Direct Enzyme-Linked Immunosorbent Assay and a Radioimmunoassay for Melatonin Compared

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A novel commercially available ELISA for direct measurement of melatonin concentration in serum was evaluated and compared with an RIA routinely used in our laboratory. The direct ELISA is technically simpler, requires a smaller sample volume (0.1 mL), and compares well with RIA in terms of stability of the calibration curve and intra- and interassay CVs. Correlation with RIA measurements is, however, suboptimal \( y = 0.39x + 56; r = 0.65, P<0.001; n = 138 \), which may be due to a serum effect, as evidenced by dilution studies. Furthermore, the detection range of the ELISA does not cover the physiological daytime melatonin concentrations in humans. Adding an extraction and 10-fold concentration step shifted the detection range of the ELISA to include low physiological concentrations as well. Correlation with RIA measurements also improved significantly \( y = 0.97x-23; r = 0.95, P<0.001; n = 105 \), probably due to removal of the serum effect. Although extraction increases the required sample volume (1.5 mL), work load, and procedure time, this step is necessary for the ELISA to compete successfully with RIA.

Indexing Terms: pineal gland/biorhythm regulation/intemethod comparison

Melatonin, the major hormone of the pineal gland, is involved in the regulation of circadian and annual biorhythms in seasonal breeders through adjustment of the endogenous biological clock to the diurnal and seasonal photoperiodic cycles (7). In such species it also regulates the onset of sexual maturation and seasonal reproductive activity (2, 3). The potential role of melatonin in humans is still unclear and currently under intense investigation. Depression, seasonal affects disorder, schizophrenia, sleep and sleep disturbances, aging, human sexual maturation, and regulation of the immune system have all related to melatonin (3-5). However, the lack of a simple, sensitive, and reliable method for measuring melatonin has been an obstacle to the elucidation of its physiological and pathological role (6-8).

After initial attempts with bioassay, gas chromatography-mass spectrometry for quantifying melatonin in body fluids was tried, followed by HPLC (9-11). The major limitations to these techniques were expense, low throughput, extensive sample work-up before assay, a need for sophisticated equipment, and technical expertise by the analyst (6, 7). With the raising of antiserum to melatonin in the mid-1970s, several laboratories, including ours, developed RIAs of melatonin and refined this technique to achieve an acceptable degree of specificity, sensitivity, and precision (12-16). RIA is currently the method of choice for most laboratories concerned with pineal function (6-8). However, the need for radioisotopes is an inherent disadvantage of RIA, precluding its utilization in laboratories that are not equipped for handling radioactive materials.

ELISA techniques offer a nonradioactive method for melatonin measurement that—by allowing the direct use of serum—is simpler, faster, and more economical than most RIAs. Various ELISAs for melatonin have recently been developed (17, 18), one even allowing direct use of human serum (Bühlmann Laboratories, Schönenbuch, Switzerland). To determine the suitability of this ELISA for routine application, we assessed its major operational characteristics, i.e., stability, sensitivity, reproducibility, accuracy, and ease of handling.

Materials and Methods

Clinical Specimens

Blood specimens were collected from healthy drug-free individuals at the Children’s Hospital of the University of Vienna. After coagulation, serum was separated by centrifugation at 1200 g for 10 min. Sera were frozen and stored at \(-20^\circ\text{C}\) until thawed for melatonin assay. Informed consent was obtained from all subjects and (or) their parents, and the guidelines of the Declaration of Helsinki 1975 were followed.

Reagents

The melatonin ELISA kit was purchased from Bühlmann Laboratories. Coated plates, calibrators from 43 to 8610 pmol/L (10–2000 ng/L), blanking reagents, rabbit antiserum to melatonin, supplemented sera as internal quality control (IQC), enzyme label buffer, buffered peroxide solution, and stop solution (H₂SO₄, 1.5 mol/L) were included, ready to use. The wash buffer was supplied as a 10-fold-concentrated solution, and melatonin-peroxidase conjugate and peroxidase substrate were in lyophilized form. (For details see the user’s manual of the ELISA kit, Bühlmann Laboratories.)

Melatonin antiserum (R-158) (19) was obtained from CID-Tech, Hamilton, Ontario, Canada; [³H]melatonin and Biofluor scintillation fluid were from New England Nuclear, Boston, MA. Melatonin, RIA-grade bovine
serum albumin, and Trizma base (Tris) were purchased from Sigma Chemical Co., St. Louis, MO. Chloroform (for chromatography) and all other chemicals (analytical grade) were from Merck, Darmstadt, Germany.

Apparatus

The absorbance of wells in ELISA microtiter plates was determined with a spectrophotometer (Bio-Rad 2550 EIA Reader; Bio-Rad, Hercules, CA). Chloroform containing extracted melatonin was evaporated in a heated evacuated centrifuge (Univap 150 H; Uni-equip, Martinsried, Germany), and the bound radiation in the RIA was quantified in a beta counter (Beta LS 8100; Beckman Instruments, Fullerton, CA).

Procedures

Melatonin ELISA. The melatonin concentration in our samples was measured in duplicate with the commercial ELISA kit, which uses goat anti-rabbit Ig-coated plates and rabbit antiserum to melatonin, raised and previously described by Manz et al. (20). The competitive binding of melatonin in samples vs peroxidase-conjugated melatonin aliquots added to each well is measured by reading the absorbance after a chromogenic reaction according to the manufacturer’s directions. The nonspecific binding is accounted for by using a blanking reagent.

Each well of the goat anti-rabbit-Ig-coated plate was filled with 50 μL of rabbit antiserum to melatonin and 50 μL of either calibrators, IQC, or specimens in duplicate. After an incubation period of 16 h at 2–8°C, 25 μL of peroxidase-conjugated melatonin was added to each well, and the plate was incubated for another hour at 2–8°C. The plates were washed four times with wash buffer and subsequently filled with 100 μL of fresh enzyme substrate solution per well. After 20 min of incubation, the reaction was stopped by adding 50 μL of stop solution, and the absorbance at 492 nm was determined immediately. Melatonin concentration was calculated from the absorbance readings by a computer program that uses a four-parameter equation to generate the calibration curve.

Extraction of melatonin for ELISA. Melatonin was extracted into 8 mL of chloroform from 1.5 mL of serum mixed with 1 mL of NaOH (1 mol/L) in a disposable glass tube. The aqueous phase was removed after vortex-mixing for 10 s and centrifuging at 1000g for 20 min. The tubes were left overnight at room temperature for serum residues to dry out and adhere to the tube wall. The organic phase was then transferred to a disposable 13 × 100 mm glass tube and the chloroform was evaporated in a heated evacuated centrifuge at 40°C. The dry residue was dissolved in 150 μL of an aqueous solution containing NaCl (0.154 mol/L) and RIA-grade bovine serum albumin (3.5 g/L). Lipids were removed by adding 1 mL of petroleum benzine to each tube, vortex-mixing, and centrifuging as described above. Petroleum benzine residues were allowed to evaporate by standing at room temperature for >2 h. The melatonin concentration was increased 10-fold by this extraction procedure. Duplicates (50-μL) of each extracted specimen were used for measuring melatonin by ELISA in the same way as for unextracted serum samples in the direct ELISA.

Melatonin RIA. Melatonin concentration was measured with an RIA developed in our laboratory as described previously (16). NaOH (0.5 mL of 1 mol/L solution) was added to 1 mL of serum, and the melatonin was extracted into 5 mL of chloroform, the extract being separated and dried as described above for the ELISA. We then dissolved the residue in 0.55 mL of a buffer containing (per liter) NaCl (0.14 mol), Tris (0.01 mol), sodium azide (15.4 mmol), and gelatin (5 g) and washed the solution with 1 mL of petroleum benzine. Calibrator or extract (0.5 mL) was mixed with 100 μL of antiserum at 1:18 000 dilution and 100 μL of [3H]melatonin (~1750 counts/min), both diluted in a buffer containing (per liter) NaCl (0.14 mol), Tris (0.01 mol), sodium azide (15.4 mmol) and gelatin (15 g). The mixture was vortex-mixed and incubated at 35°C for 60 min. After addition of 1 mL of saturated ammonium sulfate, the mixture was incubated overnight at 4°C and centrifuged at 2000g for 1 h. The precipitate was dissolved in 200 μL of 0.1 mol/L NaOH and its radioactivity counted.

Statistical Methods

Within- and between-run CVs, SD, and SEM were calculated as described by Krouwer and Rabinowitz (21). The slope of the calibration curves was calculated after logit/log transformation of B/B0 as described by Rodbard (22). Analysis of variance with split-plot design was used to assess any difference between the slope of the ELISA and RIA calibration curves (23). To compare the agreement between measurements by ELISA and RIA, we calculated the correlation coefficient (r) and linear regression statistics.

Results

Direct Melatonin ELISA

Stability, slope, and range of calibration curves for ELISA and RIA. Mean (and SD) melatonin calibration curves generated from 15 and 10 individual calibration curves by ELISA and RIA, respectively, are depicted in Fig. 1. The average SDs over the entire curves were similar for ELISA and for RIA: 3.82 (SEM 0.53) and 4.24 (SEM 0.29), respectively. The slope of the typical calibration curve (1.4 vs 2.0) and the range of the 80% effective dose (ED80) to the 20% effective dose (ED20) differed considerably [135–>8610 vs 35–1205 pmol/L (32–>2000 vs 8–280 ng/L)].

Precision of melatonin measurements by direct ELISA and RIA. The precision of the direct ELISA was judged first by its within-assay CV, which was assessed by repeated measurements in serum samples supplemented with different amounts of melatonin in a single run. Fig. 2 shows the within-assay precision profiles, defined as a plot of the CV against the concentration
Fig. 1. Two mean calibration curves (±SD) calculated from individual calibration curves generated either by ELISA (○, n = 15) or by RIA (●, n = 10).

Note the difference in range and slope between the curves but the similarity of the SD values at each concentration.

(24) for direct ELISA in comparison with RIA, the latter having been elaborated earlier in our laboratory (25). The lower and upper cutoff limits yield the working range in which meaningful measurements can be obtained (26); we defined the limits as the concentrations at which the CV is <15%. This yields a range from 52 to 710 pmol/L (12–165 ng/L) for RIA and 168 to >6460 pmol/L (39–1500 ng/L) for direct ELISA.

The day-to-day variation was examined by calculating interassay CVs for repeated measurements of three supplemented serum samples in different runs; our previously published data for RIA (25) are included for comparison (Table 1). The between-assay CVs for the ELISA estimated in our laboratory are very close to the data provided by the manufacturer: 11% at melatonin concentration of 2200 pmol/L (511 ng/L); 13% at 1300 pmol/L (302 ng/L); and 23% at 270 pmol/L (63 ng/L).

Parallelism of the calibration curve and serum dilution curves of the direct ELISA. Pooled daytime serum [1 mL; <35 pmol/L (<8 ng/L)] supplemented with melatonin to 12,920 pmol/L (3000 ng/L) was serially diluted either with assay buffer or with the pooled serum to evaluate assay cross-reactivity. In previous examinations of the antiserum we used, no cross-reactivity to numerous indoles was observed (20). Dilution with assay buffer resulted in a curve that was superimposable on the calibration curve, thus indicating lack of cross-reactivity. However, dilution with serum resulted in a curve that ran basically parallel to the calibration curve but below it, with a tendency to growing deviation at lower concentrations (Fig. 3A).

Daytime serum samples (1 mL) from six different volunteers were supplemented with melatonin to either 1076 or 4305 pmol/L (250 or 1000 ng/L) and subsequently diluted serially with each respective serum for further investigation of this topic. Again, all serum dilution curves deviated from the calibration curve in the lower concentration range (Fig. 3B).

Melatonin concentration in serum samples measured by direct ELISA. Melatonin content in 138 sera from 19 different individuals was assessed both by RIA (x) and direct ELISA (y). Agreement between measurements was rather poor (y = 0.39x + 56; r = 0.65, P <0.0001; Fig. 4).

Large interindividual differences in the agreement between melatonin measurements with RIA and direct ELISA were noted. Fig. 5 displays melatonin concentrations measured over 24 h in three subjects. The course of the regression lines for the individuals apparently differed considerably. This indicates that the serum effect in the direct ELISA varies among subjects.

Melatonin ELISA After Extraction

Recovery of melatonin. The recovery of cold (unlabeled) melatonin added to daytime pooled serum varied, being 86.0% (addition of 258 pmol/L melatonin; n = 8), 82.6% (addition of 431 pmol/L melatonin; n = 7), 75.0% (addition of 517 pmol/L melatonin; n = 7), and 77.5% (addition of 689 pmol/L melatonin; n = 8).

Table 1. Interassay variation.

<table>
<thead>
<tr>
<th>n</th>
<th>Melatonin conc, pmol/L (ng/L)</th>
<th>CV, %</th>
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<tbody>
<tr>
<td>Direct ELISA</td>
<td>13 270 (63)</td>
<td>23.0</td>
</tr>
<tr>
<td></td>
<td>12 1393 (324)</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>12 2286 (531)</td>
<td>9.3</td>
</tr>
<tr>
<td>RIA*</td>
<td>32 56 (13)</td>
<td>25.9</td>
</tr>
<tr>
<td></td>
<td>32 224 (52)</td>
<td>18.0</td>
</tr>
<tr>
<td></td>
<td>32 508 (118)</td>
<td>15.8</td>
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</tbody>
</table>

* Data elaborated in our laboratory and published previously (25).
Simplicity, Speed, and Sample Size

The direct ELISA requires three incubation steps, for 17 h altogether. The total hands-on time for the procedure is ~3 h for 40 samples, with all three steps being completed by the next working day. The required amount of specimen is 0.1 mL for measurements in duplicate.

With the extraction step included, the ELISA requires ~30 h of incubation and 7 h of labor, increasing the total time needed for delivering results to 2 working days and the required amount of serum to 1.5 mL for duplicates.

RIA always involves extraction of melatonin from 1.5 mL of serum for the assay in duplicate. In total, RIA requires 36 h for incubation and ~9 h of labor for the same number of samples. The procedure takes 2 full working days and requires a laboratory equipped for handling radioactive material.

Discussion

The calibration curves of both assays show high stability, as indicated by a similarly low SD value for ELISA and RIA measurements (Fig. 1). However, the calibration curves differ greatly in their slope and detection range. The calibration curve of the ELISA is
rather flat, with a slope of 1.4, and extends from 138 to >8610 pmol/L (ED_{90} to ED_{20}). RIA displays a much steeper slope of 2.0 with a detection range from ~35 to 1200 pmol/L (Fig. 1).

Judged from the within-assay CV, the useful detection range for RIA extends from 52 to 710 pmol/L. The direct ELISA is a little more precise than the RIA and permits useful measurements over a wider concentration range, i.e., from 170 to >6500 pmol/L (Fig. 2). However, this wider detection range of the direct ELISA cannot be used in practice: At its upper half, the range applies to concentrations beyond the range of physiological serum melatonin concentrations in humans; at its lower end, the curve does not cover the low daytime concentrations expected.

The excellent parallelism of the calibration curve with the curve of supplemented pooled serum diluted by buffer indicates a lack of cross-reactivity (Fig. 3A). However, the deviation of the curves for supplemented serum diluted with nearly melatonin-free serum from the calibration curve (Fig. 3) suggests a serum effect. This assumption is consistent with the poor correlation between measurements with the RIA and the direct ELISA (Fig. 4) and the apparent interindividual differences in correlation between measurements with direct ELISA and RIA (Fig. 5).

Extraction and concentration of melatonin before measurement by ELISA improves some of the features of this assay considerably. The useful working range shifts by one order of magnitude towards lower concentrations without a major loss of precision. This makes measurements possible over the entire physiological range in humans, similar to RIA. The excellent correlation between melatonin measurements with ELISA after extraction and RIA in unselected individuals (Fig. 6) indicates removal of the serum effect and justifies the need for a larger sample size and the greater workload associated with extraction.

Although the direct melatonin ELISA displays some good features (i.e., stable calibration curve, speed, small sample volume), in our hands it is not suitable for physiological studies in humans mainly because of the flat and insensitive calibration curve and some evidence of serum effect. Similar effects have been noted in other ELISAs of melatonin (17, 18) and also for monoclonal antibodies to melatonin (27). However, extraction and concentration before the assay make this ELISA a more useful tool in pineal examination, comparable with most of the melatonin RIAs. Introduction by the manufacturer of a more avid antisera in this ELISA might improve its sensitivity and perhaps eliminate the concentration step, which would then permit a reduction in sample size.

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References