Development and validation of a melatonin radioimmunoassay using radioiodinated melatonin as tracer

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Abstract. A new radioimmunoassay (RIA) using $^{125}$I-melatonin as tracer for determination of melatonin in biological fluids has been developed. Melatonin antisera were raised in rabbits by immunization with bovine thyroglobulin conjugate of N-acetyl-5-methoxytryptophan. Two high-affinity and specific antisera were obtained. Unlike in previous studies melatonin was radioiodinated directly. Iodo-Gen was used as the oxidant. Radioiodinated melatonin was purified by TLC for use in RIA. Melatonin was extracted from plasma, serum and urine samples (1 ml) with chloroform. Using the extraction the sensitivity of the RIA method was 18 fmol/ml of original sample. Plasma, serum and urine extracts diluted parallelly with synthetic melatonin in RIA. HPLC analysis of plasma and serum extracts showed only one immunoreactive peak co-eluting with synthetic melatonin. Majority of urine immunoreactivity co-eluted with synthetic melatonin, but 7–23% contaminating immunoreactivity was also observed. Daytime values for rat plasma, human serum and urine melatonin were 30–60, 20–40 and 50–120 fmol/ml and the respective night values were 160–300, 180–370 and 230–470 fmol/ml. Thus a characteristic diurnal rhythm of melatonin was observed in all cases. The urinary excretion of immunoreactive melatonin during the day was 3–9 and during the night 11–28 pmol/h. Thus we have developed a specific and valid RIA method for the determination of plasma and serum melatonin. Despite the incomplete specificity of the RIA for urine determinations, a clear diurnal rhythm for urine melatonin was observed. The distinct advantage of the utilization of $^{125}$I-melatonin as tracer is that the costly and cumbersome scintillation counting can be avoided.

The first quantitative determination of melatonin utilized the effect of melatonin on dermal melanophores of tadpoles (Lerner & Wright 1960). Despite great specificity the bioassay was too tedious and insensitive for routine use. Later, gas chromatography-mass spectrometry (Pelham et al. 1972; Smith et al. 1976) and RIA (Arendt et al. 1975; Levine & Riceberg 1975) methods have become available for melatonin determinations (reviewed by Waldhauser & Wurtman 1983).

Most of the RIAs hitherto developed for melatonin use tritiated melatonin as tracer. A radioiodinated tryptamine derivative is used as tracer only in two RIAs (Rollag & Niswender 1976; Geffard et al. 1982). We observed in iodination experiments of a melatonin derivative that melatonin itself can be iodinated. This paper presents the preparation of $^{125}$I-melatonin and its application in the RIA of melatonin of rat plasma, human serum and urine.

Material and Methods

Bovine thyroglobulin, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl, melatonin and indole analogues except 5-methoxytryptamine hydrochloride and 5-hydroxy-D,L-tryptophan (Aldrich, B 2340, Beerse, Belgium) were purchased from Sigma Chemical Company, St. Louis, Mo, USA. Freund’s complete and incomplete adjuvant from Difco Laboratories, Detroit, Mi, USA, Na$^{125}$I (IMS 30) from The Radiochemical Centre Amersham, Buckinghamshire, UK, N-(2-aminoethyl-2-3H)-acetyl-5-methoxytryptamine (PH)melatonin with a specific activity of 32.3 Ci/mmol from New England Nuclear, Boston, Mass., USA, Sephadex G-50 from Pharmacia Fine Chemicals, Uppsala, Sweden, chromatography columns from Bio-Rad Laboratories, Richmond, Ca, USA, silica gel sheets No. 13179 from Eastman Kodak Co., Rochester, N.Y., USA, 1,3,4,6-tetrachloro-3a, 6a-diphenylglycoluril (Iodo-Gen®) from Pierce Chemical

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Co., Rockford, IL, USA, Lumagel from Lumac B.V., Schaesberg, Holland. Chloramine-T, chloroform, ethyl acetate, ammonium sulphate and all ordinary chemicals were obtained from E. Merck AG, Darmstadt, Germany. The tubes with dimensions of 1 x 7 cm used in assays were made of polystyrene.

Production of antisera

N-acetyl-5-methoxytryptophan used as hapten was prepared according to the method of Berg et al. (1929–1930) and coupled to bovine thyroglobulin by carbodiimide according to the method of Arendt et al. (1975). The immunogen was purified by gel filtration on a 1.5 x 20 cm Sephadex G-50 column eluted with 0.15 mol/l NaCl. Spectrophotometrical analysis at 280 nm showed that 26% of the hapten was coupled to bovine thyroglobulin giving the molar ratio of the hapten to protein carrier 210:1.

Five rabbits were injected sc in the back at multiple sites with 0.8 mg of N-acetyl-5-methoxytryptophan-thyroglobulin conjugate in 0.15 mol/l NaCl (0.8 ml) emulsified with Freund's complete adjuvant (0.8 ml). Five booster injections (0.4 mg conjugate emulsified with Freund’s incomplete adjuvant) were given 3 weeks later and then at monthly intervals following the primary immunization. Blood samples for testing the production of antibodies were withdrawn 10–14 days after each booster injection and screened for binding of [3H]melatonin and [125I]melatonin. The antisera obtained after the fourth booster were taken for further characterization.

Iodoindination of melatonin

Iodo-Gen (1 µg in 10 µl of chloroform) was added to an Eppendorf tube and the chloroform was allowed to evaporate to dryness. Melatonin (10 µg in 10 µl of 0.05 mol/l sodium phosphate, pH 6.0) and Na[125I] (7.4 mBq in 2 µl of NaOH solution) were added simultaneously. After 1 min the reaction solution was extracted with chloroform (100 µl) and the chloroform phase subjected to TLC using silica gel sheet (20 x 20 cm) with ethyl acetate as solvent. Following TLC the sheet was sectioned to 1 cm segments and their radioactivity and immunoreactivity (after elution with 2-propanol) were determined. Chloramine-T in amounts 10 ng–100 µg was also used with different exposure times (15 s–15 min) according to the method of Greenwood et al. (1963).

Blood and urine samples

For plasma samples outbred male Sprague-Dawley rats (200–300 g) maintained in a room lit between 07.00 and 19.00 h (12L:12D) were bled at 02.00, 06.00, 10.00, 14.00, 18.00 and 22.00 h (n = 4–6) by decapitation (in the scotophase using a dim red light) into heparinized test tubes. Human sera were obtained at 01.00, 07.00, 13.00 and 19.00 h from healthy male volunteers aged 20–40 years. Human urine was collected at 01.00, 07.00, 13.00 and 19.00 h. In addition spot urine samples (4–6 ml) were taken 10–15 min after each miction.

Extraction and radioimmunoassay

Plasma, serum and urine samples (1 ml) were extracted with chloroform (4 ml) vortexing lightly (30 s). After centrifugation at 4°C (20 min 1400 x g) the aqueous phase was discarded and the chloroform phase washed with distilled water (2 ml). After the second centrifugation (10 min 1400 x g at 4°C) the aqueous phase was discarded and the chloroform phase was evaporated in house vacuum. The residue was resuspended for RIA in 220 µl of PBS-gelatin buffer (0.2 mol/l sodium phosphate, 0.15 mol/l NaCl, 0.05% gelatin, 10 mmol/l EDTA and 0.15% NaN₃, pH 6.0). In the extraction procedure the recoveries of 40 pg melatonin added to 1 ml of rat plasma, human serum and human urine were 88 ± 12, 94 ± 12 and 92 ± 9% (mean ± sD, n = 5).

Standards or samples, antiserum (a-MT-K1 in routine use) and tracer (each 100 µl) diluted with PBS-gelatin buffer were incubated for 12–20 h at 4°C. The immunocomplex was precipitated by 1.0 ml of 3 mol/l ammonium sulphate and counted in a 12-channel gamma counter (Multigamma, LKB-Wallac, Turku, Finland). The [3H]melatonin precipitates were dissolved in distilled water (0.3 ml) and mixed with Lumagel (2 ml) for liquid scintillation counting (Ultrobeta 1210, LKB-Wallac, Turku, Finland).

![TLC profile](image)

**Fig. 1.**

TLC profile for purification of radioiodinated melatonin developed on silica gel with ethyl acetate. The mobilities of synthetic melatonin (MT) and immunoreactive tracer [125I-MT] are shown.
Table 1.
The titre and sensitivity values for different melatonin antisera as determined with \(^3\text{H}\)-melatonin and \(^{125}\text{I}\)-melatonin tracers.

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Titre of antiserum [^\text{[3H]}\text{melatonin}]</th>
<th>Titre of antiserum [^\text{[125]}\text{I}\text{melatonin}]</th>
<th>Sensitivity [^\text{[3H]}\text{melatonin}]</th>
<th>Sensitivity [^\text{[125]}\text{I}\text{melatonin}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>a-MT-K1</td>
<td>1: 4500</td>
<td>1: 15 000</td>
<td>28</td>
<td>8</td>
</tr>
<tr>
<td>a-MT-K2</td>
<td>1: 800</td>
<td>1: 2600</td>
<td>2600</td>
<td>1300</td>
</tr>
<tr>
<td>a-MT-K3</td>
<td>1:47 000</td>
<td>1:170 000</td>
<td>52</td>
<td>22</td>
</tr>
<tr>
<td>a-MT-K4</td>
<td>1: 7200</td>
<td>1: 440</td>
<td>37</td>
<td>470</td>
</tr>
<tr>
<td>a-MT-K5</td>
<td>1: 2100</td>
<td>1: 7800</td>
<td>230</td>
<td>100</td>
</tr>
</tbody>
</table>

Titre = dilution of antiserum at 40% binding of tracer. Sensitivity (fmol/tube) = intercept of maximal binding – 2 SD.

Results

TLC of chloroform extract of the iodination mixture gave several radioactive peaks (Fig. 1), of which only one was immunoreactive. The \(R_f\) value of this proposed \(^{125}\text{I}\)-melatonin was 0.54 and that of melatonin 0.38. The iodination efficiency determined as percentage amount of immunoreactive tracer of total radioactivity was 1–4% with the chloramine-T method regardless of the amount of chloramine-T or the iodination time and 20–50% with the Iodo-Gen method.

As seen in Table 1 the titres used and sensitivities of the assays obtained with antisera a-MT-K1, a-MT-K2, a-MT-K3 and a-MT-K5 were 2–4 times higher using \(^{125}\text{I}\)-melatonin than \(^3\text{H}\)-melatonin as tracer. On the other hand the sensitivity for antisera a-MT-K4 was over 10 times higher with \(^3\text{H}\)-melatonin than with \(^{125}\text{I}\)-melatonin. Antisera

Table 2.
Cross-reaction of various analogues of melatonin and other related compounds with melatonin antisera a-MT-K1, a-MT-K3 and a-MT-K4 determined at 50% displacement of \(^3\text{H}\)-melatonin and \(^{125}\text{I}\)-melatonin (only a-MT-K1 and a-MT-K3) on weight basis.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Percentage cross-reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a-MT-K1</td>
</tr>
<tr>
<td>Melatonin</td>
<td>100</td>
</tr>
<tr>
<td>6-hydroxymelatonin</td>
<td>0.8</td>
</tr>
<tr>
<td>(N)-acetyltryptophan</td>
<td>0.4</td>
</tr>
<tr>
<td>6-methoxyharmalan</td>
<td>0.2</td>
</tr>
<tr>
<td>5-methoxytryptophan</td>
<td>0.01</td>
</tr>
<tr>
<td>(N)-acetylserotonin</td>
<td>0.004</td>
</tr>
<tr>
<td>5-methoxytryptophol</td>
<td>0.004</td>
</tr>
<tr>
<td>5-methoxyindole acetic acid</td>
<td>0.003</td>
</tr>
<tr>
<td>5-methoxytryptamine</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>5-hydroxytryptophan</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Serotonin</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>5-hydroxytryptophol</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
a-MT-K1, a-MT-K3 and a-MT-K4 were characterized with respect to specificity (Table 2). Cross-reactivity determined with [3H]melatonin and [125I]melatonin (a-MT-K1 and a-MT-K3) were fully comparable with each other. Parallel displacement was observed between synthetic melatonin and rat plasma, human serum and urine extracts (Fig. 2). In HPLC studies the immunoreactive melatonin in plasma, serum and urine extracts (Fig. 3) co-eluted with synthetic melatonin. In HPLC profile of human urine extracts a minor peak eluting before melatonin was detectable (Fig. 9C). This corresponded to 7–23% of total immunoreactivity. In rat plasma, human serum and human urine melatonin concentrations were higher during the scotophase than during the photophase (Fig. 4). Urinary excretion of melatonin has the same diurnal rhythm: 3–9 pmol/h in the photophase and 11–28 pmol/h in the scotophase. Intra- and inter-assay coefficients of variation were 6.7–9.5 and 9.8–12.5%, respectively, at the dose level 110–440 pmol/l. Non-specific binding was 7–9%.

Discussion

In the RIA described in this paper for determination of melatonin from biological fluids the immunogen was prepared by coupling the synthesized N-acetyl-5-methoxytryptophan to bovine thyroglobulin with carbodiimide according to the method of Arendt et al. (1975). All 5 rabbits produced melatonin antibodies demonstrating suitability of the coupling method. Thoresen (1978), using the
same method, also obtained a specific antiserum for melatonin.

We found in this study that melatonin can be directly iodinated. Using Iodo-Gen as the oxidant a yield of 20–50% of iodinated melatonin was obtained, but only a yield of 1–4% with chloramine-T. The ability of the $^{125}$I-tracer to bind to highly specific melatonin antiserum, and the reversibility of the binding by small quantities of melatonin strongly suggest that the tracer is $^{125}$I]melatonin (since the submission of this paper we have established by MS and NMR that the iodinated product is 2-iodomelatonin, Vakkuri et al., submitted). In spite of the incorporation of the bulky radiiodine to melatonin no steric hindrance was seen in the immunologic reaction between four of the five antisera and $^{125}$I]melatonin, which can be concluded from titre and sensitivity values for antisera determined with $[^3]$H]melatonin and $^{125}$I]melatonin. Because antiserum a-MT-K4 recognized $^{125}$I]melatonin only weakly, it is suggested that in this case radioiodine had been incorporated to the antigen determinant. Thus we have been able to utilize $^{125}$I]melatonin as tracer in melatonin RIA, which has improved the RIA method with respect to both antiserum titre and sensitivity. This is probably due to a greater specific activity of $^{125}$I as compared with $^3$H. Moreover, expensive and cumbersome scintillation counting could be rejected. Since radioiodination is a routine procedure in many laboratories, the direct radioiodination of melatonin presented here may increase the practicality of RIA methods developed for melatonin.

The antisera a-MT-K1 and a-MT-K3 were shown to be very specific. Both antisera had detectable but only negligible cross-reaction against N-acetyl- and 5-methoxytryptophan. This indicates that N-acetyl and 5-methoxy groups are included in the antigen determinant. The highest cross-reaction was against 6-hydroxymelatonin, the principal metabolite of melatonin. This does not, however, invalidate plasma or serum determinations of melatonin as seen in HPLC profile of plasma and serum extracts. In HPLC of urine extracts an earlier eluting immunoreactive peak (which is not 6-hydroxymelatonin) is responsible for 7–23% of total immunoreactivity.

Parallel displacement between synthetic melatonin and extracts of rat plasma, human serum and urine, and similar elution of plasma, serum and the bulk of the urine immunoreactive material with synthetic melatonin in HPLC provide validation for the present RIA method. Diurnal rhythm of melatonin with low day and high night levels in rat plasma, human serum and urine are in agreement with previous gas chromatography-mass spectrometry and RIA studies. Quantitatively, rat plasma melatonin levels of this study are similar to those of Ozaki et al. (1976), Wilson (1978), Grotta et al. (1981) and Kawashima & Nagakura (1982). However, Pang et al. (1980) have reported 10 and Geffard et al. (1982) 1000 times higher levels for rat plasma melatonin. The reason for these discrepancies is not known. Different levels have also been reported for human serum melatonin, especially for the low day levels. In this study melatonin concentrations in human serum are somewhat lower than those reported by Arendt et al. (1975, 1977), but agree with the results of Smith et al. (1977) and Kennaway et al. (1977) and with the mean results determined by gas chromatography-mass spectrometry (Wilson et al. 1977; Lewy &
Markey 1978). With respect to urinary excretion of melatonin our results are similar to those presented by Lynch et al. (1975), Jimerson et al. (1977) and Lang et al. (1981). The present melatonin RIA is valid for use in measurement of melatonin in biological fluids. The method has the advantage of being simpler and more sensitive than most previous melatonin RIAs based on 3H-tracer.

Acknowledgments
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References