determination of the M2, M1(ala), and M1(val) genotypes. This will allow the evaluation of their role in the development of bronchial asthma in a large number of well-defined patients.

References

Improved Enzyme Immunoassay Method for Melatonin: Application to the Determination of Serum Melatonin in Rats, Sheep, and Humans, Shai K. Shavali, Michiharu Samejima, Katsuhiro Uchida, Yukitomo Morita, and Atsuo Fukuda (Department of Physiology, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka 431-3192, Japan; * author for correspondence: fax 81-53-435-2245, e-mail samejima@akiha.hama-med.ac.jp)

Melatonin influences physiological processes such as reproduction, immune regulation, and aging. Recent findings have also indicated its potent antioxidative properties. This hormone is known to exhibit a circadian rhythm, with high concentrations during the dark phase. Pinal function can be evaluated by the measurement of melatonin in body fluids by the use of specific and sensitive immunoassays. Although RIA is widely used, Ferrua and Masseyeff (8) in 1985 first reported an immunonoassay for melatonin with enzyme-labeled antibodies. They used anti-melatonin IgG coupled with horseradish peroxidase as a nonisotopic label. Although their assay procedure was simple and rapid, the detection limit of the assay was 5 pg/well, which may be inadequate to measure daytime melatonin concentrations. In 1993, Yie et al. (9) reported a competitive solid-phase enzyme immunoassay (EIA) for melatonin with melatonin-sodium-p-carboxybenzyl-alkaline phosphatase (MT-pcb-AP) as a nonisotopic label. They validated the assay with a detection limit of 1.0 fmol/well (0.2 pg/well), but the assay required overnight incubation steps over several days.

Our aim was to modify the method of Yie et al. (9) so that it could be completed within 1 or 2 days. We describe here the method and its validation in rat, sheep, and human serum samples after extraction with dichloromethane.

Polystyrene multiwell plates (modified flat bottom; Corning 25805-96), serum samples [rat (cat. no. S7648), sheep (cat. no. S2263), and human (cat. no. H1388)], bovine albumin (cat. no. A9418), thimerosal (cat. no. T8784), diethanolamine (cat. no. D8885), and melatonin (cat. no. M5250) were purchased from Sigma Chemical. MT-pcb-AP (cat. no. CIA101a) and purified anti-melatonin serum (cat. no. CIA101, lot no. S#380, 24-4-89) were obtained from CIDTech Inc. The specificity of the purified anti-melatonin serum has been reported (9). p-Nitrophenyl phosphate, disodium salt (cat. no. 1.0685) was from Merck. All other chemicals used in this study were of analytical grade.

Blood from six healthy volunteers was collected at 1400–1500 h and at 0200–0300 h. Blood was also collected from 10 laboratory rats (male Sprague–Dawley rats; body weight, 260–295 g) after decapitation during the same time periods. Nighttime samples were collected under a dim red light. After clotting, the blood was centrifuged at 1500g at 4 °C for 20 min. The serum was separated and stored at −20 °C until being assayed for melatonin.

The coating buffer for the anti-melatonin serum was 0.05 moles of carbonate-bicarbonate buffer, pH 9.6. The
assay buffer was 0.05 mol/L sodium phosphate (Na₂HPO₄ and NaH₂PO₄), pH 7.5, containing 9 g/L saline, 2 g/L bovine albumin, and 0.1 g/L thimerosal. The washing solution contained 9 g/L NaCl, 0.5 mL/L Tween 20, and 0.1 g/L thimerosal. The substrate solution contained 1.6 g/L p-nitrophenyl phosphate in 1.0 mol/L diethanolamine buffer, pH 9.8.

We followed the extraction procedure described by Yie et al. (9). Briefly, serum and dichloromethane were combined in a 1:5 ratio by volume and mixed gently for 30 s. The solution was then centrifuged at 2000 g at 4 °C for 20 min. The upper phase was removed without disturbing the dichloromethane phase. For smaller volume samples, the upper phase was not removed, and the tubes were kept directly in dry ice. The dichloromethane phase was transferred into new glass tubes and evaporated to dryness in a SpeedVac concentrator (Savant Instruments); a volume of assay buffer equal to the original volume of the serum was then added to the dried tubes and mixed well before being assayed.

The polystyrene plate wells were coated with purified anti-melatonin serum (1:7500 dilution) in 0.05 mol/L sodium carbonate-bicarbonate buffer, pH 9.6, at 30 °C for 6 h. The wells for nonspecific binding were coated with bovine albumin in the same buffer. After the wells were coated, each plate was washed three times; the washing step included the addition of 200 μL of washing solution to each well, agitation of the plate for 5 s, and removal of the solution. After the plate was washed three times, the wells were blocked with 10 g/L bovine albumin (200 μL/well) at 30 °C for 2 h. The plate was then washed once, and melatonin calibrator or sample (100 μL), MT-pcb-AP (50 μL), and assay buffer (50 μL) were added to each well. The total volume in each well was 200 μL. The plate was shaken gently for 5 min in a mini shaker (MS1S1; IKA Works), covered with aluminum foil, and incubated at 8 °C for 12–14 h. After incubation, the plate was washed three times with washing buffer. After a final wash, the plate was inverted on a filter paper to remove the remaining washing solution, after which substrate solution (200 μL) was added to each well and incubated at 37 °C under complete darkness for 8–10 h. Finally, the absorbances were read at 405 nm in a ELISA plate reader (EL340; Bio-tek Instruments).

Serum samples were also assayed by an RIA (10), with 3H-melatonin (Amersham Life Sciences) and anti-melatonin serum (Stockgrand Ltd.).

Inhibition curves were obtained with 5–80 μL of the extracts of rat, sheep, and human serum. Because the serum melatonin in the above samples was found to be low, a known amount of melatonin was added. The displacement and calibration curves were parallel (Fig. 1).

The lowest detectable amount of the melatonin by the improved EIA method was 0.8 pg/well, similar to the detection limit of 0.2 pg/well reported by Yie et al. (9). The displacement with 0.8 pg of melatonin in the assay buffer was significantly (P < 0.001) different from that with the melatonin zero calibrator. The intra- and inter-assay CVs for low and medium melatonin concentrations in rat, sheep, and human serum extracts were 6.5–18% (Table 1).

The mean (± SD) recoveries for 6.3, 12.5, 25.0, 50.0, and 100.0 pg of melatonin added to rat, sheep, and human serum extracts were 112.8% ± 14.1%, 122.6% ± 14.0%, and 108.5% ± 13.5%, respectively, as assessed by linear regression of measured vs expected melatonin. As reported by Di et al. (11), the recovery was also better in our experiment when the sera were extracted.

The day-night variations in serum melatonin in six healthy volunteers (four men and two women; ages, 24–47 years) and in five male Sprague–Dawley rats were

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraassay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>14.6</td>
<td>1.6</td>
<td>11</td>
</tr>
<tr>
<td>Sheep</td>
<td>57.8</td>
<td>8.6</td>
<td>15</td>
</tr>
<tr>
<td>Human</td>
<td>8.0</td>
<td>0.5</td>
<td>6.4</td>
</tr>
<tr>
<td>Interassay</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>13.8</td>
<td>1.7</td>
<td>13</td>
</tr>
<tr>
<td>Sheep</td>
<td>54.0</td>
<td>3.7</td>
<td>7.0</td>
</tr>
<tr>
<td>Human</td>
<td>8.5</td>
<td>1.4</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>48.5</td>
<td>3.4</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>10.3</td>
<td>1.9</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>56.0</td>
<td>4.2</td>
<td>7.5</td>
</tr>
</tbody>
</table>

* Each well contained 100 μL of calibrator or sample.
16.4 ± 1.3 ng/L (day)/63.3 ± 24.6 ng/L (night) and 18.8 ± 10.0 ng/L (day)/95.3 ± 34.0 ng/L (night), respectively, by an EIA. Those by an RIA were 11.5 ± 2.6 ng/L/50.1 ± 18.9 ng/L and 15.5 ± 6.5 ng/L/76.5 ± 17.9, respectively. There was no significant difference between values obtained by the EIA and the RIA (P > 0.5 for day samples, P > 0.3 for night samples from rats, and P > 0.3 for night samples from humans). Significant differences were observed between daytime values and nighttime values in all cases by both methods (P < 0.01).

The EIA for melatonin is sensitive and reliable, and is relatively faster than the method reported previously. We modified the assay in several ways, including coating the melatonin antibody to the polystyrene wells by incubating the antibody in a carbonate-bicarbonate buffer at 30 °C for 6 h. During the course of our investigation, we observed that the incubation with sucrose solution (25 g/L) was not necessary. The sensitivity, precision, stability of the calibration curves, and sample estimations were found to be the same without this step. The color development was also achieved by incubating the wells with the substrate at 37 °C for 8–10 h. These modifications decreased the assay time to 30 h.

The serum melatonin results were similar to those already published for rat and human serum (10, 12, 13). In addition, the serum melatonin results obtained by this method were not significantly different from those obtained by a standard RIA method for rat and human sera, except for the daytime values in humans, where a difference was observed (P < 0.01).

The MT-pcb-AP label is convenient to handle, and it is more stable when stored at 4 °C without loss of activity for several months. The melatonin antiserum has already been characterized as having low cross-reactivity with several methoxy and hydroxy indoles (9). Thus, we suggest that the improved EIA developed with MTPcb-AP is a simple, convenient, and valid method for the estimation of serum melatonin in rats, sheep, and humans. Because the EIA method does not require any radioactive material, it will be available to many investigators who do not use the RIA method at present.

This study was supported in part by grants-in-aid from the Ministry of Education, Science, Sports and Culture of Japan (grants 07839002 and 960285). S. Shavali was supported by a postdoctoral fellowship for foreign researchers in Japan from the Japan Society for the Promotion of Science (JSPS).

References

OVX1 Radioimmunoassay Results Are Dependent on the Method of Sample Collection and Storage, Estrid V S. Høgdall,1* Klaus K. Høgdall,2 Susanne K. Kjaer,³ Fengji Xu,4 Yinhua Yu,4 Robert C. Bast,4 Jan Blaakaer,2 and Ian J. Jacobs3 (1 Laboratory of Molecular Biology, Department of Clinical Biochemistry, State Serum Institute, Artillerivej 5, Denmark DK-2100; 2 The Gynaecologic Clinic, The Juliane Marie Centre, Rigshospitalet, Copenhagen, Denmark DK-2100; 3 Institute of Cancer Epidemiology, Danish Cancer Society, Copenhagen, Denmark DK-2100; 4 MD Anderson Cancer Center, Houston, Texas, 77030; 5 Department of Obstetrics and Gynaecology, Aarhus University Hospital, Skejby, Denmark DK-8200; 6 Gynecology Cancer Research Unit, Department of Gynaecological Oncology, St. Bartholomew’s Hospital, London, UK EC1A 7BE; * author for correspondence: fax 0045 3268 3878, e-mail hogdall@dadmnet.de)

OVX1 is a tumor marker that may be of value in the management of ovarian cancer and appears to be complementary to CA125 measurement (1–3). The results of a study by Woolas et al. (2) suggested that OVX1 is increased in the serum of a major proportion of patients with stage I ovarian cancer who have serum CA125 concentrations within the health-related reference interval. We, therefore, had planned to include OVX1 measurement in a prospective study of screening for ovarian cancer. Because the screening study involved sending blood samples by post to a central laboratory, we undertook this preliminary study to assess the stability of OVX1 in the 2- to 3-day time period required for postal delivery.

All subjects in this study were apparently healthy premenopausal women. Venipuncture was performed on 20 subjects in the UK and 9 subjects in Denmark. The collection tubes, the storage temperature before centrifugation, and the length of storage before centrifugation were varied. All samples were then centrifuged at 2000g for 10 min, and the plasma or serum samples were stored at −20 °C until OVX1 analysis was performed. In addi-