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A simple, specific high-throughput enzyme-linked immunosorbent assay (ELISA) for quantitative determination of melatonin in cell culture medium

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Abstract

A simple, specific, high-throughput enzyme-linked immunosorbent assay (ELISA) for quantitative determination of melatonin was developed for directly measuring melatonin in cell culture medium with 10% FBS. This assay adopts a commercial monoclonal melatonin antibody and melatonin-HRP conjugate, so it can be applied in multiple labs rapidly with low cost compared with commercial RIA and ELISA kits. In addition, the procedure is much simpler with only four steps: 1) sample/conjugate incubation, 2) plate washing, 3) TMB color reaction and 4) reading of results. The standards of the assay cover a wide working range from 100pg/ml to 10ng/ml. The sensitivity was 68pg/ml in cell culture medium with 10% FBS and 26pg/ml in PBS with as little as 25μL sample volume. The recovery of melatonin from cell culture medium was 101.0%. The principal cross-reacting compound was 5-methoxytryptophol (0.1%). The variation coefficients of the assay, within and between runs, ranged between 6.68% and 15.76% in cell culture medium. The mean linearity of a series diluted cell culture medium sample was 105% (CV=5%), ranging between 98% and 111%, $y = 5.5263x + 0.0646$, $R^2 = 0.99$. The assay enables small research and teaching labs to reliably measure this important neurohormone.

Keywords

Melatonin; ELISA; melatonin-HRP conjugate; chicken pineal

1. Introduction

Melatonin, an important hormone involved in the regulation of circadian and seasonal rhythms in vertebrates, is a direct output of circadian oscillators within the retinae and pineal gland [1]. In non-mammalian vertebrates such as birds and lizards, pineal melatonin is critical for the expression of overt circadian rhythms of behavior and physiology. There is extensive evidence that melatonin is also important in mammalian sleep: wake cycles. In seasonally breeding mammals, the hormone is critical for the transduction of photoperiodic

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information in the seasonal control of gonadal function [1–3], as well as seasonal control of birdsong and migration in passerine birds [4–6].

Melatonin is synthesized in pinealocytes in the pineal gland and in retinal photoreceptors [7]. These cells take up the amino acid tryptophan (TRP) and convert TRP to 5-hydroxytryptophan (5HTRP) through the action of tryptophan hydroxylase (TrH; EC 1.14.16.4). Then, aromatic amino acid decarboxylase (AAADC; EC 4.1.1.28) converts 5HTRP to 5-hydroxytryptamine (serotonin). With the action of aryl alkyl amine N-acetyltransferase (AANAT; E.C.2.3.1.87), serotonin is converted to N-acetylserotonin, which is finally converted to melatonin via hydroxyindole-O-methyltransferase (HIOMT; E.C. 2.1.1.4).

The chick pineal gland has been used as a model system for studying the generation of circadian rhythms [8–17]. Chick pineal cells synthesize melatonin rhythmically in organ and cell culture [18–21]. The production/secretion of melatonin by pineal cells *in vitro* is rhythmic in both light/dark cycles and in constant environmental lighting conditions, with melatonin peaking in the night or dark phase. The melatonin rhythm from cultured pineal cells is acutely responsive to light and is entrained by light: dark cycles [22,23]. Therefore, the chick pineal gland remains an excellent experimental model for circadian rhythms, which directly perceives light as an input, contains a circadian clock and rhythmically releases melatonin as an output. Dispersed pineal gland cells in static culture and perfusion systems facilitate the studies of the mechanisms underlying circadian rhythm phenomena.

Analytical chemical methods of melatonin determination such as gas chromatography–mass spectrometry (GC–MS) and high performance liquid chromatography (HPLC) require a high cost of apparatus, complex sample preparation, large sample volume, and are not suitable for measuring large numbers of samples [24–26]. In contrast, a number of radio- (RIA) and enzyme immunoassay (ELISA) methods have been developed to enable endocrinologists to process many biological samples [27–31]. In RIAs, an anti-melatonin antibody is typically mixed with samples or standards, and radioactively labelled ($[^{125}\text{I}]$ or $[^3\text{H}]$) melatonin or a melatonin analog is used as tracer to compete for anti-melatonin antibody binding. The antibody binding melatonin and radioactive tracer are then precipitated for measurement of radioactivity by scintillation or γ counter, depending on the tracer. The more the cold melatonin in samples binds the antibody, the fewer binding sites are available to the tracer, and therefore the less radioactivity will be read. RIA is widely used because it is sensitive, and the procedure is relatively simple; however, the RIA is also expensive and the potential health risks for researchers and students preparing and handling the radioactive antigen are not inconsequential.

Enzyme-linked immunosorbent assays (ELISA) employ antibodies and colorimetric enzymes [32]. The enzymes catalyze color reactions with an enzyme-specific chromogen as an indicator. ELISA is simple to perform, and the reagents are relatively inexpensive. There are different forms of ELISA, such as direct ELISA, indirect ELISA, competitive ELISA and sandwich ELISA [33]. In direct ELISA, the antigen is attached to a solid phase and is recognized by an enzyme labeled antibody. In indirect ELISA, the antigen is also attached to a solid phase, but it is recognized by an unlabeled primary antibody and detected with an

enzyme-labeled secondary antibody directed against the primary antibody. Competitive ELISA involves the simultaneous addition of 'competing' antibodies or proteins in different ways. In sandwich ELISA, capture antibodies are attached to a solid phase to capture the antigen, and then a specific antibody is added to detect the antigen, forming a sandwich structure. The specific antibody can be enzyme labeled or can be detected by an enzyme-labeled antibody.

ELISA is specific and sensitive for melatonin measurement [34,35]. However, commercial ELISA kits are usually very expensive and therefore not suitable for high throughput measurement. Customized melatonin ELISAs usually involves development of melatonin antibodies and melatonin conjugates, which are generally non-trivial to produce and/or unavailable for most researchers. Therefore, we developed an easy and direct ELISA based on a readily available commercial supply of a monoclonal melatonin antibody and a melatonin-horseradish peroxidase (HRP) conjugate, which is available and convenient for most of the researchers. Our ELISA is a competitive ELISA in which the melatonin antibody is coated on 96-well plates as the solid phase, and the enzyme-labeled melatonin is mixed with sample melatonin to compete for the antibody. The ratio of sample melatonin versus enzyme-labeled melatonin determines the intensity of the color reaction. The assay can directly measure melatonin in cell culture medium and, after extraction, reconstitution in PBS.

2. Materials and methods

2.1 Samples

Pineal cells were cultured as described in previous studies with minor modification [8–10,36]. Briefly, pineal glands were collected from embryonic day 21 chick (*Gallus gallus domesticus*) brain as previously described [36]. The pineal glands were transferred to sterile Dulbecco's phosphate-buffered saline (PBS) with D-glucose (1.8%), and digested by 0.25% trypsin (10 pineals/mL of trypsin) for 30 min with trituration every 10 min. The debris was removed and then the dispersed cells were spun down for 10 min at 1000 g. The pellet was re-suspended to 4 pineal/mL (10^6 cells/mL) in M-199 medium with 10% FBS, 10% chicken serum and 1% penicillin/streptomycin. 30 μ L of the cells were loaded into one channel of the 6-channel multi-well slide (IBIDI Cat.80606) and cultured for 3 days. The multi-well slide was loaded on the customized perfusion system for 6 days either under 6 days under light/dark cycle (LD) or 3 days under light/dark cycles followed by 3 days in constant darkness (DD). The samples with melatonin from the pinealocytes were collected every hrs. and then measured by the ELISA.

2.2 Plate preparation

Monoclonal melatonin antibody (CalBioReagents, Inc, San Mateo, CA, USA) was diluted (0.5 μ g/ml) in Plate Coating Buffer (ph7.4 PBS with 5% sucrose) and then loaded as 50 μ l antibody solutions per well of the 96-well plates (15041, Thermo Pierce, Rockford, IL, USA). The plates were kept in 4°C overnight (12~20hrs). On the second day, the antibody solution was removed and rinsed with 300 μ l of washing buffer (ph7.4 PBS with 0.5% Tween 20) in each well for 4 times. Then the plates were blocked with 50 μ L of protein-free

(PBS) blocking buffer (37516, Thermo Pierce, Rockford, IL, USA) and incubated at 4°C for 1~2hr. After removing the blocking buffer, the plates were rinsed 2 times with washing buffer and placed under vacuum for drying. Then the plates are ready to use or store. Stored at 4°C in a vacuum storage cabinet with desiccant, the plate can be kept 1 month.

2.3 Assay procedure

A stock melatonin (M-5250, Sigma-Aldrich, St. Louis, MO, USA) standard of 1 mg/mL was prepared by dissolving 10 mg melatonin in 100µL dimethyl sulfoxide (DMSO, D2650 Sigma-Aldrich, St. Louis, MO, USA) first and then in 9.9ml PBS. The stock solution was further diluted with PBS or cell culture medium (M-199, M7528, Sigma-Aldrich, St. Louis, MO, USA) to give 6 individual standards ranging from 50 pg/mL to 10,000 pg/mL. 25 µL of the melatonin calibrators or experimental samples were loaded respectively into each well of the plate, followed by 25µL of Mel-HRP (1:2000 dilution in PBS, CalBioReagents, Inc, San Mateo, CA, USA). Then the plate was incubated at 4°C for 3 hrs. The plate was emptied, and 50 µL of TMB solution were added into each well for 15 min after the plates were washed for 4 times with washing buffer. The color reaction was ceased by 50 µL stop solution (50-85-06, Kpl Inc, Baltimore, MD, USA). OD readings were recorded at 450 nm wavelength by a plate reader.

2.4 Data Analysis

All data were presented as means, standard deviation (SD), and coefficients of variation (CV). Curve fitting programs (4-parameters) from the plate reader software (Microplate Manager 6, Biorad, Hercules, CA, USA) are described in the user manual. Linear regression and EC50 were analyzed by Sigmaplot12.3 (Systat Software, Inc.). All the plots were created also in Sigmaplot12.3. Time course data for validation were subjected to cosinor analysis utilizing linear harmonic regression (CircWave software) [37].

3. Results

3.1 Antibody titration

The optimum amount of melatonin antibody immobilized on the plates was determined from dilution curves (Figure 1). Figure 1 shows the dilution curves for each dilution of melatonin antibody in cell culture medium (M-199 w/ 10% FBS). Although the estimated concentrations at 50% maximal binding, EC₅₀, was very similar in each curve, 0.5µg/mL dilution was selected as a compromise between the highest absorbance and the lowest possible concentration of antibody.

3.2 Analytic sensitivity

The analytical sensitivity or lower limit of detection (LOD) of the assay was determined by the absorbance of the minimum concentration (-2 SD) that did not overlap with the absorbance at zero concentration (2 SD). The sensitivity (defined as the lowest detectable amount of melatonin) was 68pg/mL in cell culture medium and 26pg/mL in PBS with as little as 25µL sample volume.

3.3 Recovery

Culture medium (M-199 with 10% FBS) was enriched with different amounts of melatonin (0, 75, 150, 250, 750, 2000, and 10000 pg/mL). The analytical recoveries were estimated at six different concentrations by using the expected and measured values. The mean recovery for all concentrations was 101.0% (CV=7.5%), ranging between 91.0% and 109.0%, $y=1.071x-56.4$, $R^2=0.99$ (Table 1).

3.4 Specificity

Compounds with chemical structures related to melatonin were investigated to determine possible interferences as shown in Table 2 and Figure 2. The principal cross-reacting compound was 5-methoxytryptophol (0.1%).

3.5 Precision

Assay precision was measured at three samples of low, middle and high concentrations. Inter-assay-precision was analyzed by comparing ten different assay runs (Table 3). Intra-assay-precision was measured (n=20) within one plate. Three pooled samples at different concentrations (low, middle and high) were used as controls.

3.5 Linearity

Series dilution of samples was conducted with cell culture medium. The melatonin concentrations for all dilutions were measured and analyzed by regression analysis. The mean linearity for cell culture medium was 105% (CV=5%), and ranged between 98% and 111%, $y = 5.5263x + 0.0646$, $R^2 = 0.99$.

3.6 Melatonin rhythm of chicken pineal cells from a perfusion system

Melatonin concentration of samples from 6 channels of cultured pineal cells in 6 light: dark cycles (LD) or 3 LD and then 3 cycles in constant darkness (DD) experiments were measured every two hours and plotted in a time line (Figure 3A, B). In LD cycles, the melatonin rhythm from cultured pineal cells was rhythmic (period= 24 hours, $p<0.05$ by Circwave) and robust. Once in DD, the melatonin rhythm damped out in two cycles (Not significantly rhythmic by Circwave).

4. Discussion

Here we report a simple, specific, high-throughput ELISA for quantitative determination of melatonin in cell culture medium and in PBS. This assay is suitable for batched sample measurements with a wide working range of melatonin concentrations, high precision and linearity, and low cross reactivity.

The antibody titer for this ELISA is about 1/10000, depending on the batch of production. Usually, a lower titer of antibody gives better sensitivity. But as a commercial monoclonal antibody, the titer is not comparable to those with the antiserum produced polyclonally in animals (e.g. 1/500[38], 1/2000[39], 1/4000[40], 1/5000[41]). The sensitivity of this ELISA (10pg/mL level) is not as high as those assays (0.1pg/mL to 1pg/mL), but this is not an issue in pineal culture experiments. The sensitivity differences might derive from the properties of

the antibody. However, the antibody of our assay provides a wide working range (68pg/mL to 10ng/mL in M-199 with 10% PBS), and is therefore able to reliably measure melatonin in cell culture medium (usually about 100pg/mL to 10ng/mL) without sample dilution. In addition, the assay adopts melatonin-HRP as the tracer, which saves time and simplifies the procedure, so it's suitable for the batched sample measuring. However, the simplified steps also sacrifices sensitivity, because the signal is not amplified such as in a biotin-streptavidin amplification system.

This assay has high specificity. The major cross-reacting compound is 5-methoxytryptophol at only 0.1%. The high specificity of the antibody may derive from the antigen used to produce the antibody, which is melatonin conjugated BSA as antigen. This is different from common methods in which melatonin derivative conjugates are used as the antigen, such as N-acetyl-5-methoxytryptophan [42], N-acetyl serotonin [28], or 5-methoxytryptamine [30,41]. In these cases, N-acetylated derivatives (N-acetyltryptamine or N-acetylserotonin) are typically the major cross-reactants.

The main advantage of this assay is that it's direct and simple. The working range covered the melatonin concentrations of the outputs from cell culture, so the sample can be directly loaded to the assay without dilution or any other processes. In addition, the plates can be prepared in batches and stored in compact stacks, which saves experimental time, labor and space. Users could prepare enough plates for high throughput samples. Furthermore, the procedure for measurement is simplified to four steps: 1) sample/conjugate incubation (incubating for only 3 hrs), 2) plate washing, 3) tetramethylbenzidine (TMB) color reaction and 4) results reading. In the first step, the competitor melatonin labeled with HRP is mixed with samples at the same time. Therefore, there is only one step before the color reaction, which saves time and labor for batched sample processing. In addition, the assay requires as low as 25µL of sample, potentially the sensitivity can be higher with a larger volume.

Many published methods of melatonin RIA or ELISA involve developing antibodies and radiation or enzyme linked tracer, which are actually non-trivial for general lab use [28,38,40,41,43–45]. On the other hand, commercial products in which the antisera are already produced are usually very expensive. This assay could be conducted easily and quickly under general research or teaching laboratories. All the supplies necessary in this assay are listed in table 4.

The data obtained using this assay are consistent with published results [9–23]. We measured the melatonin rhythm of cultured pineal cells under LD cycles and DD. In LD cycles the rhythm is robust and in DD, the rhythm damps out. These data are consistent with previous studies but with a better temporal resolution [9,10,22]. The results obtained show that the ELISA is applicable in high throughput measuring of melatonin from cultured pineal cells.

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Highlights

A high-throughput, selective and simple enzyme-linked immunosorbent assay for melatonin was developed.

Melatonin from cultured chicken pineal cells on a perfusion system was measured for validation.

The melatonin rhythm of the chicken pineal cells was rhythmic in light/dark cycle but damped in constant darkness.

Figure 1. Dilution curves of melatonin antibody ranging from 0.125ug to 1ug were applied in coating the plates. Standard curves were plotted for determining the optimal concentration for coating, the reading of blank is not show on the plot because of the log-scale of X axis.

Figure 2.
The dilution curves of the seven melatonin analogues with melatonin.

Figure 3. Melatonin output profile (n=6) for 6 days under light/dark cycles. The white bars indicate 12 hours lights on; the black bars indicate 12 hours dark. 0 indicates time of lights on, 12 indicates time of lights off. The time interval between consecutive points is 2 hours. B. Melatonin output profile (n=6) under 3 light/dark cycles and 3 days in constant darkness. Hatched bar indicates constant darkness.

Table 1

Recovery

Expected (added)[pg/mL]	Measured by ELISA[pg/mL]	Recovery [%]
10000	10700±541	107
2000	1827±70	91.4
750	823±39	109.7
250	267±43	106.8
150	136±15	90.7
75	76±12	101.3
0	7±16	--

Table 2

Cross reactivity determined by comparing the dilution curves of the eight indole compounds with those of melatonin.

Compounds	Cross reaction [%]
Melatonin	100
5-Methoxytryptophol	0.1
5-Hydroxytryptophol	0.03
N-acetyltryptamine	0.03
N-Acetyl-5-hydroxytryptamine	0.02
5-Methoxy-DL-tryptophan	0.001
Serotonin	<0.001
DL-Tryptophan	<0.001

Table 3

Intra- and inter-assay-precision for three concentrations of melatonin samples.

	Mean±SD [ng/mL]	CV [%]
<u>Inter-assay-precision, n=10</u>		
Low concentration	0.58±0.09	15.76
Middle concentration	1.30±0.11	8.51
High concentration	2.20±0.35	15.68
<u>Intra-assay-precision, n=20</u>		
Low concentration	0.66±0.10	15.64
Middle concentration	1.91±0.20	10.48
High concentration	4.74±0.32	6.68

Table 4

Order List

Name	Company	Cat #	Number of Samples
Melatonin monoclonal antibody	Calbioreagents	M467	40000
Melatonin-HRP	Calbioreagents	C063	9600
96 Well Plates Thermo Pierce	VWR	PI15041	9600
Protein-Free (PBS) Blocking Buffer	VWR	PI37516	10000
TMB	VWR	PI34028	2500
Stop Solution	VWR	95059-198	4000
Plates Sealer	VWR	53515-986	4800