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Radioimmunoassay for Melatonin and its Application to Fowl Pineal Research

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A radioimmunoassay for melatonin has been developed after raising anti-melatonin antibodies in rabbit. Melatonin was extracted from serum or pineal gland of chickens (*Gallus domesticus*). The radioimmunoassay was performed by using ^{3}H-melatonin as tracer. The standard curve covered the range 0.022-0.345 pmol/vial and the K_D value for melatonin was estimated at 1.37×10^{10} l/mol. The antiserum specificity has been analysed, none of the common melatonin analogues influencing this method of melatonin measurement. The intra-assay variability was 7.2 % for serum samples and 8.6 % for pineal extract. The inter-assay variability for this biological sample was 15.3 % and 6.4 % respectively.

Key words: Melatonin, Pineal, Radioimmunoassay.

The pineal factor, melatonin (MT), has been a subject of interest since LERNER *et al.* (6) isolated and identified it as N-acetyl-5-methoxytryptamin. Subsequently, many attempts have been made to demonstrate a possible central role of this substance in the endocrine system. MT has generally received wide support as the primary substance secreted from the pineal gland which affects the gonadal function in a great number of vertebrate species (10, 11). Furthermore, dramatic effects of MT upon the functional status of the adenohypophysial-thyroidal axis have been reported in several mammalian species (14, 15). Other effects of the pineal gland through MT mediation have been reported to occur in relation to the secretion of growth hormone from the pituitary gland (9) or in relation to the adrenal function (10).

The interest in indolamines as neurotransmitters or neuromodulators has given rise to the development of new analytical techniques, among which the combination of high performance liquid chromatography and electrochemical detection (5, 8) and immunoassay are the most promising. However, the radioimmunoassay technique (RIA) is one of the most extensively employed in the MT determination (1). When a specific anti-

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body is used, RIA is simple and rapid, and can be applied to large number of samples. In this way, we have developed a RIA method and examined its potential ability to determine the MT content in serum and pineal extract of chicken (*Gallus domesticus*).

Materials and Methods

Melatonin (MT) and various MT analogues, bovine serum albumin (BSA), triethylamine and ethylchloroformate were obtained from Sigma and Freund's Complete Adjuvant from Behringwerke A. G. G-25 Sephadex was obtained from Pharmacia Fine Chem. and tritiated MT, n-(2-amino-ethyl-2-^{3}H) acetyl-5-methoxytryptamine, with a specific activity of 40.7 Ci/mmol, from New England Nuclear. The scintillation fluid was 0.018 M-2,5 diphenyloxazole and 5.49×10^{-4} M-1, 4-bis-2-(5-phenyloxazolyl) benzene solution in toluene: triton X-100 (63:33).

Preparation of antigen for immunization.

Synthesis of N-succinyl-5-methoxytryptamine. — One hundred milligrams of 5-methoxytryptamine (5-MT) were dissolved in 20 ml benzene (0.3 mM). Fifty milligrams of succinic anhydride in 5 ml benzene and 250 μl of triethylamine were then added. The mixture was stirred until the components had been dissolved completely. Completeness of the reaction was checked by chromatography on silica gel plates (60G) in chloroform/ethanol/acetic acid (80:19:1 by vol.). N-succinyl-5-methoxytryptamine (succinyl-5-MT) was obtained from waterness phase and crystallized by slow evaporation in vacuum atmosphere. Identity was confirmed using infrared spectroscopy and elemental analysis to carbon, hydrogen and nitrogen.

Synthesis of immunogen. — Immunogen synthesis was carried out by anhydrous mixture method (1). The succinyl-5-MT was redissolved in dioxan at a final concentration of 2×10^{-2} M. Seven hundred microliters of this solution were mixed with 40 μl of 1/11 dilution of triethylamine in dioxan and 20 μl of 1/6 dilution of ethylchloroformate in dioxan. All the operations were carried out at 12° C. After 10 min, 500 μl of the mixture were added to 1 ml of a solution of BSA (13 mg), which had been made slightly alkaline with 10 μl of a 1/11 solution of triethylamine. The mixture was left for 30 min at 30° C and loaded onto a G-25 Sephadex column with 2×10^{-2} M NaCl as eluent. The succinyl-5-MT-BSA conjugate was analyzed by u.v. spectrometry using the following molar coefficients: 5-MT:$\epsilon(300)=4000$; $\epsilon(280)=5490$: albumin: $\epsilon(300)=0$: $\epsilon(280)=34{,}500$. About 40 mol of 5-MT were found to be coupled per mol of albumin.

Immunization procedure. — Male New Zealand White Rabbits were immunized according to the method of VAITUKAITIS *et al.* (13), with slight modification. Each rabbit received 500 μg of immunogen emulsified in 0.75 ml of 0.15 M NaCl and 0.5 ml of Complete Freund's adjuvant. One booster injection was made monthly during 5 months.

Radioimmunoassay. — Extraction of serum and pineal gland melatonin. This was based on the method of WETTERBERG *et al.* (16). Samples of 0.5 ml serum or pineal gland homogenized in phosphate buffer solution (PBS) were placed in plastic vials and, after addition of 5 ml methylene chloride, the tubes were stoppered with plastic closures and shaken at 1,300 rpm for 2 min avoiding emulsions. The two phases were separated by centrifugation at 2,500 × *g* and 4° C for 10 min, and the aqueous phase was aspirated and discarted. Methylene chloride phase was

then put on dry ice for 20 min in order to freeze any of the remaining aqueous phase. After that, the methylene chloride extract was spilling into the assay tubes and evaporated to dryness under a stream of nitrogen at 40° C.

Radioimmunological incubations. — The RIA for MT was performed at 0-4° C as follows: 50 µl PBS-0.1 % gel, 100 µl antiserum (1:3500) and 50 µl ^{3}H-MT (12 pg) were added to the dry residues in the assay tubes; the mixture was gently shaken and then incubated for 16 hours at 4° C. After that, saturated ammonium sulphate (200 µl) was added and, besides being, C, thoroughly mixed and subjected at 4° C for 45 min, the tubes were centrifuged for 30 min at 2,500 × *g* and 4° C. The supernatant (300 µl) was separated and mixed with 10 ml scintillation cocktail, and the radioactivity of the samples was counted in a Packard Tricarb Scintillation Counter for 10 min.

For the antiserum specificity studies, the compounds to be tested and the MT standards were dissolved in 50 µl PBS and processed through the radioimmunoassay without a preliminary extraction.

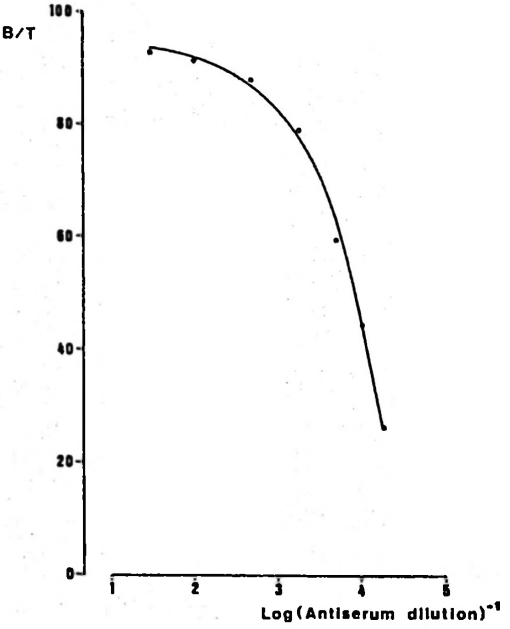

Fig. 1. *Melatonin antiserum dilution curve.* The dilution of the melatonin antiserum are represented on a logaritmic scale and are plotted against the percent binding relativeness to the total of ^{3}H-melatonin.

Results

Antibody titer. — The antibody dilution curve is shown in figure 1; this is the curve obtained when percent binding of the label MT is plotted against antiserum concentration on a logarithmic scale. All of our studies were performed at approximately 60 % binding, using a final antiserum dilution of approximately 1:7000.

Standard curve and reproducibility. — Figure 2 shows the mean and 95 % confidence limits of the extracted standard curve derived from eight assays. The regression of log ($B/B_O - B$) (y) on log pmol MT/vial (x) gave the linear equation $y = -1.38\ x - 1.31$ with a standard error for the slope of ± 0.180. The minimum sensitivity, defined as B_O −2 S.D., was equivalent to about 0.013 pmol/vial.

From this standard curve the equilibrium constant was calculated with a «Scatchard plot». The value obtained was K_D 1.37 × 10^{10} l/mol.

A test with a serum sample containing 0.21 nmol MT/l gave within-assay (n = 10) and between-assay (n = 12) coefficients of variation of 15.3 and 7.2 % respectively. These coefficients of variation were 6.4 and 8.6 % respectively to a pineal extract sample containing 0.22 nmol MT/l.

Antiserum cross-reactivity. — Cross-reactivity is defined as the amount of compound producing a 50 % drop in the binding of ^{3}H-MT by antiserum.

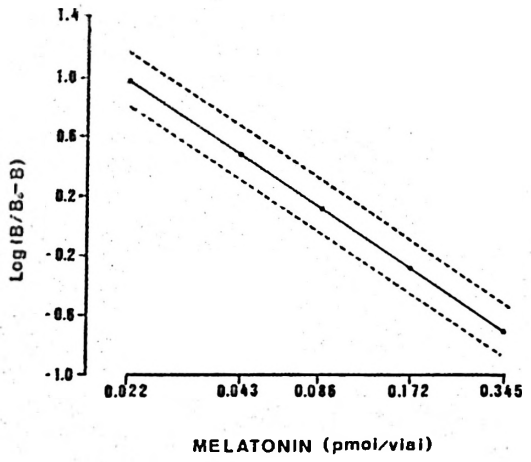

Fig. 2. *Melatonin standard curve: combined standard curve of eight assays with 95 % confidence limits.*
The regression of log (B/B_O − B) (y) on log pmol melatonin/vial (x) gave the linear equation $y = -1.38 \times 1.31$ with a standard error for the slope of ± 0.180.

Table I shows the analogue dilution factor for each of them, which factor causes the MT dose to be inferior to that of the analogue; both doses produce an equivalent reduction in percentage of bound radioactivity. These results show that 5-methoxytryptophol had the highest cross reactivity followed by N-acetylserotonin. All the other compounds tested showed much less cross-reactivity.

Table I. *Cross reaction of various compounds with melatonin antiserum.*

Compound	Analogue dilution factor*
Melatonin	1
N-Acetylserotonin	0.0010
5-Methoxytryptophol	0.0117
5-Hydroxytryptophan	0.0002
5-Hydroxytryptamine	< 0.0002
5-Methoxytryptamine	< 0.0002
Tryptamine	< 0.0002
Tryptophan	< 0.0002
Fluorotryptamine	< 0.0002
5-Methoxy-N,N-dimethyltryptamine	< 0.0002

* Factor by which the dose of melatonin is less than the dose of analogue; both doses producing an equivalent reduction in percentage of radioactivity bound.

Recovery of melatonin added to serum or pineal extract. — Quadruplicated samples of serum (0.5 ml) or pineal extract (0.5 ml) to which different amounts of MT were added (0.022-0.345 pmol) were taken through the extraction and RIA procedures. The results show that recoveries from serum of chicken ranged from 57-89 % and from pineal extract of chicken ranged from 68-98 % over the different concentrations used (table II).

Table II. *Recovery of melatonin added to 0.5 ml of serum or pineal extract.*
Values (pmol/0.5 ml) are means ± S.E.M. of quadruplicates.

Melatonin added (pmol)	Serum		Pineal extract	
	Melatonin measured	Fraction recovered	Melatonin measured	Fraction recovered
0	0.035 ± 0.003	—	0.068 ± 0.004	—
0.022	0.051 ± 0.003	0.89	0.088 ± 0.006	0.98
0.043	0.060 ± 0.004	0.77	0.099 ± 0.006	0.89
0.086	0.082 ± 0.009	0.68	0.123 ± 0.009	0.80
0.172	0.133 ± 0.013	0.64	0.175 ± 0.014	0.73
0.345	0.216 ± 0.023	0.57	0.281 ± 0.020	0.68

Discussion

The experiments presented in this paper were intended to illustrate the possibilities of the radioimmunological method for MT in serum and pineal extract samples of chicken.

The major analytical challenge in quantifying MT concentration is to measure these small amounts in the presence of relatively large concentrations of other indole derivatives such as serotonin and tryptophan. The chicken pineal gland procedures MT in a rhytmic fashion: MT is synthesized at night (in dark-time) (3). The maximum serum levels of this pineal factor are lower than 0.86 nmol/l (10). It is necessary, therefore, to use a specific and very sensitive method of RIA to study the levels of circulating MT in blood. The usefulness of a particular RIA is dependent upon the quality of the antiserum which has been elicited against MT. MT by itself is not antigenic. However, the antibody formation can be elicited by a hapten covalently linked to a large protein molecule. GROTA *et al.* (2) have investigated the conjugation of indole substances to protein in order to stimulate antiserum that would bind MT; the small size of the indolamine molecules turned the conjugation used to produce the immunogen into critical nature. In this investigation, the anhydrous mixture method has been used to produce immunogen, coupling succinyl-5-methoxytryptamine-BSA. This immunogen was able to raise antibodies in a short period of time and its titer was higher than antiserum of others (4, 12, 18). Furthermore, our antiserum recognized unmodified MT fairly well. Therefore, the K_D value for MT ($K_D = 1.37 \times 10^{10}$ l/mol) was higher than the K_D value in the RIA procedure described by GEFFARD *et al.* (1) ($K_D = 2 \times 10^9$ l/mol) which utilizes a radioiodinated MT analog to monitor binding of MT to antibody.

The present method sensitivity was 0.013 pmol/vial (B_O-2 S.D.), which was similar to the method of MARTIN *et al.* (7) and higher than that of WETTERBERG *et al.* (16). The method sensitivity and the standard curve range allow, therefore, to detect small variations, in serum MT concentration due to daily fluctuations of the chicken pineal gland activity.

In addition to MT, our antiserum also recognizes 5-methoxytryptophol, but such recognition was 85.7 times lower than that of MT. This metabolite has been identified as a product of a minor route of serotonin metabolism, present in the pineal gland of cows and hens (17). N-acetyl-serotonin was the other compound which was found to cross-react with MT antiserum, but its binding to the antiserum was 1,000 times less efficient than that of MT. MARTIN *et al.* (7) have verified the extraction recovery of MT analogues with methylene chloride. The extraction efficiency to 5-methoxytryptophol and N-acethylserotonin was 41.9 and 1.4 % respectively. Thus 2 μg/ml of 5-methoxytryptophol in serum would be interpreted as 10 pg/ml of MT. In this way, interference would occur only in the presence of large amounts of 5-methoxytryptophol. All the other compounds indicated that the sensitivity of the present RIA method is more than 1,000 times in favor of MT.

The above mentioned features allow the present RIA method to evalute chicken serum and pineal extract MT with an acceptable degree of accuracy that turns it into a valuable method for the measuring of MT levels in chicken.

Resumen

Se desarrolla un radioinmunoensayo para melatonina, después de obtener anticuerpos específicos anti-melatonina a partir de conejos. La melatonina se extrae de suero y de glándulas pineales de pollos (*Gallus domesticus*). El radioinmunoensayo se rea-

liza utilizando melatonina tritiada como trazador. La curva estándar cubre la escala que va de 0,022 a 0,345 pmol/vial, estimándose el valor K_D para la melatonina en $1,37 \times 10^{-10}$ l/mol. También se analiza la especificidad del antisuero, comprobándose que ninguno de los análogos de la melatonina influye sobre este método de medida. La variabilidad intra-ensayo para las muestras de suero es del 7,2 % y, para los extractos de pineal, del 8,6 %. La variabilidad inter-ensayo para estas muestras biológicas es del 15,3 y del 6,4 % respectivamente.

Palabras clave: Melatonina, Pineal, Radioinmunoensayo.

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