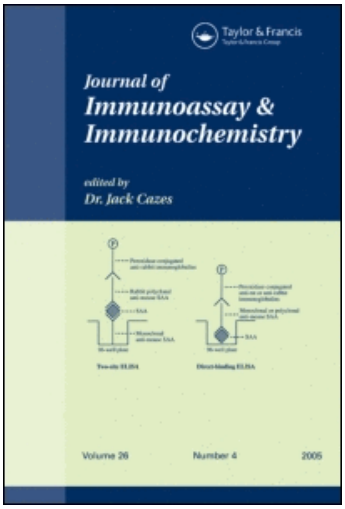

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IMMUNOASSAY OF MELATONIN WITH ENZYME-LABELED ANTIBODIES

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ABSTRACT

This paper reports a non equilibrium competitive enzyme immunoassay method using enzyme-labeled antibodies, for the quantitation of melatonin in chloroform-extracted samples. Its principle is as follows : methoxytryptamine hemisuccinate-human serum albumin conjugate physically absorbed onto a polystyrene sphere and melatonin to be measured, compete for a limited and fixed amount of peroxidase-labeled anti melatonin IgG. After incubation and washings the enzymatic activity bound to the sphere was measured with a chromogenic substrate. This simple method can detect as low as 22 fm of melatonin and is fairly precise. We also present its application to the determination of melatonin in serum and pineal gland.

KEY WORDS : Enzyme immunoassay, Melatonin.

INTRODUCTION

Melatonin, (N acetyl 5 methoxytryptamine M.W. : 232.3) has been identified as the principal hormone of the pineal gland (1). Its presence could be demonstrated in serum (2) retina (3), Harderian gland (3, 4) and brain (5). It is synthesized, at least in mammals, in parenchyme cells of the pineal and is metabolized in the liver by hydroxylation and conjugation (6). Its assay in

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serum shows a circadian rhythm (3). Although the exact role of this hormone is not fully elucidated, it is generally admitted that melatonin controls the sexual maturation through the pineal gland and influences several physiological processes (7).

Melatonin has been measured by sensitive radioimmunoassays (2, 8, 9, 10, 11, 12, 13, 14), but until now no non-isotopic immunoassay method has been published. We have developed an enzyme immunoassay method (EIA) for melatonin, similar to that which we have already published for methotrexate (15). It is based on the measurement of the distribution of an enzyme-labeled anti hapten antibody (anti melatonin-IgG coupled to horse radish peroxidase) between solid phase-hapten (human serum albumin-5 methoxytryptamine hemisuccinate conjugate physically absorbed onto polystyrene sphere) and liquid phase hapten. This procedure is simple to perform, has a sensitivity comparable to that of radioimmunoassay (RIA) with an acceptable precision. It also allows for the determination of melatonin in chloroform-extracted samples.

MATERIALS AND METHODS

5 methoxytryptamine (MT), melatonin and all its analogue compounds, succinic and maleic anhydrides, o-phenylene diamine dihydrochloride (OPD 2 HCl), human serum albumin (HSA), bovine serum albumin (BSA) and thimerosal were obtained from SIGMA, St Louis, Mis. (USA). Hydrogen peroxide, chloroform, perchloric acid, lysine, sodium m-periodate, sodium borhydride were purchased from MERCK, Darmstadt (W. Germany). Horse radish-peroxidase (HRP) grade 1, 250 U/mg, was purchased from Boehringer Mannheim, Mannheim (W. Germany), and was dialyzed against distilled water before coupling. Polystyrene spheres, 6.5 mm diameter, were obtained from Precision Plastic Ball Co. Chicago, Il. (U.S.A).

Methoxytryptamine Derivatives and Immunogens

MT hemisuccinate (S-MT) and hemimaleate (M-MT) were prepared (Fig. 1) and coupled to HSA (S-MT-HSA ; M-MT-HSA) or lysine 5 (S-MT-lysine ; M-MT-lysine) by the mixed anhydride method as described by GEFFARD et al. (8). The concentrations of MT derivatives and the substitution degrees of immunogens were calculated from UV spectrometry measurement taking as molar coefficients (8) MT (300) = 4 000 ; (280) = 5 490, HSA (300) = 0 ; (280) = 34 500.

Substitution degrees were 14.2 and 5.1 for S-MT-HSA and M-MT-HSA respectively.

Antimelatonin Antiserum

The anti melatonin antiserum kindly provided by Pr. M. Delaage, Centre d'Immunologie Luminy, Marseille, France, has been raised in the rabbit with the S-MT-HSA conjugate prepared as described above (8). The titer measured by RIA was 1 : 200000 and the Kd calculated from Scatchard analysis was 10^{-8} M.

Antimelatonin IgG Fraction

An IgG fraction was purified by ammonium sulphate precipitation followed by DE 52 WHATMAN cellulose chromatography in 0.015 M phosphate buffer pH 8.0. From 3 ml of antiserum, 13.3 mg of IgG were prepared. The IgG solution was concentrated to 8g/L by dialysis against polyethylene glycol MW 35 000. It was checked by immunoelectrophoretic analysis and stored at -20° C until conjugation.

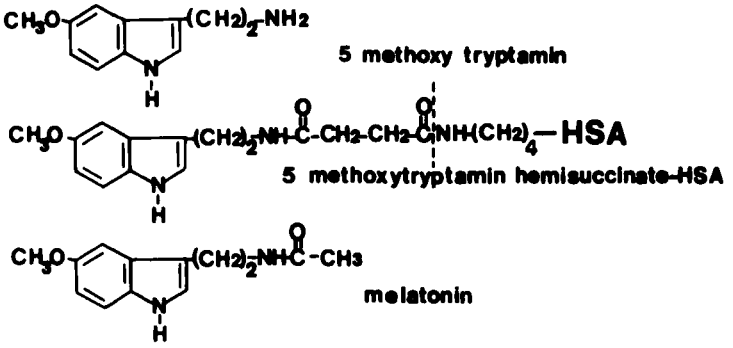

Figure 1 Chemical structure of 5 methoxytryptamine, melatonin and the immunogen 5 methoxytryptamine hemisuccinate-human serum albumin conjugate used for immunisation and to coat the polystyrene spheres.

Antimelatonin IgG-HRP

Eight mg of anti melatonin IgG were conjugated to 4 mg of sodium m-periodate-treated HRP, following the method of WILSON and NAKANE (16). After sodium borhydride reduction, the enzyme-antibody conjugate was precipitated with 1 volume of neutral saturated ammonium sulphate solution, washed twice with 50 % ammonium sulphate and finally dissolved in 8 ml of PBS containing 2 % BSA and 0.01 % thimerosal. After filtration on 0.45 μ Millipore membrane, this solution was aliquoted and kept at -20° C. In these conditions the conjugate was stable at least one year.

Immunogen-coated Polystyrene Spheres

Polystyrene spheres, in batches of one hundred, were rinsed twice with ethanol and distilled water and placed in clean glass beakers. Each batch was covered with 50 ml of 0.1 M phosphate buffer pH 7.2 and allowed to equilibrate for 30 min at + 45° C in a water-bath.

Variable aliquots of the immunogen S-MT-HSA were added under stirring to the different batches (final concentration ranging 0-2.5 µg/L). The spheres were incubated for 2 hours at + 45° C. The immunogen-coated spheres were then washed five times with PBS and kept at + 4° C in this last buffer containing 0.01 % of thimerosal, until use. Under these conditions, the coated spheres were stable at least one month.

Melatonin Standard

Melatonin was first dissolved at 10^{-3}M in ethanol and then at 10^{-4}M in PBS 0.1 % HSA (PBS-HSA). This stock solution was kept at -20° C and further diluted in PBS-HSA in order to prepare a calibration curve ranging from 10^{-11}M to 10^{-6}M.

Samples

Pineals were dissected out from Sprague Dawley rats (strain OFA) after light ether anaesthesia and stored at -20° C until extraction. Rat sera were obtained by aortic puncture. Randomly chosen human sera and cerebrospinal fluids (CSF) were kept at -20° C until the assay.

Extraction Procedure

The whole procedure was done in polypropylene tubes. To 1.2 ml of serum, 0.12 ml of perchloric acid (11.67 N) was added under stirring. The mixture was centrifugated for 5 min at 13 000 g and the supernatant was neutralized with 0.166 ml of 9 N KOH and 0.38 ml of 0.5 M phosphate buffer pH 8.0. The precipitated

potassium perchlorate was eliminated by a short centrifugation and the liquid phase was gently agitated on a rotative shaker with two volumes of chloroform, for 30 min at room temperature. The emulsion was centrifugated for 30 min at 25 000 g and the organic phase was carefully transferred in another tube and evaporated under nitrogen pressure. The dry residue was dissolved with 0.3 ml of PBS-HSA and the solution was kept for 1 hour at room temperature before the assay.

A similar procedure was used to extract the pineal glands and the CSF. In order to estimate the yield of extraction, a pool of serum was made free of melatonin by stirring with 100 mg/ml of charcoal, overnight at +4° C. After centrifugation and filtration on 0.45 μ Millipore membrane, this sample was spiked with a known amount of melatonin, extracted with chloroform as described before and assayed for melatonin content. The recovery was in the range of 60 to 70 %.

Assay Protocol

Immunogen-coated spheres were saturated for 30 min. with 1 % BSA in PBS before use. In order to measure the non specific binding of the enzyme-antibody conjugate, some spheres coated only with 1 % BSA were included in each serie (blank). In disposable polystyrene tray wells, the followings were successively distributed in duplicate : 0.1 ml of calibrators or unknown extracted samples and 0.1 ml of anti melatonin IgG-HRP diluted from the stock solution in PBS-HSA. Immediately, one immunogen-coated sphere was dispensed into each well with a forceps and the tray was incubated for 1 hour at room temperature. The spheres were then washed three times with 5 ml of deionized water using an automatic device (PENTAWASH^{TM}, ABBOTT) and quickly transferred into disposable polystyrene tubes (75 x 13 mm). 0.3 ml of freshly prepared substrate solution (H_2O_2 0.02 % ; OPD 2HCl 3 g/L in 0.1 M

phosphate-citrate buffer pH 5.5 final pH = 5.0) was delivered into each tube and the enzymatic reaction proceeded for 30 min. in the dark, at room temperature. The color development was stopped by addition of 1 ml of 1 N HCl. After vortexing for 10 sec. the absorbances were read directly through the tubes in a computerized automatic photometer (QUANTUM™ ABBOTT) against the blank. Absorbances ratios B/B_o were plotted versus melatonin concentrations on semilog scale paper.

RESULTS

Optimization of the Coating Procedure

The effect of varying the immunogen concentration of the coating solution, on the binding capacity of the immunogen-coated spheres towards labeled antibodies is shown in Fig.2. Maximal capacity was achieved with spheres prepared with 2.5 µg/L solution, but as predictible, better sensitivity was observed with solid phases coated with lower concentration of ligand. Routinely, immunogen concentration resulting in spheres giving an absorbance value equal to 50 % of that of plateau was chosen. The labeled antibodies were then diluted in order to give a maximal absorbance (Bo) in the range of 1.3 - 1.5. Under theses conditions, the immunogen concentration used for coating and the amount of HRP-labeled antimelatonin IgG used par test were 0.2 µg/L and 200 ng respectively.

Standard Curve

Fig. 3 represents a typical calibration curve for the melatonin enzyme immunoassay. The 50 % intercept corresponds to

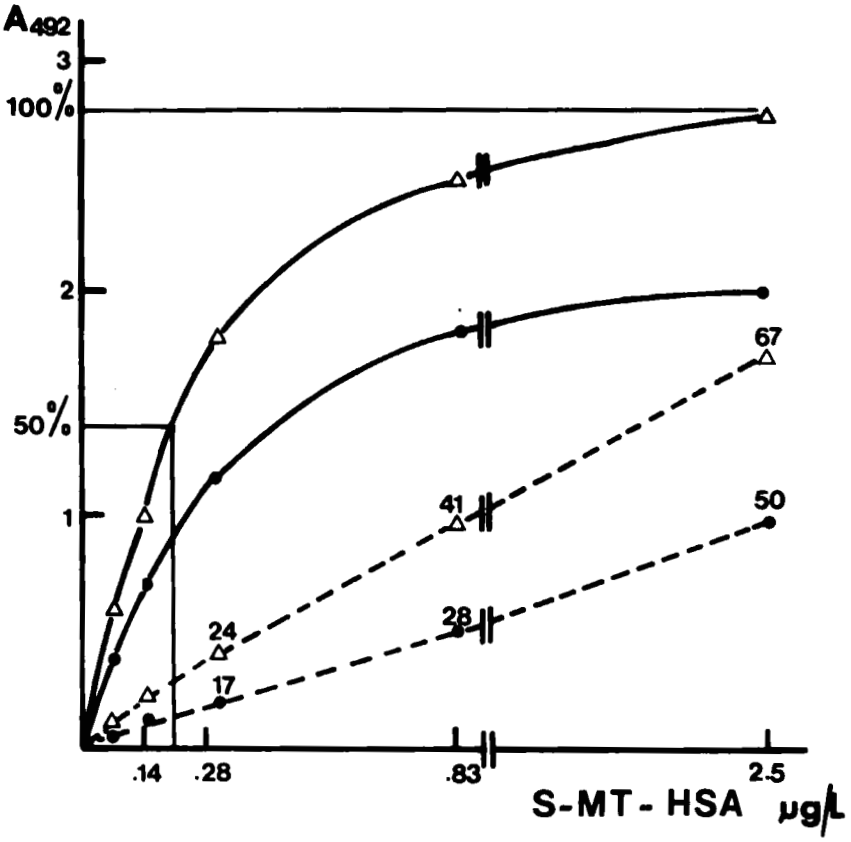

Figure 2 Optimization of the assay. Solid lines : relationship between immunogen concentration used for preparing the spheres and the capacity of the coated spheres to bind 120 ng (dots) or 200 ng (triangles) of anti-melatonin IgG-HRP in absence of melatonin (Bo). The dotted lines represent the corresponding absorbances (B) of the calibrator at 10^{-7} M and the numbers the percent of ratios B/Bo. The immunogen concentration giving spheres having around 50 % of the maximal uptake capacity were selected for the assay.

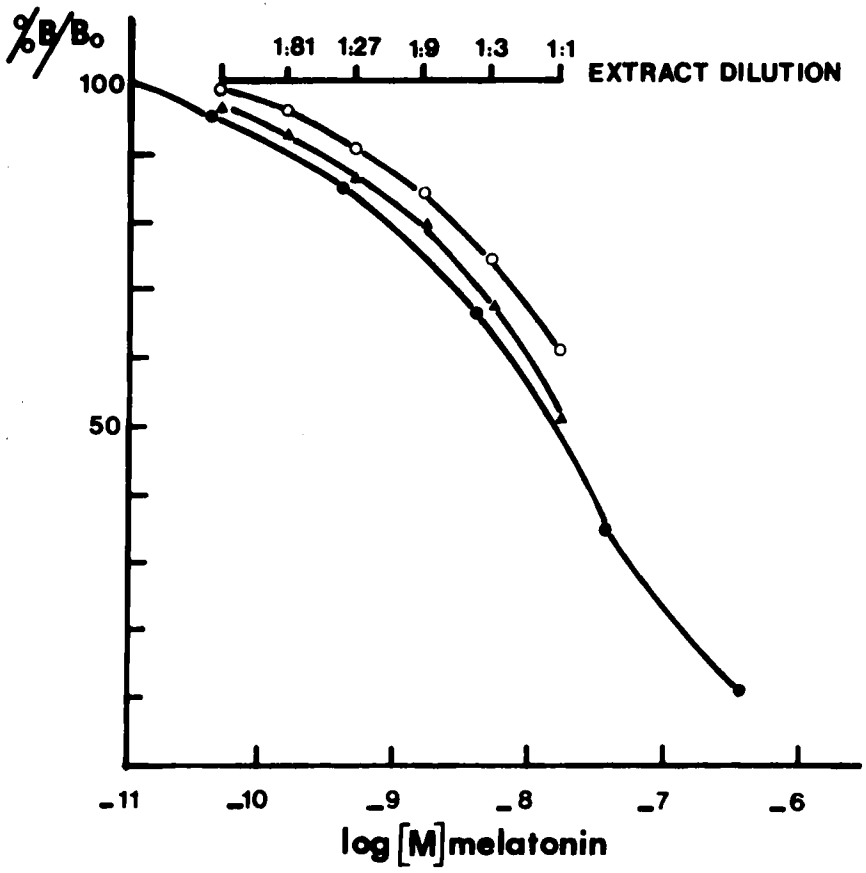

Figure 3 Calibration curve for melatonin enzyme immunoassay. Dots : curve built with melatonin. Triangles : curve drawn with dilutions of a 0.5 ml chloroformic extract obtained from 50 ml of human serum. Open circles : Curve built with dilutions of a perchloric extract of ten pineal glands.

1.7×10^{-8}M of melatonin (1.7 pm) and the smallest quantity detectable (absolute amount of melatonin giving an absorbance value which significantly differs from standard zero with 95 % confidence) was 22 fm (5 pg). In comparison the RIA method (8) using the same antibody was twice as sensitive.

Precision

Four different melatonin samples were assayed ten times in the same serie and four other samples were measured in duplicate on five different occasions. Table 1 shows that the intra and inter assay precisions were acceptable with coefficients of variation ranging from 7.9 % to 13.7 %.

Specificity

The cross reactivity of different chemical analogues of melatonin is shown in Table 2. Because of its small size, all the chemical groups of the molecule contribute to the specificity of the antibody used in this test. All the analogues devoid of N acetyl or methoxy group were poorly recognized. In addition, the 6 position must be free since 6 OH melatonin which is the main metabolite cross-reacted at only 0.64 % rate.

Reasonable parallelism was noted between curves drawn with melatonin and those built with dilutions of perchloric extract of pineals or chloroform-extracted pooled sera (Fig. 2).

Homology Effect

The cross reactions between melatonin and its synthetic derivatives S-MT, M-MT, S-MT lysine and M-MT lysine are listed in Table 3. The results indicate that, in the assay conditions, the lysyl residue which is involved in the covalent binding between S-MT and HSA contributed to the affinity of the antibody since the compound S-MT-lysine was the best inhibitor. The presence of an hemimaleyl group, the configuration of which differs from succinyl, partly prevented this interaction to occur. Thus M-MT lysine and melatonin exhibited a similar affinity towards the

TABLE 1

Intra and Inter Assay Precision :

	N° of replicates	Mean 10^{-10} M	Standard deviation 10^{-10} M	Coefficient of variation %
	10	2.15	0.27	12.5
INTRA	10	74.8	10.3	13.7
ASSAY	10	288	25	8.6
	10	1120	130	11.6
	5	5.3	0.72	13.5
INTER	5	31.7	3.6	11.3
ASSAY	5	214	17	7.9
	5	770	82	10.6

TABLE 2

Cross Reactivity of Chemical Analogues of Melatonin :
cross reactivity was expressed as :

$$\frac{\text{moles of melatonin at 50 \% inhibition}}{\text{moles of analogue et 50 \% inhibition}} \times 100$$

ANALOGUE	CROSS REACTIVITY
Melatonin	100
5 methoxy tryptophol	2.5
N acetyl tryptamine	2.0
6 hydroxy melatonin	0.64
N acetyl 5 hydroxytryptamine	0.25
5 methoxy tryptamine	0.16
5 methoxy indole 3 acetic acid	0.015
5 hydroxy DL tryptophan	< 0.0009
5 methoxy DL tryptophan	< 0.0009
Tryptamine	< 0.0009
N acetyl L tryptophan	< 0.0009
5 hydroxytryptamine (serotonin)	< 0.0009

TABLE 3

Cross Reactivity of Synthetic Derivatives of 5 Methoxytryptamine :

Synthetic derivative	Cross reactivity
Melatonin	100
M-MT	66
M-MT-Lysine	83
S-MT	142
S-MT-Lysine	232

TABLE 4

Concentrations of Melatonin in Pineal and Biological Fluids :

	N° of cases	Mean 10^{-9}M	S.D. 10^{-9}M
Human serum	29	1.35	1.0
Human CSF	4	0.78	0.19
Rat serum	4	0.60	0.08
	N° of cases	Mean pm/gland	S.D.
Rat pineal	5	0.65	0.18

antibody. Consequently an improved sensitivity could be expected by selecting a solid phase, coated with an immunogen prepared from the hemimaleyl derivative. This hypothesis was confirmed and the assay developed with M-MT-HSA as solid phase ligand was found to be twice as sensitive as the reference enzyme-immunoassay.

Assay of Melatonin in Biological Samples

This method was applied to the measurement of melatonin in various conditions. Values found for sera (Table 4) were in the

range of those already published (2). Levels obtained for pineals must be considered as a specificity test, without any physiological significance, as the rats were randomly chosen with respect to their living conditions.

DISCUSSION

The non equilibrium and competitive assay design using enzyme labelled antihapten IgG antibodies as tracer, presented here, for the measurement of melatonin, has been rarely used in the past in the field of low molecular weight compounds. Its mechanism has been discussed elsewhere (15).

Its main advantage lies in the easiness of tracer preparation. The IgG fraction of an antiserum is readily obtainable and there exist several and well codified procedures of enzyme-antibody coupling (17) irrespective to the chemical nature of the hapten to be assayed. In addition enzyme-antibody complexes are generally stable. Conversely, enzyme-hapten coupling requires much attention with regard to parameters such as hapten solubility, availability of amino or carboxylic groups on the enzyme to allow conjugation, substitution degree of the conjugate, loss of enzymatic activity and tracer stability. All these factors can influence to a variable extent the performances of the assay.

However this design which necessitates the use of solid phase-hapten was not in our experience applicable to every compound. Surprisingly, some hydrophobic haptens such as thyroxine and adriamycin conjugated to classical protein carriers by current chemical methods, and physically absorbed onto polystyrene surfaces, were unable to bind the corresponding labelled antibodies. To overcome this problem, some basic work concerning the chemical structure of the solid phase-hapten is required.

When applied to melatonin determination, this method, in spite of its short incubation period, showed a sensitivity

comparable to that of the published RIAs (8, 9, 10, 11, 12, 13, 14). A ten fold increase might be expected by using a fluorogenic substrate (17) such as p-hydroxy phenyl propionic acid. In spite of the absence of a cross validation study and an absolute arbiter of accuracy, the validity of the method was assumed on the basis of the cross reactions study, parallelism tests and measurement of melatonin in biological samples which fell in the expected range. In addition, two parameters of this assay have already been explored in a previous study aimed at the development of a radioimmunoassay of melatonin (8). The first parameter was the specificity of the antibody since we used the same batch. The second was the extraction procedure that we adapted with only minor modifications.

Assay of melatonin in unextracted sera revealed the presence of at least one cross-reacting contaminant which has not been identified and which does not occur in pineal gland. The chloroform extraction eliminated also the possible interference of antiglobulinic factors (18) or the matrix effect due to protein content of serum.

The homology effect which has been discussed elsewhere (15) was partly circumvented in this case by the procedure of DRAY et al., (19).

The sensitivity of this melatonin enzyme-immunoassay is likely to be improved by the selection of other markers such as enzyme-labeled second antibody or protein A, avidin-biotin system or the use of monoclonal antibodies.

However as it stands this EIA provides a simple mean to measure melatonin and using the same design, many other haptens.

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