N-SGA-5HT, N-succinyl-glycinamide serotonin; PEG, polyethylene glycol; PFP, platelet-poor plasma; PRP, platelet-rich plasma; TFA, trifluoroacetic acid; and UE, Ellman unit.

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of a proprietary program. Microtiter plates (Model 4.68667) were from Nunc (Roskilde, Denmark). The HPLC equipment, purchased from Waters (Millipore, Milford, MA), consisted of two Model 510 pumps, a V6K injector with a 2-mL sample loop, and a Waters 3.9 mm × 30 cm C18 µBondapack column (particle diameter 10 µm). The controller was a Waters Model 680, the detector was a Waters Model 990 photodiode ultraviolet detector, monitored by an APC IV computer (Nec, Tokyo, Japan) and a Model 460 electrochemical detector with a potential set at +600 mV vs Ag/AgCl. Radioactivity was measured with a Betamatic beta counter (Kontron, Zürich, Switzerland) and a Packard (Downers Grove, IL) gamma counter.

Reagents

Unless otherwise stated, all reagents were of analytical grade and purchased from Merck (Darmstadt, F.R.G.). Solvents were of spectroscopic grade (SDS, Peypin, France). 5HT and its metabolites, glycine, acetylthiocholine iodide, 5,5'-dithiobis(2-nitrobenzoic acid), and BSA were purchased from Sigma Chemicals (St. Louis, MO). [3H]5HT binoxylate (26.8 kCi/mol) was purchased from New England Nuclear (Boston, MA). AChE from electric organs of Electrophorus electricus was purified by Pradelles and colleagues (CEA, Gi/ Yvette, France). AChE was used as the globular form, G4, consisting of four catalytic subunits. AChE activity was measured by the method of Ellman et al. (38). The substrate consisted of 0.75 mmol of acetylthiocholine iodide, 0.5 mmol of 5,5'-dithiobis-2-nitrobenzoic acid, and 30 mmol of NaCl per liter of 10 mmol/L potassium phosphate buffer, pH 7.4. In lyophilized form, the reagent was stable for one year when stored at 2–8 °C.

One Ellman unit (UE) is defined as the amount of enzyme that produces an absorbance increase of 1.000 min⁻¹ at 412 nm, in 1 mL of solution (1-cm light path). The concentrations of AChE mentioned in this paper are expressed as the molarity of the catalytic subunit of AChE (Mr 80 000).

Serotonin standard. Serotonin oxalate was dissolved in either water or 0.2 mol/L HCl reagent to a final concentration of 0.2 mmol/L. Concentrations were checked by ultraviolet absorbance, with the use of the following molar absorbivities (L·mol⁻¹·cm⁻¹): ε₂₅₀nm = 3135, ε₂₆₀nm = 4480, ε₃₂₅nm = 4152.5, ε₃₆₅nm = 3265. The 5HT standard was stored at −20 °C in vials containing 10 nmol of lyophilized 5HT.

Procedures

Synthesis of N-succinylated serotonin. The 5-hydroxytryptamine oxalate (10 mg) and succinic anhydride (23 mg) in 0.5 mL of water containing 370 µmol of potassium hydroxide were incubated with shaking at room temperature in the dark. After 10 min, 90 µmol of potassium hydroxide was added; the solution was stirred in the dark for an additional hour. Then the medium was rapidly acidified with trifluoroacetic acid (TFA) to pH 6 and applied to a µBondapack C₁₈ column. The column was eluted with water/methanol (85/15 by vol) (Figure 2). The N-succinylated serotonin was lyophilized after purification and stored at −20 °C.

Immunogen synthesis and immunization. We synthesized the immunogen by linking N-succinylated 5HT to BSA, to which glycyrl residues had been coupled as previously described for 5-hydroxyindoleacetic acid (39). We purified the immunogen by passage through a Sephadex G25 (Pharmacia, Upsala, Sweden) column, analyzing the fraction eluted in the void volume by ultraviolet spectroscopy. About 5 mol of 5HT was coupled per mole of albumin. Rabbits were immunized by multiple intradermic injections into the backs of the animals, as described by Vaitukaitis et al. (40).

Preparation of tracers. [3H]N-Succinylated 5HT was obtained by incorporation of [3H]5HT during the synthesis of N-succinylated 5HT. 125I-labeled N-succinylated 5HT-glycyltyrosinamide was prepared as described earlier (29).

The enzymatic tracer was obtained as follows: 600 nmol of N-succinylated 5HT was activated for 4 min at 4 °C in 60 µL of a mixture containing 878 nmol of ethyl chloroformate and 600 nmol of triethylamine in anhydrous dimethylformamide (DMF). Then 12 nmol (75 000 UE) of AChE in 500 µL of borate buffer (50 mmol/L, pH 8.8) was added. After 30 min of incubation at 4 °C in the dark, we purified the reaction product by elution from a 10 × 1 cm Sephadex G25 Superfine column with an eluent of 3 mmol of ascorbate, 3 g of BSA, and 0.4 mol of NaCl per liter of 0.1 mol/L citrate buffer, pH 6.2. The product, recovered in the void volume, had a total enzyme activity of 64 000 UE. About 2 mol of 5HT was bound to 1 mol of AChE, as determined.
by using [3H]N-succinylated 5HT as tracer.

Acylation of 5HT in samples. The acylating reagent, NHS-SGA, was made by linking succinylglycinamide to N-hydroxysuccinimide in DMFA, after activation by ethyl chloroformate (37). The acylating reagent, NHS-SGA, was lyophilized.

We acylated 5HT by adding 100-\(\mu\)L aliquots of 5 mmol/L 5HT solution, at different pH and salt concentrations, to 1 mg of acylating reagent with the simultaneous addition of acylating buffer (50 \(\mu\)L of 50 mmol/L borate buffer, pH 8.2). The yield and the rate of coupling were analyzed by HPLC; 25 \(\mu\)L of acylated sample was acidified with TFA (0.5 g/L, 475 \(\mu\)L), applied to a \(\mu\)Bondapack C18 column, and eluted with 0.5 g/L TFA solution/methanol (85/15 by vol) at a flow rate of 1 mL/min.

In biological samples, such as platelet-poor plasma (PPP), serum, etc., containing [3H]5HT (purified on a Sephadex G25 Superfine column), the yield of the acylation was verified after separating the converted product by elution from a Mono-S column (Pharmacia), with a linear gradient of NaCl (0–2 mol/L) in 50 mmol/L 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 6.

Testing of antisera. Several dilutions of antisera were incubated overnight at 4°C with a fixed amount of I\(^{125}\)I-labeled \(N\)-succinylated serotonin-glycyltryptosinamide. The antigen–antibody complexes were precipitated in the presence of 50 \(\mu\)L of normal human plasma with polyethylene glycol (PEG) 6000 (125 g/L, final concentration) as described by Desbuquois and Aurbach (41).

Solid-phase assay in avidin-coated wells. We used Sepharose–Protein A affinity chromatography to purify IgG from selected antisera. The IgG was then coupled to \(N\)-hydroxysuccinimidyld biotin (Boehringer, Meylan, France). The biotinylated antibodies were purified on a Sephadex G25 column and tested at different dilutions in avidin-coated wells (Immunotech patent).

Processing of samples. To avoid both enzymic oxidation and cellular uptake of serotonin, the use of an inhibitor solution, though not essential, is recommended, if blood samples are to be stored longer than 2 h at room temperature. To 2.5 mL of sample add 100 \(\mu\)L of a solution containing 250 \(\mu\)mol of clorgyline, 250 \(\mu\)mol of chlorimipramine, 250 \(\mu\)mol of pargyline, 150 mmol of NaCl, and 3 mmol of ascorbate per liter of 0.1 mol/L citrate buffer, pH 6.2.

To assay 5HT in PPP, collect 2.5 mL of blood into a cold (4°C) polystyrene tube containing disodium EDTA, 5 mmol/L. Within 20 min, centrifuge the samples at 1700 \(\times g\) and 4°C for 30 min. Transfer the upper two-thirds (~0.8 mL) of the PPP to a fresh polystyrene tube and store at ~20°C until assay.

For serum assays, collect 2.5 mL of blood into a clean glass tube and let this stand at room temperature for 1 h. Centrifuge (1700 \(\times g\), 30 min, 4°C), then aspirate the upper two-thirds (~0.9 mL) of the supernate and store at ~20°C until assay. For analysis of whole blood, collect 2.5 mL of blood into a polystyrene tube containing lithium heparinate. Mix gently by inversion and store at ~20°C.

To assay platelet-rich plasma (PRP), collect 2.5 mL of blood into a cold (4°C) polystyrene tube containing disodium EDTA, 5 mmol/L. Mix gently by inversion and centrifuge without delay (120 \(\times g\), 10 min, room temperature). Remove the upper three-quarters (~0.9 mL) of the supernate for storage at ~20°C until assay.

For cerebrospinal fluid (CSF), collect 1 mL into a cold (4°C) polystyrene tube and centrifuge (1700 \(\times g\), 30 min, 4°C). Transfer the upper two-thirds of the supernatant fluid (~0.7 mL) to a cold tube and store at ~20°C until assay.

For urine specimens, collect 24-h urine into a clean plastic container containing a bacteriostat (e.g., boric acid, toluene). Determine the total volume, then remove a 1-mL aliquot to store at 4°C for assay within one week (store at ~20°C, if the sample is to be assayed later).

For assays of platelets, collect 9 mL of blood into a polystyrene tube containing disodium EDTA, 5 mmol/L; mix gently by inversion; and centrifuge without delay (120 \(\times g\), 3 min, room temperature). Apply 2 mL of the supernate to a 10-mL column of Sepharose 2B, equilibrated in Tyrode’s buffer (per liter, 0.138 mol of NaCl, 29 mmol of KCl, 12 mmol of NaHCO\(_3\), 36 mmol of NaH\(_2\)PO\(_4\), 1 g of glucose, and 1 mmol of EDTA, pH 7.4). After counting the platelets, resuspend the opalescent
fraction to $10^6$ platelets/mL in Tyrode’s buffer containing 3.5 g of BSA per liter. The endogenous 5HT content is obtained by sonication or by perichloric precipitation of the suspension (cf. solid tissue, below). After sedimentation of the platelets, acylate the 5HT in the Tyrode’s buffer and assay.

For solid tissue samples, homogenize in 0.2 mol/L HClO₄ reagent (10 µL of HClO₄ per milligram of tissue), then centrifuge (5 min, 10,000 × g, 4 °C). Neutralize the supernate with an equal volume of potassium borate solution (1 mol/L, pH 9.25). After a 1-min centrifugation at 10,000 × g and 4 °C, store the supernatant at −20 °C until assay. To remove 5HT from human serum, pass the serum through a 100% carbon cartridge (Millipore, Bedford, MA) (unpublished method).

**Diluent for standards and samples.** Standards and samples are diluted in a solution containing 6 mmol of ascorbate, 10 g of BSA, 0.4 mol of NaCl, and 0.25 mmol of thimerosal per liter of 50 mmol/L citrate buffer, pH 6.2.

**Enzyme Immunoassay**

Under standard conditions, we acylate 100 µL of samples or standards with 1 mg of acylation reagent in 50 µL of acylating buffer. To each well of the microtiter plate, coated with anti-serotonin antibodies, we add 20 µL of this mixture and 200 µL of a solution containing the 5HT-AChE conjugate at an appropriate concentration. The plate is then incubated for 3 h in the dark with shaking; after rinsing the wells three times with 250 µL of wash solution (0.5 g of Tween 80 and 0.15 mol of NaCl per liter), we add 200 µL of substrate solution. The reaction product is determined spectrophotometrically at 405–414 nm after 15–20 min of incubation, which is terminated by adding 50 µL of 0.1 mmol/L tarcine solution. The absorbance of samples is converted to concentration by using a standard curve, constructed from six standards as processed by ELIOT software.

**Cross-Reactivities**

We measured the cross-reactivities of various test compounds after their acylation in depleted human serum. The percentage of cross-reactivity is calculated from the ratio of the concentration of the cross-reactant at 50% B/B₀ to the concentration of acylated 5HT at 50% B/B₀.

**Results**

**Development of the Immunoassay**

*Immunogen and antibody.* The yield in the succinylation of 5HT was 99% (88.3% N-succinyl-5HT and 11.7% di succinyl-5HT) (Figure 2); only the N-succinyl-5HT was used in the linkage to glycy1 albumin.

The succinyl-5HT-glycylalbumin conjugate (5 mol/ mol) was highly immunogenic. Two rabbits gave antibodies, detectable at 1000-fold dilution, after the second injection. One of the rabbits (AST74) gave antisera usable in the radioimmunoassay at a dilution of 10 000-fold after the third injection. The antiserum finally selected (AS74) contained 4.3 mg of IgG antibody protein per milliliter. We immobilized the antibody on 96-well microtiter plates at 200 µL per well of a solution containing 200 ng of the anti-5HT antibody protein per milliliter. The antibody was stable for at least one year in this form.

**Binding of tracer.** The binding of the 5HT enzymic tracer was examined in various buffers over a pH range from 4 to 9. The binding was optimal in the pH range of 5–6.5. The optimal concentration of 5HT enzymic tracer was 0.012 fmol/well. We diluted the enzymic tracer in a solution containing 6 mmol of ascorbate, 1 g of BSA, and 0.4 mol of NaCl per liter of 50 mmol/L citrate buffer, pH 6. Lyophilized enzymic tracer was stable for more than one year when so prepared.

**Acylation of samples.** The sensitivity and specificity of this EIA lie in the ability of the reagent, NHS-SGA, to acylate 5HT directly and reproducibly in samples. The acylation of 5HT at various salt concentrations and pH values was analyzed by HPLC (Figure 3, Table 1). The reaction was almost instantaneous and the yield was reproducibly 70%. Acylation in plasma gave identical yields for between 25 and 200 µL of plasma per milligram of acylating reagent (Figure 4). To confirm this result, we compared the curves obtained after direct acylation of 5HT with the curve obtained by diluting acylated 5HT that had been prepurified by HPLC (Figure 5).

**Antibody specificity.** The degree of cross-reactivity of compounds structurally related to 5HT is presented in Table 2. The highest cross-reactivity ratio was observed with N-succinyl 5HT, a compound not found in nature, but used in the synthesis of the enzymic tracer. Endogenous metabolites of 5HT are at least 1000-fold less well recognized than is acylated 5HT.

**Analytical range and detection limit.** A typical standard curve is shown in Figure 5. The same standard curve was obtained irrespective of whether the standard was diluted in the diluent or in depleted human serum. The day-to-day CV of the zero standard was 2%. Therefore, the minimum detectable concentration at which

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**Fig. 3. HPLC of product of acylation: 1, 5HT; 2, acetylated 5HT**

HPLC conditions as described in Materials and Methods
Table 1. Effect of Salt Concentration and pH on Acylation of 5HT

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Acylation yield, % (and CV, %)</th>
<th>Chromatographic recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acylation times &lt;1 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate, 10 mmol/L, pH 6.2</td>
<td>70 (1.3)</td>
<td>98.0</td>
</tr>
<tr>
<td>Phosphate, 10 mmol/L, pH 7.2</td>
<td>70 (1.2)</td>
<td>98.0</td>
</tr>
<tr>
<td>Borate, 10 mmol/L, pH 8.7</td>
<td>70 (0.8)</td>
<td>98.0</td>
</tr>
<tr>
<td>Phosphate, 10 mmol/L, pH 7.2; NaCl, 0.15 mol/L</td>
<td>70 (1.2)</td>
<td>98.0</td>
</tr>
<tr>
<td>Citrate, 0.1 mol/L, pH 6.2; thimerosal, 0.25 mmol/L; ascorbate, 3 mmol/L; NaCl, 0.4 mol/L</td>
<td>69 (1.0)</td>
<td>98.4^b</td>
</tr>
<tr>
<td>Acylation time 30 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate, 0.1 mol/L, pH 6.2; thimerosal, 0.25 mmol/L; ascorbate, 3 mmol/L; NaCl, 0.4 mol/L</td>
<td>71 (1.0)</td>
<td>98.5^p</td>
</tr>
</tbody>
</table>

* For acylation of 5HT, 5 mmol/L, determined by absorbance at 275 nm.
^b n = 4; all others, n = 3.

Fig. 4. Mono S column chromatograms of [3H]5HT added to patients’ plasma before acylation: 1, acylated 5HT; 2, 5HT. Different volumes of patients’ plasma—25 µL(B), 100 µL(B), 200 µL (C)—containing the same concentration of [3H]5HT were treated with 1 mg of acylating reagent.

Table 2. Specificity of 5HT Enzyme Immunoassay

<table>
<thead>
<tr>
<th>Analogs</th>
<th>Cross-reactivity ratio</th>
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<tbody>
<tr>
<td>Acylated serotonin</td>
<td>1.0</td>
</tr>
<tr>
<td>N-Succinyl serotonin</td>
<td>2.8</td>
</tr>
<tr>
<td>Acylated tryptamine</td>
<td>1 000</td>
</tr>
<tr>
<td>Acylated 5-methoxytryptamine</td>
<td>3 400</td>
</tr>
<tr>
<td>Acylated 5-hydroxytryptophan</td>
<td>6 700</td>
</tr>
<tr>
<td>Acylated 5-hydroxyindoleacetic acid</td>
<td>150 000</td>
</tr>
<tr>
<td>Acylated melatonin</td>
<td>172 000</td>
</tr>
<tr>
<td>Serotonin</td>
<td>230 000</td>
</tr>
<tr>
<td>Acylated tryptophan</td>
<td>1 000 000</td>
</tr>
</tbody>
</table>

* Analog concentration (nmol/L) at 50% of B/B₀ divided by acylated serotonin concentration (nmol/L) at 50% of B/B₀.

Fig. 5. Standard curve for determinations of 5HT. Dilution of 5HT in medium (V) and of 5HT acylated and then purified by HPLC (B).

Measurement of 5HT in biological samples. Serum, PPP, PRP, CSF, urine, and tissue homogenates were diluted and enriched with 5HT at concentrations ranging from 5 to 1000 nmol/L. Analytical recovery was 99%

increased to 200 µL per well (Figure 6).

Fig. 6. Effect of sample volume on standard curve: 20 µL (V), 100 µL (V), 200 µL (C). (A): Results expressed in fmol/well; (B): in nmol/L pg/0.175 = fmol

the B/B₀ is equal to 0.96 (2 SD) is 0.5 nmol/L (1.8 pg of 5HT per well). For samples with low 5HT contents, the incubation volume of samples and standards may be
(SD 4%) in all cases. Samples were aliquoted and stored at −20 °C and used to estimate the intra- and interassay variation (Table 3). The interassay study was done by several technicians using different lots of reagents. We routinely diluted samples before acylation: 100-fold in the case of PRP or blood, 50-fold for urine, 20-fold for serum, and 10-fold for platelet extracts. PPP and CSF samples were assayed undiluted. Normal values for 5HT found by EIA in human material were <4 nmol/L for CSF, 4–15 nmol/L for PPP, 300–700 nmol/L for male serum, 500–900 nmol/L for female serum, 1000–2500 nmol/L for PRP, and 900–1300 nmol/L in 24-h urine samples.

The concentration of serotonin in platelets was 0.32 (SD 0.11) nmol/10^8 platelets (n = 8); the percentage of serotonin release after thrombin stimulation (10 NIH units/10^8 platelets) was 85.1 (SD 9.5)% (n = 8). These results are in agreement with other reports [42, 43]. The concentration of 5HT in platelet-free Tyrode's buffer after sedimentation was 0.006 (SD 0.001) nmol/10^8 platelets.

Diet affected the concentrations of 5HT. Values were lower after a tryptophan-poor diet (no mollusks, bananas, chocolate, pineapples, plums, or nuts) maintained for 24 h before PPP sample collection: i.e., 10 nmol/L (CV 5%, n = 6) vs 19 nmol/L (CV 10%, n = 4) after a “normal” diet.

The manner of collecting the samples also affected the concentrations of 5HT in PPP. When a tourniquet was used, 20 nmol/L (CV 6%, n = 7) was measured, whereas with hand pressure the concentration was 12.5 nmol/L (CV 5%, n = 6) and by free flow it was 8 nmol/L (CV 5%, n = 7).

**Correlation with HPLC.** The correlation between 5HT values, measured by HPLC with electrochemical detection (x) and by EIA (y) for 21 samples of CSF, 37 of PPP, and 12 of serum, was excellent: $y = 0.954x + 2.5$ nmol/L ($r = 0.998$). The small positive intercept reflects the greater sensitivity of the EIA: two samples of CSF were below the detection limit of the HPLC method. For the 41 samples that gave results between 0 and 30 nmol/L, the linear-regression equation was $y = 1.011x - 0.127$ nmol/L ($r = 0.996$).

**Discussion**

The present study illustrates the efficacy of chemical modification for improving hapten detection. The enhancement of sensitivity and specificity subsequent to the chemical addition of a spacer was first described for cAMP [44]. The succinylation used in that case increased the affinity for the antibody 100-fold. The succinylation of 5HT increased its affinity for the antibody by ~1000-fold [28]. A glycinamide arm, used with vasopressin [45] and 5-hydroxyindoleacetic acid [39, 46], increased the affinities for their respective antibodies about 500-fold. Combining both modifications, i.e., SGA conversion and elongation by the addition of a succinylglycinamide arm to histamine, the smallest of these haptons, increased the affinity of the antigen for the cognate antibody by $5 \times 10^5$-fold [39]. The same modification increased by 2.3 $\times 10^5$-fold the affinity of 5HT for its antibody. Because most other indole derivatives are not susceptible to SGA acylation, their affinities are thus not affected and the problem of cross-reactivity does not arise; i.e., specificity is increased.

The aliquoted, lyophilized acylation reagent facilitates the acylation of biological samples over a broad range of volumes and concentrations. None of the metabolites of 5HT, at concentrations found in vivo, interfere with the assay. The detection limit of the EIA (1.8 pg or 10 fmol/well) is better than that of any previously published RIA or HPLC for 5HT. Furthermore, the incubation volume may be varied from 20 to 200 μL per well. As many as 200 samples can be assayed readily by one worker in one working day.

The assay of 5HT has several applications in both clinical routine and in research, e.g., as an aid in the diagnosis of carcinoid tumors. Because intestinal chromaffin cells are a major source of 5HT, effectiveness of treatment of the abnormal proliferation of these cells may be monitored by the measurement of 5HT. Measurement of 5HT is also used in cardiology and other relevant branches of clinical medicine for the quality control of blood and platelets used in transfusions: the damage of blood platelets leads to an increase of 5HT in the plasma, and the thrombin-induced 5HT release from platelets is used as an indicator of the functional integrity of stored platelets. A sensitive assay for 5HT is evidently of potential usefulness in the ongoing search for pertinent markers in various neurological abnormalities, including Parkinson's and Alzheimer's diseases. The assay has been used also in research on the neuronal regulation of corticotropin [47] and on the cellular origin of 5HT in the islets of Langerhans [48].

In summary, this new EIA greatly facilitates the routine determination of 5HT in clinical practice and

<table>
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<th>n</th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
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<td></td>
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<tr>
<td>10</td>
<td>420–550</td>
<td>450*</td>
<td>65.5</td>
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<td>50.9</td>
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<td>27.7</td>
<td>2.66</td>
<td>9.4</td>
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* Before measurement, samples were diluted: * fivefold, b 50-fold, c 20-fold.
may well open up new areas of research where involvement of 5HT is suspected.

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References