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Radioimmunoassay of Human Serum Serotonin

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Summary: A radioimmunoassay for extracted, N-acetylated human serum serotonin (5-hydroxytryptamine) is described. Antisera were raised in rabbits against a conjugate of bovine serum albumin with serotonin hemisuccinamide. Polyethylene glycol in combination with anti-rabbit immunoglobulins was used to separate bound and unbound ^{125}I-*Bolton Hunter*-serotonin conjugate. Ethanol precipitation of serum proteins was used to extract serotonin, which was subsequently acetylated with acetic anhydride to N-acetyl serotonin. The average recovery was 66%. The minimal detectable concentration of N-acetyl serotonin was 0.012 µmol/l serum (25 fmol per tube). The intra-assay precision (CV) was 6.8% (n = 20) at a level of 0.9 ± 0.06 µmol/l. The inter-assay CV was 10% at a level of 0.49 ± 0.049 µmol/l, and 25% (n = 10) at a level of 2.16 ± 0.53. Analytical recovery of serotonin, corrected for losses during extraction and acetylation, was 99 ± 13%. The only substance cross-reacting with the antibody was endogenous N-acetyl serotonin. This was detectable when the acetylation step was omitted, and it can be removed by extraction before the acetylation. The observed range for the concentration of serotonin in serum was for 59 women 0.45 – 3.46 (mean ± SD: 1.37 ± 0.63 µmol/l) and for 59 men 0.19 – 2.8 (mean ± SD: 1.18 ± 0.56 µmol/l). All values are corrected for endogenous N-acetyl serotonin: observed range 0 – 0.18 (mean ± SD: 0.03 ± 0.03 µmol/l).

Radioimmunologische Bestimmung des Serotonins in Humanserum

Zusammenfassung: Wir beschreiben die radioimmunologische Bestimmung des extrahierten und N-acetylierten Serotonins (5-Hydroxytryptamin) im Serum des Menschen. Antiseren wurden gegen das an Rinderserumalbumin gebundene Bernsteinsäuremonoamid-Derivat des Serotonins bei Kaninchen erzeugt. Polyethylenglycol und anti-Kaninchen Immunglobulin wurden zur Trennung des freien und gebundenen ^{125}I-markierten Serotonins verwendet. Serotonin wurde durch Ethanolfällung der Serumproteine extrahiert und anschließend mit Essigsäureanhydrid zum N-Acetylserotonin acetyliert. Gesamtausbeute: 66%. Die Nachweisgrenze für N-Acetylserotonin beträgt 0,012 µmol/l Serum (25 fmol pro Assayröhrchen). Der Intra-Assay-Variationskoeffizient (VK) bei einer Serotoninkonzentration von 0,9 ± 0,06 µmol/l beträgt 6,8% (n = 20), die Inter-Assay VK bei 0,49 ± 0,049 und 2,16 ± 0,53 µmol/l betragen 10 bzw. 25% (n = 10). Künstlich erhöhte Serotoninkonzentrationen werden nach der Korrektur auf Extraktions- und Acetylierungsverluste zu 99 ± 13% wiedergefunden. Nur endogenes N-Acetylserotonin zeigt eine Kreuzreaktion mit dem Antikörper und kann durch Extraktion ohne Acetylierung nachgewiesen werden.

Die beobachteten Referenzwerte für Serotonin im Serum betrugen: Frauen (59 Seren) 0,45 – 3,43 ($\bar{x} \pm s$: 1,39 ± 0,63 µmol/l) und Männer (59 Seren) 0,19 – 2,8 ($\bar{x} \pm s$: 1,18 ± 0,56 µmol/l). Alle Werte wurden um den jeweiligen Anteil endogenen N-Acetylserotonins korrigiert: (Referenzwerte: 0 – 0,18 ($\bar{x} \pm s$: 0,03 ± 0,03 µmol/l).

Introduction

Serotonin, a well-known neurochemical transmitter, has been strongly implicated in a number of mental and physical disorders (1). Serotonin is located primarily in the enterochromaffin cells of the intestine, serotonergic neurons of the brain, and blood platelets. Increased concentrations of circulating serotonin have been shown in several pathological conditions that may involve peripheral serotonergic mechanisms: migraine (2), schizophrenia (3), carcinoid syndrome (4), essential hypertension (5), *Huntington*'s chorea (6), and *Duchenne*'s muscular dystrophy (7).

Classical methods for its determination are based on fluorescence measurements (8, 9). Recently, more specific and sensitive methods – radioenzymic asssay (10), mass spectrometric assay (11), liquid chromatography with electrochemical detection (12), and radioimmunoassay (13–15) – have been described. For a routine laboratory each of the above analytical methods has significant drawbacks either being low in specificity or time consuming. Although a sensitive direct radioimmunoassay for serotonin has been reported (15), the generally low titers of anti-serotonin antibodies (13, 15) and the instability of serotonin in aqueous solution has strictly limited its applicability. It was therefore decided to establish a radioimmunoassay for N-acetyl serotonin instead of serotonin using a homologous [^{125}I]-N-acyl analogue as radioactive ligand. The specific conversion of serotonin into N-acetyl serotonin by acetic anhydride (16), the use of ^{125}I instead of ^{3}H (17) and the double antibody technique allow the determination of serum serotonin in a routine laboratory.

Materials and Methods

Reagents

Chemicals

5-[1,2-^{3}H(N)]Hydroxytryptamine (creatinine sulphate complex, 1.1 TBq/mmol) and N-succinimidyl-3-(4-hydroxy-5(3)-[^{125}I]iodophenyl)propionate (^{125}I-*Bolton-Hunter* reagent, 74 TBq/mmol) were from New England Nuclear Corp. (Dreieich, F. R. G.). Bovine serum albumin was from Behring Institut (Marburg, F. R. G.). 5-Hydroxytryptamine and its analogues were from Sigma (München, F. R. G.). Analytical grade chemicals and glass-distilled water were used throughout.

Buffers

Buffers used are identified by the following abbreviations:

buffer A (10 mmol/l potassium phosphate, pH 7.4, containing 1.5 mmol/l EDTA-disodium salt (Merck, Darmstadt, F. R. G.), 3 mmol/l sodium azide, 100 ml/l glycerol);

buffer B (10 mmol/l potassium phosphate, pH 7.4, containing 1 g/l gelatine, 3 mmol/l sodium azide).

Isotope solution

Appropriate amounts of HPLC-purified, iodinated serotonin were diluted with buffer B to a final radioactivity of 400,000 counts/min (6660 Bq) per ml.

Antiserum solution

Each antiserum was tested at different dilutions in buffer A. The dilution which was able to bind 30% of the isotope under assay conditions was used.

Precipitating antiserum reagent

The precipitating antiserum (goat anti rabbit gamma globulin, polyethyleneglycol 6000, sodium azide as preservative) was purchased from DRG Biochemie (Marburg, F. R. G.).

Preparation of immunogen and immunisation

The immunogen used consisted of serotonin hemisuccinamide bound to bovine serum albumin as described (14). The final reaction mixture was dialysed against 0.15 mol/l NaCl, and insoluble reaction products were removed by filtration. Spectrophotometric analysis at 280 nm showed that 13–15 mol of serotonin were coupled per mol of albumin. The immunogen was stored at –20 °C in the dark. It is stable for years.

Rabbits were immunized with an initial dorsal injection of an emulsion of 0.5 ml (1 mg) of immunogen and 0.5 ml of complete *Freund*'s adjuvant. Booster injections were given in the same way at 4 week intervals.

Iodination of serotonin (^{125}I-*Bolton-Hunter*-serotonin conjugate)

Ten mg of serotonin were dissolved in 1 ml dry pyridine. Five µl aliquots of this stock solution were mixed with appropriate amounts (10–50 µl) of commercially available ^{125}I-*Bolton-Hunter* reagent and the reaction mixture was kept for 1 h at room temperature. Solvents were removed under a gentle stream of nitrogen in a fume cupboard, redissolved in water/methanol (9 + 1 by vol.) and purified by use of reversed phase high performance liquid chromatography (PEP RPC, Pharmacia, Uppsala). Authentic unlabeled I-*Bolton-Hunter*-serotonin conjugate was prepared as described above except that non-radioactive *Bolton-Hunter* reagent (19) was used (tab. 2).

Sample collection

To avoid clotted fibrin, blood samples were collected by use of Monovette® syringes (Sarstedt, Nümbrecht, F. R. G. or similar products), and centrifuged at 1500 g. The serum was separated and frozen at −20 °C before analysis (within two weeks). All samples were taken only during normal waking hours, but at no specific time.

Serotonin extraction and sample preparation

Sera were diluted with double-distilled water (1 + 4). One hundred µl of diluted serum and 2 ml of ethanol were added to a glass tube and carefully vortexed. The mixture was kept for 30 min at 4 °C to complete the precipitation of proteins and centrifuged at 2000 *g*. The clear supernatant was decanted into a glass tube and 50 µl of a pyridine/acetic anhydride (1 + 1; by vol.) solution was added. The tube was carefully vortexed, placed in a heated water bath (50 °C), and the organic solvent removed by evaporation under a gentle stream of nitrogen. One ml of buffer A was added and the tube heated (70 °C) for 30 min, then vortexed for no less than 30 seconds. The extracts were stored at 0−4 °C (no longer than 1 week).

Radioimmunoassay procedure

One hundred µl of serum extract, 100 µl of isotope solution, and 100 µl of diluted antiserum were added to a polystyrene tube and incubated overnight at 4 °C (1 h at 37 °C, respectively). One ml of precipitating antibody was added, the mixture incubated for a further 10 min at room temperature and centrifuged (1500 *g*, 15 min, 4 °C). The tube was decanted and the pelleted radioactivity determined in a gamma-counter.

Each series consisted of a standard curve (0.12−9.26 pmol serotonin), unspecific binding, quality control samples and the samples to be assayed.

Results

Immunisation

Ten rabbits were immunised. During the process of immunisation the binding capacities and the equilibrium constants for the antisera were estimated according to *Scatchard* (19, 20). Concentrations of free and protein bound ligand were determinated by the dextran-coated charcoal technique (20). Useable antisera were obtained from two rabbits after six months of immunisation. The antiserum with the highest equilibrium constant ($K_d = 3 \cdot 10^{-11}$ mol/l) was diluted 1 : 1000 with buffer A (final assay dilution 1 : 3000) and used in the experiments described in this paper.

Conditions for storage of serum

Many previous reports have commented on the appropriate storage for serotonin blood samples. The long-term stability of frozen serum serotonin was examined by determination of the inter-assay coefficient of variation of our radioimmunoassay system. There was no significant loss of serotonin content of 20 different samples when stored for two weeks at −20 °C.

Extraction and acetylation of serotonin

Twenty different sera (including haemolytic and lipaemic samples) were enriched with 33.3 kBq of [^{3}H]serotonin per ml. After five-fold dilution with water and precipitation of proteins with ethanol, 100 µl aliquots of the ethanolic extracts were withdrawn and counted for radioactivity. The analytical recovery of [^{3}H]serotonin from all sera was 71% (SD 2%) (tab. 1). In a second set of experiments the ethanolic extracts were acetylated with acetic anhydride and 1 ml aliquots were withdrawn for thin-layer chromatography (tab.1). No side reactions of [^{3}H]serotonin with acetic anhydride were observed by either thin-layer chromatography (data not shown) or reversed phase high performance liquid chromatography (fig. 2). The analytical recovery of [^{3}H]N-acetyl serotonin was 93% (SD 3%) (tab. 1).

The remaining 1 ml of acetylated extracts was dried by evaporation under a gentle stream of nitrogen. The residue was carefully redissolved in 500 µl of buffer A and 100 µl aliquots withdrawn for both radioactivity measurements and radioimmunosassay. The generally observed turbidity of the buffered extracts did not interfere with the subsequent radioimmunoassay procedure.

Tab. 1. Analytical recoveries of extraction and acetylation of [^{3}H]serotonin in twenty different sera.

Step	Recovery (%)[a]	
	Mean	SD
Ethanol extraction	71	2
Acetylation[b]	93	3
Buffer solution	93	4

[a] The overall recovery of N-acetylated [^{3}H]serotonin is the product of mean recoveries of extraction and acetylation and was approximately 66%. The final value for serotonin is obtained by multiplying the radioimmunoassay results by 1.5, which corrects for the average 66% recovery.

[b] The recovery of acetylated [^{3}H]serotonin was determined as follows: One ml of acetylated ethanolic extract (1.6 kBq) was enriched with 1 mg of serotonin and 1 mg of N-acetyl serotonin respectively. The solvent was removed under nitrogen and the residue redissolved in 50 µl of ethyl acetate. Preparative thin-layer chromatography was performed on Merck F-254 precoated silica gel plates in solvent systems ethanol/ethylacetate/NH_3 (5+5+1, by vol.) and n-butanol/acetic acid/H_2O (12+3+4, by vol.). Serotonin and N-acetyl serotonin spots were scraped off and counted for radioactivity.

Because of the analytical recoveries of both extraction (71 ± 2%) and acetylation (93 ± 3%) a *constant factor* of 1.5 was introduced to correct the serotonin values of unknown sera for losses during sample preparation (tab. 1).

Endogenous N-acetyl serotonin

The N-acetyl serotonin content of all sera was determined by the same extraction procedure except that the acetylation step was omitted. The levels were 0.03 ± 0.03 µmol/l (0–0.18 µmol observed range). All values in this study are corrected for endogenous N-acetyl serotonin.

Standard curve

A typical standard curve is shown in figure 1. The standard curve is sigmoid over the range of 0.12–9.26 pmol/tube. As the sample preparation includes a 50-fold dilution of sera, the standard curve actually covers the range of 0.06–4.63 µmol/l serum.

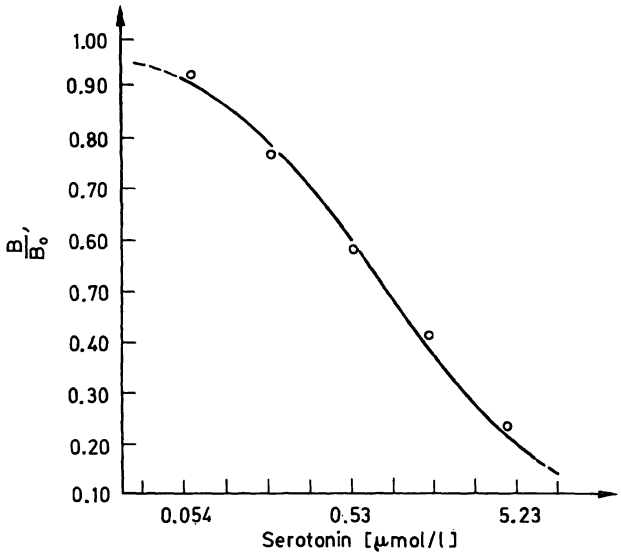

Fig. 1. Typical standard curve for the radioimmunoassay of N-acetylated serotonin.
The abscissa represents the concentrations of N-acetyl serotonin in the standards corrected for the fifty-fold dilution of serum during sample preparation.

Minimum detectable concentration

The within-day CV of the zero-dose standard is < 2%. Therefore, the minimal detectable concentration is the concentration at which B/B_0 is approximately 0.96 (1–2 CV), or 0.012 µmol/l serum (25 fmol/tube).

The sensitivity of the assay is enhanced 20-fold if 100 µl of normal sera instead of 1 : 5 diluted sera are extracted and the acetylated extracts are redissolved in 250 µl instead of 1 ml of buffer A. This "enhanced sensitivity extraction" procedure uses the same standard, isotope, and antibody solutions except that the standard curve now covers the range of 3–231 nmol/l (0.6 nmol/l serum minimal detectable concentration).

Specificity

The specificity of antibody for N-acetyl serotonin is demonstrated in figure 2. Reversed phase high pressure liquid chromatography of [^{3}H]serotonin-enriched acetylated human serum extracts reveals only one peak detectable by radioimmunoassay, which also contains more than 90% of total radioactivity. The retention time of this peak is identical with that of authentic N-acetyl serotonin.

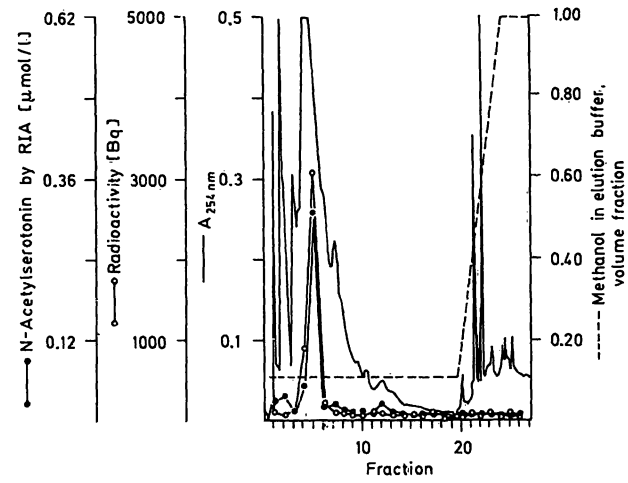

Fig. 2. High performance liquid chromatography of a human serum extract enriched with [^{3}H]serotonin. Localization of N-acetyl serotonin by RIA.
One ml of human serum was enriched with 33.3 kBq of [^{3}H]serotonin per ml, and serotonin was extracted and acetylated as described in Materials and Methods. In contrast to the RIA procedure, the acetylated extract was redissolved in water/methanol (9 + 1, by vol.) and submitted to isocratic reversed phase high performance liquid chromatography on a PEP RPC column (Pharmacia, Freiburg, F. R. G.). Fractions of 1 ml were collected for the determination of radioactivity and N-acetyl serotonin (by RIA).

The inhibition, by structurally related compounds, of the binding of ^{125}I-*Bolton-Hunter*-serotonin conjugates by antibody is shown in table 2. The data show that the acylation of the free amino group of serotonin is a prerequisite for the specific recognition of the 5-hydroxindole nucleus by antibody (due to the "bridge" effect of the immunogen recognition!).

Tab. 2. Inhibition by serotonin analogues of binding of [^{125}I]serotonin by antibody.

Compound	Cross-reactivity (%)[a]
I-*Bolton Hunter*-serotonin conjugate[b]	100
N-Acetyl serotonin	98
N-Propionyl serotonin	34
Serotonin	<0.01
Melatonin	<0.01
5-Methoxytryptamine	<0.01
5-Hydroxy-3-indolylacetic acid	<0.01
Trpytamine	<0.01
N-Acetyltryptamine	<0.01
Tryptophan	<0.01
Tyrosine	<0.01
5-Hydroxytryptophan	<0.01
5-Methoxytryptophan	<0.01
L-Dopa	<0.01
Dopamine	<0.01
N-Acetyl dopamine	<0.01

[a] The potency of serotonin analogues to inhibit 50% of the ^{125}I-*Bolton Hunter*-serotonin conjugate binding by antibody. The non-radioactive I-*Bolton Hunter*-serotonin conjugate is set at 100% and the cross reaction of serotonin analogues is by weight. The radioimmunoassay was performed as described under "Material and Methods".

[b] N-(3-(4-hydroxy-3(5)-iodophenyl)-propionyl) serotonin.

Recovery

Thirty different sera (including haemolytic and lipaemic probes) were tested. Aliquots were enriched with 0.62 µmol/l serotonin, and both normal and enriched sera were prepared for radioimmunoassay. The values thus obtained were corrected for losses during sample preparation (*factor 1.5*) and the differences between enriched and normal sera determined. Analytical recovery was 99% (SD 13%).

Precision

Sera were stored in aliquots at −20 °C and used to estimate the intra- and inter-assay precision. The intra-assay CV at the levels of 0.9 ± 0.06 µmol/l and 1.7 ± 0.18 µmol/l was 6.8% and 11% for 20 consecutive determinations respectively.

Tab. 3. Reference intervals for serotonin in serum of healthy donors.

	Men	Women
Number	59	59
Age (years)	18–60	19–55
Serotonin concentration (µmol/l)		
Mean	1.18	1.37
SD	±0.56	±0.63
Observed range	0.19–2.8	0.45–3.43

The inter-assay CV for 10 consecutive assays at a level of 0.49 ± 0.049 µmol/l was 10%, and 25% at a level of 2.16 ± 0.53).

Reference intervals

The observed range for the concentration of serotonin in serum of healthy male and female probands is shown in table 3. All values are corrected for endogenous N-acetyl serotonin.

Discussion

We have determined serotonin by radioimmunoassay, because this method is faster, more sensitive and more specific than previously described methods (9–15). The determination of N-acetylated serotonin has several additional advantages: firstly, antigen, isotope and N-acetylserotonin, are N-acyl homologues of serotonin; secondly, there is no cross-reactivity of the antibody with endogenous serotonin; thirdly, the need of acetylation prior to radioimmunosassay strictly limits the number of possibly cross-reacting compounds to amines. Endogenous N-acetyl serotonin, a metabolite of serotonin (21), which could falsify the assay is detectable by direct comparison of acetylated and nonacetylated extracts.

The major "compartment" for serotonin in the blood is in the platelets, from which it is released during blood coagulation (22). It has previously been shown that the concentration of serotonin in plasma is only on the average 2% of that in serum. The addition of monoamine oxidase inhibitors (23) and an inhibitor of the platelet uptake of serotonin (24) to the blood specimen just after sampling increased the yield of serotonin only by about 10%, whereas inducers of the platelet release reaction had no detectable effect on the serum concentrations of serotonin (15).

The concentration of serotonin in serum is a measure of the serotonin content of the platelets, provided that appropriate precautions are taken. We find that blood coagulation in the presence of fibrin-adsorbing surfaces (serum Monovette) is a prerequisite to a constant recovery of platelet serotonin, because clotted fibrin may cause erroneous results (data not shown). Blood samples should not be stored at elevated temperatures and no longer than two hours.

Fluorometrically, the serotonin content of platelets has been found to be about 700 ng/10^9 platelets (15, 25, 26). These results lead one to expect a concentration of serotonin in serum of about 1.5 µmol/l. We obtain a quite similar value for women, whereas

the value for men is about 15 percent lower. A similar sex-related difference has also been observed by other authors (15, 27). Only one third (15) or one-half (26) of this concentration was found by previously described methods. However, in both these cases, clotted fibrin might have caused the low results.

The present investigation shows that the radioimmunoassay of N-acetylated serotonin is highly sensitive for the analysis of serotonin in a variety of blood samples. The acceptable coefficients of variation exhibited by the method should facilitate the examination and comparison of groups that differ only slightly from one another with respect to serotonin levels.

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