Direct Radioimmunoassay of 6-Sulfatoxymelatonin in Plasma with Use of an Iodinated Tracer

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We describe here a direct radioimmunoassay (RIA) for the determination of 6-sulfatoxymelatonin (aMT6s) in plasma, with iodinated aMT6s as tracer. The aMT6s antiserum was raised in rabbit by immunization with a bovine serum albumin conjugate, giving negligible cross-reactivities for related compounds. The low limit of detection (15 pmol/L) allowed a direct assay that required only a 100-µL plasma sample. Dilutions of plasma and of synthetic aMT6s gave the same parallel response in the RIA. A preliminary study showed a circadian variation in healthy volunteers, with mean concentrations ranging from 52 (at 1600–2100 h) to 378 pmol/L (at 0400 h), whereas this rhythm was abolished in pinealectomized patients. After administration of melatonin orally, or by infusion, the aMT6s concentrations in plasma concorded with previous data on aMT6s production and pharmacokinetics, with aMT6s being cleared from plasma more slowly than melatonin. This assay should have practical application in the development of new pharmaceutical formulations that minimize the hepatic metabolism of melatonin.

Additional Keyphrases: pharmacokinetics · melatonin · circadian pineal activity

Melatonin, the major indole compound synthesized by the pineal gland, is metabolized mainly by hydroxylation at the 6-position (1) and conjugated with sulfate and glucuronide (2). 6-Sulfatoxymelatonin (aMT6s) is the major melatonin metabolite in humans (3).⁴ Similar to melatonin, the concentration of aMT6s in plasma increases at night and can be used as a marker of circadian pineal activity. The first radioimmunoassay of aMT6s in plasma involved a tritiated tracer and needed a relatively large plasma sample (0.5 mL) (4). Because iodinated tracers of higher specific activity allow more-sensitive assays than do the tritiated tracers, we developed an ¹²⁵I RIA for aMT6s in plasma. The analytical and physiological validations are reported here.

Materials and Methods

Materials

¹²⁵I]NaI (IMS 30) was purchased from Amersham International (Amersham, Bucks., U.K.) and Iodogen from Pierce Chemical Co. (Rockford, IL). Bovine serum albumin (BSA; Fraction V) was purchased from IBF (Villeneuve-la-Garenne, France). Chlороsulfonic acid was from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade from Sigma (St. Louis, MO) or Merck (Darmstadt, F.R.G.). The radioimmunoassay buffer (pH 7.5, 50 mmol/L) consisted of 1.3 g of KH₂PO₄, 7.2 g of Na₂HPO₄ · 2H₂O, and 5 g of BSA per liter. The second antibody reagent, purchased from CEA (Saclay, France), consisted of a mixture of polyethylene glycol and an insoluble complex of sheep anti-rabbit gamma-globulins and nonimmunized rabbit immunoglobulins. Plates for thin-layer chromatography (TLC; cellulose or Silica G60, 20 × 20 cm) were obtained from Merck.

aMT6s was removed from pooled heparinized plasma as follows: charcoal (100 g/L) was mixed with plasma for 4 h at 37 °C, then centrifuged (2 h at 8000 × g) and filtered through a 0.45-µm (pore size) filter (Millex; Millipore Corp., Bedford, MA).

The concentration of melatonin in plasma was determined according to the method of Claustrat et al. (5).

Procedures

Synthesis of 6-sulfatoxymelatonin. We synthesized aMT6s according to the method of Leone et al. (6) and Jones et al. (7). In brief, we dissolved 6-hydroxymelatonin in dimethylformamide (DMF), and cooled the solution to 4 °C. We then added slowly a solution of chlorosulfonic acid in DMF at 4 °C. After 30 min at 4 °C, we purified the reaction mixture by passing it through a Florisil column. Using TLC on silica gel, we located and identified, by reference to the solvent front (Rᶠ), the reaction products after separation on the Florisil column. The chromatographic system was butanol/acetic acid/water (4/1/1 by vol). After being sprayed with Prochaska's reagent—a mixture of 10 mL of hydrochloric acid (250 mL/L), 10 mL of formaldehyde (350 mL/L), and 20 mL of ethanol—and heating to 100 °C, aMT6s was visible as a blue spot.

The aMT6s identification (Rᶠ = 0.53) was based on a aMT6s standard extracted from urine from a healthy volunteer who had been given 1 mg of melatonin orally. After TLC analysis, the fractions containing aMT6s were pooled. Removal of the solvent left a white powder, which we recrystallized in a methanol-ethyl acetate mixture and then purified by TLC. Infrared analysis of the purified product gave a spectrum similar to that obtained by Leone et al. (8) for aMT6s isolated from urine.

Synthesis of the immunogen. The synthesized aMT6s was bound to BSA via Mannich's reaction (9). We did

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⁴ Nonstandard abbreviations: aMT6s, 6-sulfatoxymelatonin; BSA, bovine serum albumin; DMF, dimethylformamide; and TLC, thin-layer chromatography.

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not determine the molar ratio of hapten conjugated to protein.

**Production of antisera.** We immunized three rabbits by subcutaneous dorsal injection of immunogen (500 µg per animal) in complete Freund's adjuvant. Booster injections were given by the same route at six-week intervals. We tested blood samples 10 days after each injection.

**Synthesis of 125I-labeled aMT6s.** We iodinated aMT6s directly according to Aldhous and Arendt (10) with the method of Vakkuri et al. (11). This labeling, which involves iodogen as the oxidizing reagent, yields tracer monoiiodinated in position 2. We reduced the time of the reaction to 18 h.

**RIA aMT6s.** To prepare the standards, we diluted in RIA buffer a 6 nmol/L stock solution of aMT6s in methanol to give the following concentrations: 3048, 1524, 762, 381, 190, 95, 47.5, and 23.8 pmol/L. Use of the antiserum at the initial dilution of 30 000-fold in buffer corresponded to a 50% binding of the tracer. We diluted the tracer to ~10 000 counts/min in 100 µL. We compared assayed plasma and quality-control samples directly with a standard curve produced with an equal volume of charcoal-stripped plasma. Plasma samples (100 µL), controls (100 µL), and standards (100 µL, 2.4–305 fmol/tube) were pipetted into plastic tubes. We then added the tracer (100 µL) and the antiserum (100 µL) to the standards, blank, and reference tubes and added buffer to bring the total volume in each tube to 500 µL. After incubation at 4 °C for 18–24 h, bound and free aMT6s were separated by incubation with 1 mL of second antibody reagent for 15 min at 20 °C. After centrifugation (2000 × g) for 15 min at 4 °C, we decanted the supernate and counted the pellet's radioactivity in a gamma-counter (Multigamma; LKB, Bromma, Sweden).

**Analytical Performance**

We evaluated the specificity of the RIA by determining the relative potency of the compounds listed in Table 1. To calculate the relative potency, we divided the quantity of cross-reactants displacing 50% of the antibody-bound 125I-labeled aMT6s by the quantity of aMT6s producing 50% displacement.

We assessed parallelism by comparing the displacement of a nocturnal plasma sample, diluted with charcoal-stripped plasma, with the values on the standard curve. We also assessed whether the plasma dilution curve could be superimposed on the standard curve.

The specific activity of the iodinated aMT6s was assessed by the method of Joseph et al. (12).

To ascertain analytical recovery, we added known amounts of aMT6s to a stock plasma pool, to give final concentrations of 457, 530, and 814 pmol/L, and then assayed.

Eight replicated plasma standard curves, one assay per day, were run to ascertain the precision of the assay. The least detectable concentration was defined as the concentration giving a displacement of 2 SD of counts from maximum binding.

**Physiological Studies**

In all cases, blood was collected into heparinized tubes, and plasma was separated by centrifugation and stored at −20 °C until RIA.

We studied the daily rhythm of aMT6s in four young (ages 22–28 y) healthy volunteers. Blood was collected into heparinized tubes at 2-h intervals during the day and at 1-h intervals during the night.

We also studied, under the same conditions, the 24-h plasma aMT6s profile in four pinealectomized patients and in one pinealectomized patient during and after a 5-h infusion of 20 µg of melatonin (4 µg/h) started at 2000 h. We collected blood samples at 1-h intervals.

Finally, we assayed aMT6s in the plasma of a young healthy volunteer who had ingested 1 mg of melatonin at 0800 h. Blood samples were collected at 10-min intervals.

These study protocols had previously been approved by the National Ethics Committee.

**Results**

**Assay Validation**

The overall iodination yield was 35%. The concentrated tracer (15 mCi/L in methanol at −20 °C) is stable for at least four months. We determined its specific activity to be 720 kCi/mol.

All the rabbits immunized produced antisera after the second immunization. After the fourth immunization, we killed the rabbit showing the highest titer and used the antiserum obtained for the RIA at an initial dilution of 30 000-fold.

The percentage of cross-reactivity of several indoles in this assay was very low (Table 1). Because the compounds occur in such small quantities, we consider the cross-reactivity negligible. A higher cross-reactivity is obtained with 6-chloromelatonin (0.15%), a synthetic compound.

The affinity constant was $0.64 \times 10^{10}$ L/mol, calculated by the method of Scatchard (13).

Plasma collected during the night and diluted with charcoal-stripped plasma gave the same displacement as that of the aMT6s standard curve when we assayed 100-μL samples of plasma directly (Figure 1).

Analytical recoveries of three different aMT6s concen-
Concentrations (457, 530, and 814 pmol/L) were 100 ± 7%, 103 ± 9%, and 109 ± 10%, respectively (mean ± SD, n = 6).

Only 381 pmol of aMT6s per liter was necessary for a 50% displacement of the iodinated tracer. The least detectable concentration of aMT6s was 15 pmol/L (Figure 1).

For an aMT6s concentration in plasma of 300 pmol/L, the intra-assay CV was 8% and the interassay CV was 12%.

Physiological Studies

In healthy men, aMT6s in plasma showed a circadian variation (Figure 2), with mean concentrations ranging from 378 pmol/L at 0400 h to 52 pmol/L at 1600–2100 h (Figure 2e).

The pinealomectomized patients showed a complete abolition of the aMT6s rhythm, with nocturnal concentrations in plasma remaining constantly <33 pmol/L.

Infusion of melatonin into a pinealomectomized patient induced plasma aMT6s concentrations comparable with those observed in healthy subjects (Figure 3).

Oral administration of 1 mg of melatonin produced a dramatic increase in aMT6s concentrations, similar to the increase in melatonin concentrations, but with a 30-min lag time; the clearance rate of aMT6s from plasma was slower than that of melatonin (Figure 4).

Discussion

In this paper, we describe a sensitive (detection limit = 381 pmol/L) and specific RIA of aMT6s and detail its technical validation. The good sensitivity we observed is a direct consequence of the higher specific activity of iodinated tracer compared with the tritiated tracer (720 vs 50 kCi/mol). We observed parallelism between the plasma dilution curve and the standard curve, and obtained good analytical recovery. Undetectable aMT6s concentrations in plasma in the pinealomectomized patients and the concentrations observed after melatonin infusion in one patient also support our conclusions regarding assay specificity. This assay has the advantage of being direct and requires only a small plasma sample: 0.1 mL vs 0.5 mL of plasma for the tritiated tracer. In addition, the use of an iodinated tracer avoids the preparation of [3H]aMT6s by the biological transformation of [3H]melatonin and cumbersome liquid scintillation counting. Thus, many samples can be rapidly and conveniently processed. The results obtained agree with those of other authors (14). The night/day concentration ratio in plasma was >7, confirming the use of aMT6s as a marker of the circadian pineal activity.

We recorded higher diurnal concentrations in plasma

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Fig. 1. Parallelism between aMT6s standard curve in charcoal-stripped plasma (●) and increasing amounts of nocturnal human plasma (10, 20, 30, 50, 70, and 100 μL) serially diluted with charcoal-stripped plasma (+).

Fig. 2. (a, b, c, d): Individual plasma aMT6s (●) and melatonin (+) profiles observed in four healthy male volunteers, sampled over 24 h; (e): mean of plasma profiles a, b, c, and d.

Fig. 3. Basal plasma aMT6s profile in a pinealomectomized patient (●) and plasma aMT6s profile (+) before, during, and after melatonin infusion, sampled for 14 h in the same patient. Melatonin infusion (20 μg) was begun at 2000 h and stopped at 0100 h.
in the normal subjects than in the pinealomectomized patients, possibly because of low diurnal pineal activity in the former group, and a slow clearance of aMT6s from plasma. This slow clearance from plasma was also observed after melatonin infusion, with significant aMT6s concentrations still being recorded 6 h after the hormone administration had been stopped. Similarly, 3 h after oral administration, aMT6s concentrations in plasma remained high, whereas melatonin concentrations had returned to baseline values.

The presence of aMT6s in plasma and the delay observed in the onset of the aMT6s profile compared with the melatonin profile are a consequence of the liver metabolism. Because a strong first-pass hepatic metabolism after oral administration of melatonin (15) leads to a rapid decline of the concentrations in plasma, this direct RIA, used with the assay of melatonin, should be valuable in the development of new parenteral pharmaceutical formulations that minimize liver uptake and ensure a sustained release of the hormone.

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