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Validation of a Direct Radioimmunoassay of Melatonin in the Blue Fox

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Forsberg, M. and A. Madej: Validation of a direct radioimmunoassay of melatonin in the blue fox. Acta vet. scand. 1987, 28, 73–79. – A direct radioimmunoassay procedure for the determination of melatonin in the blood of blue fox has been validated and applied.

The assay required 50 µl of sample and standard, 100 µl of antiserum and 100 µl of (^{3}H)melatonin. After overnight incubation at 4°C the antibody bound melatonin was separated from the free hormone with dextran-coated charcoal. Following centrifugation the antibody bound (^{3}H)melatonin was determined in a beta scintillation counter.

The antiserum bound 30–35 % of the (^{3}H)melatonin at a final dilution of 1:36000. The non specific binding represented less than 5 % of the total radioactivity in all assays. The lowest detectable amount of melatonin was 2.6 fmol/tube, corresponding to 52.5 pmol/l. The inter-assay coefficient of variation at 178 and 510 pmol/l was 15.6 and 8.8 %, respectively. The precision profile, calculated from a 10-replicate standard curve, showed that the coefficient of variation decreased from 43 % at 84 pmol/l to 15 % at 336 pmol/l, and remainded at or below 10 % for concentrations exceeding 670 pmol/l.

Plasma was collected from 2 male blue foxes at about hourly intervals during a 24 h period in September and assayed for melatonin. Maximum (421 pmol/l) and minimum (97 pmol/l) concentrations of the hormone were inversely related to light intensity.

reproduction.

Introduction

Melatonin seems to act as an important mediator of changes in day light length on reproductive processes in animals which have a seasonal breeding pattern. This aspect of melatonin secretion is best understood in the sheep (*Lincoln & Short* 1980, *Arendt et al.* 1983, *Almeida & Lincoln* 1984) while relatively little is known about the role of melatonin in other species with seasonal breeding. The blue fox (Alopex lagopus) is a species commonly raised in the northern hemisphere for fur production and it has a pronounced seasonal breeding pattern (*Smith et al.* 1985). Females usually conceive during March and April and have a 52 day gestation period. In theory the animals thus has the capacity to carry and give birth to another litter in the same year provided the constrain of seasonal breeding can be overcome. Such attempts will have to rely on a sound knowledge of the hormonal

factors governing seasonality of breeding in both sexes as well as the endocrine mechanisms regulating spermatogenesis and ovulation. Since melatonin may play a key function in regulating the season of sexual activity it was decided to develop, evaluate and apply a method for the determination of this hormone in the blue fox. Detailed investigations on the secretory pattern of melatonin requires frequent sampling over a 24 h period and since the blue fox is a relatively small animal the method should preferably require small amounts of sample volume for the analysis and it should have a high capacity or through-put of samples.

Materials and methods

Reagents

All common reagents were of pure analytical grade.

Buffer

Assay buffer was PBS – 0.1 % gelatin (0.5M $NaH_2PO_4 \cdot H_2O$, 0.1M $Na_2HPO_4 \cdot 7H_2O$, NaCl 8.19 g/l, merthiolate 0.1 g/l, gelatin 0.1 %). The buffer was adjusted to pH 7.5 with sodium hydroxide and stored at 4°C.

Tracer

The tritiated melatonin (Amersham International, Buckinghamshire, England) had a specific activity of 285 TBq/mmol (77 Ci/mmol). The stock solution and a 1:100 dilution of tracer were stored at –20°C. The 1:100 dilution was further diluted to 1:5000 with assay buffer before use to yield about 5000 dpm per assay tube (30–35 % counting efficiency).

Antiserum

Antiserum to melatonin raised in rabbit was kindly provided by Dr. *G. Niswender* (Dept. of Physiology and Biophysics, Colorado State University, Fort Collins, Colorado, USA; Rabbit 1055 9/16/74). The antibody was stored at 4°C at a dilution of 1:400 and diluted with assay buffer before use to give an initial dilution of 1:12000.

Standards

Melatonin was purchased from Sigma Chemical Company, USA. A stock melatonin standard of 4.3 mmol/l was prepared by dissolving 10 mg melatonin in 0.5 ml ethanol and adjusting the volume to 10 ml with distilled water. The stock solution was stored in 100 µl aliquots at –20°C and was further diluted with assay buffer before use to give a set of individual standards ranging from 84 pmol/l to 2690 pmol/l.

Ligand separation

Antibody-bound melatonin was separated from free melatonin by incubation with dextran-coated charcoal (charcoal: Merck, Darmstadt, West Germany; dextran T70: Pharmacia Fine Chemicals, Uppsala, Sweden) 250 mg charcoal plus 25 mg dextran per 100 ml of assay buffer containing no gelatin.

Scintillation fluid

Miniria 20 was used as scintillation fluid (Zinsser Analytic Ltd., England).

Preparation of melatonin-free plasma for standards

To remove melatonin, a pool of bovine plasma was stirred with 50 mg charcoal per ml of plasma at room temperature for 30 min and centrifugated at 12000×g for 60 min at 10°C. Finally, the plasma was passed through a sterile filter (pore size 0.22 µm) to remove any remaining charcoal. The melatonin-free plasma was stored at –20°C in 2 ml aliquots until use.

Radioimmunoassay procedure
Standards and samples (50 µl) were dispensed into glass vials. Bovine melatonin-free plasma (50 µl) was then added to standard vials and assay buffer (50 µl) to the sample vials. Finally 100 µl of the rabbit anti-melatonin and 100 µl of the (^{3}H)melatonin was added to samples and standards. The contents of the tubes were vortexed after all additions has been made. After overnight equilibration at 4°C the antibody bound melatonin was separated from the free hormone by incubating with 0.5 ml dextran-coated charcoal (per vial) for 15 min after vortexing. The tubes were centrifugated for 5 min and the supernatant decanted into plastic vials containing 3.5 ml scintillation fluid. Temperature was carefully controlled and kept at 4°C during charcoal incubation and centrifugation. The content of each vial was mixed and counted for 10 min in a beta scintillation counter (1215 Rackbeta 11, LKB Wallac, Turku, Finland).

Animals and sample preparation
Two blue fox males were sampled at about hourly intervals in September by venipuncture (V. saphena). Blood samples were collected into heparinized tubes. Plasma was separated by centrifugation and stored at –20°C until assay. All samples for assay were thawed the previous day and stored at 4°C overnight.

Quality controls
To calculate inter-assay variation, one quality control containing endogenous melatonin was prepared by pooling blue fox plasmas containing low concentrations of melatonin. As a second control, a reference serum from a commercial kit was utilized (KALAB, Donville, Calif., USA). Each control was measured in duplicate in 5 different assays. All quality control samples were stored at –20°C until used.

Statistical methods
Regression analysis, calculation of straight line equations and precision profiles were done using procedures available from the Statistical Analysis System (SAS) and Riacalc System (LKB Wallac, Turku, Finland).

Results

The specificity of the antiserum has been reported by *Rollag & Niswender* (1976). In developing the present assay the effects of incubation period, temperature and the addition of reagents were investigated. Incubation at 37°C for 1 h, at room temperature for 2 h and at 4°C overnight resulted in 15, 22 and 29 % binding of the tracer by the antiserum at a final dilution of 1:24000. Longer incubation periods at 4°C did not result in any significant improvement in the binding of the tracer.
A 30 min delay in the addition of the tracer did not improve the sensitivity of the assay. The addition of 50 µl bovine melatonin-free plasma to the standards prevented losses caused by adherence of antiserum to tube surfaces during the assay procedure and improved the maximum binding by about 10 %.
In general the antiserum bound 30–35 % of the melatonin tracer at a final antiserum dilution of 1:36000. Non specific binding was less than 5 % of total radioactivity in all assays. The slope of the dose response curve in 7 assays was -1.04 ± 0.05 (mean ± SD) after logit-log transformation.
Serial dilutions of blue fox and bovine plasmas containing high melatonin concentrations produced curves parallel to the standard curve (Fig. 1) and non specific binding was unaffected (< 5 % of total radioactivity)

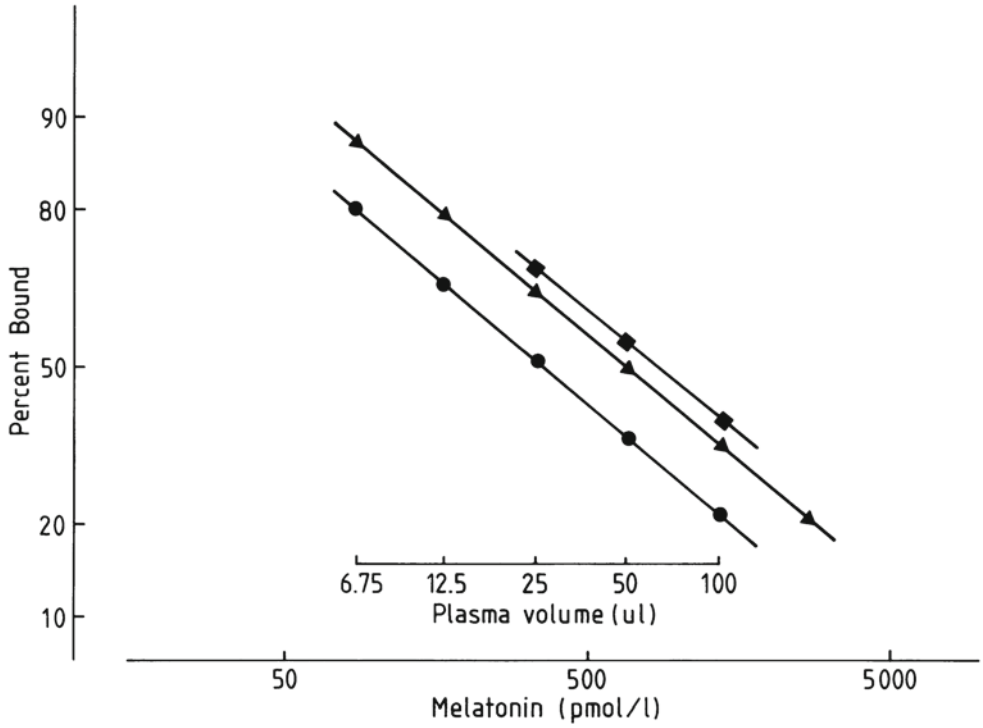

Figure 1. Standard curve (●–●) for melatonin and for dilutions of blue fox (▶–▶) and bovine (■–■) plasma with sample volumes adjusted to 100 µl using assay buffer.

by changes in sample volume. Melatonin added to plasma from blue fox and cattle was recovered in quantitiable amounts. The correlations between assayed and expected amounts of melatonin were in both cases 0.99. The corresponding regression equations were for blue fox plasma, $y = 0.94x + 186$ and for bovine plasma, $y = 1.13x + 30$. Values of the y intercept represent the endogenous concentration of melatonin in the two plasmas.

In an assay with a 10-replicate standard curve the lowest amount of melatonin detectable (defined as intercept of maximal binding –2 SD) was 2.6 fmol/tube, corresponding to 52.5 pmol/l. In the same assay a 50 % inhibition of specific tracer binding was induced by 34 fmol/tube, corresponding to 680 pmol/l of melatonin.

The precision profile calculated from a 10-replicate standard curve is shown i Fig. 2. The coefficient of variation in melatonin concentration decreased from 43 % at 84 pmol/l to 15 % at 336 pmol/l, and remained at or below 10 % for concentrations exceeding 673 pmol/l.

The inter-assay coefficient of variation for the quality control samples were 15.6 % (mean = 178 pmol/l, n = 5) and 8.8 % (mean = 510 pmol/l, n = 5).

It was found that the shelf life of the standards and the tracer when diluted to the final concentrations for use in the assay was 1 week when stored in a refrigerator at 4°C.

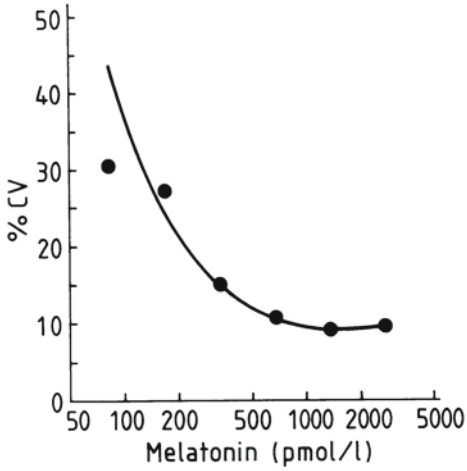

Figure 2. Precision profile calculated from a 10-replicate standard curve. The curve represents the estimated error function calculated from the observed error (●) at different concentrations of standards.

The circadian variation in plasma melatonin from two blue fox males sampled at about hourly intervals in September are shown in Fig. 3. All samples were analysed in duplicate in a single assay. A rise in plasma melatonin concentration was observed to occur in the afternoon (14.00–16.00), with elevated levels observed between 16.00 and 04.00. A decrease was observed at dawn after which melatonin levels remained low until midday (06.00–13.00).

Discussion

The sensitivity of melatonin assays employing extraction of plasma or serum varies considerably. The use of radioiodinated tracers allows an increase in sensitivity compared with that obtained with tritated tra-

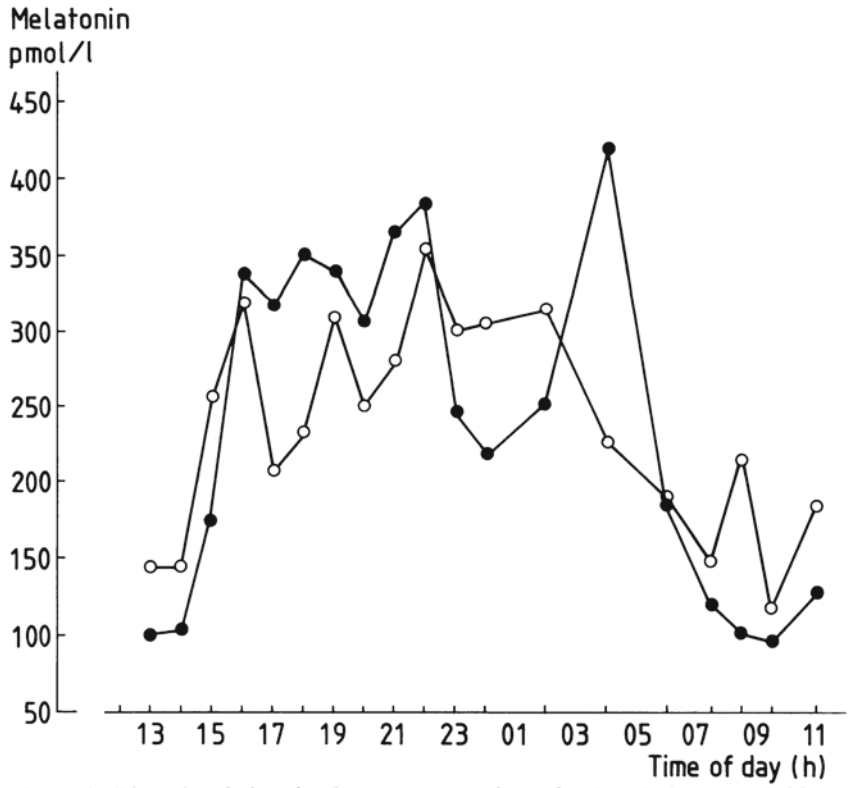

Figure 3. Diurnal variations in plasma concentrations of melatonin in two male blue foxes: fox no. 1 (●–●) and fox no. 2 (○–○).

cers (*Waldhauser & Wurtman* 1983). *Vakkuri et al.* (1984) reported a sensitivity of 8 fmol/tube and *Rollag & Niswender* (1976) 10 fmol/tube using radioiodinated tracers. Even so, when measuring melatonin in species with low levels of the hormone a large sample volume is necessary for the initial extraction.

Recently, direct radioimmunoassays utilizing tritiated melatonin of high specific activity have been described. *Webley et al.* (1985) reported a sensitivity of 2.2 fmol/tube using double antibody precipitation as a separational system, and *Fraser et al.* (1983) reported a sensitivity of 24 fmol/tube using a charcoal separation system. The sensitivity of the present assay system was 2.6 fmol/tube corresponding to 52.5 pmol/l.

Due to its simplicity, the method using dextran-coated charcoal is very attractive as long as non specific binding can be kept at a low level (e.g. $< 5\ \%$). Based on our experience, the drawbacks of charcoal as a separational system can be minimized if the incubation time is sufficiently long, allowing equilibrium to be reached, and if the time between addition of charcoal and centrifugation is tightly controlled.

Since investigations on circadian variations of melatonin secretion calls for repeated blood sampling, the present assay system only requiring 50 µl plasma as starting material lends itself to such investigations especially in small species like the blue fox, thus avoiding the reinjection of red blood cells to maintain circulating blood volume. As judged from the studies on parallelism, recovery and precision profiles in the present study the assay method is valid for determination of melatonin in the blue fox. It is, however, necessary to use ligand-free bovine plasma in the standard curve to prevent absorption losses which otherwise could lead to inaccurate low results (*Chad* 1982). The ability of the assay system to detect a circadian rythm in melatonin secretion in the two blue fox males is a further criteria of validation for the assay system developed.

It should be emphasized that the determination of the melatonin concentration in the commercial reference serum gave a mean estimate of 510 pmol/l (SD = 45, n = 5) which is similar to that stated by the manufacturer, 500 pmol/l.

We are currently trying to determine whether the method evaluated here also can be adapted for use in the mink (Mustela vison) and the silver fox (Vulpes vulpes).

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Sammandrag

Utvärdering av en direkt radioimmunologisk metod (RIA) För analys av melatonin i blod hos blåräv.

Analysmetoden erfordrar 50 µl prov respektive standard, 100 µl antiserum och 100 µl tritierat melatonin. Efter inkubation över natt vid 4°C separeras det antikroppsbundna melatoninet från det fria hormonet genom absorption till dextrankol. Efter centrifugering bestämms mängden antikroppsbundet tritierat melatonin i en scintillationsräknare.

Antiserat spätt 1:36000 band 30–35 % av det tritierade melatoninet. Den ospecifika bindningen (NSB) var lägre än 5 % av den totala radioaktiviteten. Metodens känslighet är 2.6 fmol per rör, vilket motsvarar 52.5 pmol/l.

Inomkörningsvariationen vid 178 och 510 pmol/l var 15.6 och 8.8 %. Precisionsprofilen, kalkylerad från en standardkurva med 10 replikat, visade att variationskoefficienten minskade från 43 % vid 84 pmol/l till under 10 % vid melatoninkoncentrationer överstigande 673 pmol/.

Blodprover togs med ungefär 1 timmes intervall under en 24 timmars period från två blårävshanar, och analyserades med avseende på koncentrationen av melatonin. De högsta koncentrationerna av melatonin uppmättes under dygnets mörka och de lägsta under dygnets ljusa del.

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