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TECHNICAL INFORMATION

Catalog Number: 193596
Melatonin - ELISA

Instructions for Use

Enzyme Immunoassay for the Quantitative Determination
of Melatonin in Serum and Plasma

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1.Clinical Significance

The pineal gland (“corpus pineale”) has been called a neuroendocrine transducer because of its important role in photoperiodism. The major hormone of the pineal gland is N-acetyl-5-methoxy-tryptamine or melatonin which is synthesized from the amino acid tryptophan. Melatonin has its highest levels in plasma during night time. The circulatory melatonin is primarily derived from the pineal gland. Its characteristic nocturnal surge appears to encode temporal information such as length of night. Human plasma melatonin levels show marked individual variation. Daytime levels are usually in the range of 20 pg/ml in the morning hours whereas nighttime levels generally exceed 55 pg/ml. However, the inter-individual ranges are significant.

Regulation of the melatonin secretion is under neural control. Sympathetic innervation seems to play a major role via its release of noradrenaline. High affinity binding sites specific for melatonin have been identified in the hypothalamus of the human and many other mammalian species. Altered patterns and/or levels of melatonin secretion have been reported to coincide with sleep disorders, “jet lag”, depression, schizophrenia, hypothalamic amenorrhea, pregnancy, anorexia nervosa and some forms of cancer as well as control of sexual maturation during puberty.

The physiological and pathophysiological role of melatonin is under intensive investigation in recent years.
The half-life of melatonin in human circulation is approximately 47 minutes. Most of the circulating melatonin is metabolized in the liver to 6-hydroxymelatonin and subsequently to 6-sulfatoxymelatonin which is excreted into the urine. The melatonin concentrations in saliva are approximately 30% of plasma levels showing the same typical circadian pattern.

2.Principle of the Test

The Melatonin ELISA is a competitive enzyme immunoassay for the quantitative determination of melatonin in serum. The assay procedure follows the basic principle of competitive ELISA whereby there is competition between a biotinylated and a non-biotinylated antigen for a fixed number of antibody binding sites. The amount of biotinylated antigen bound to the antibody is inversely proportional to the analyte concentration of the sample. When the system is in equilibrium, the free biotinylated antigen is removed by a washing step and the antibody bound biotinylated antigen is determined by use of anti-biotin alkaline phosphatase as marker and p-nitrophenyl phosphate as substrate. Quantification of unknowns is achieved by comparing the enzymatic activity of unknowns with a response curve prepared by using known standards.

3.References

1.Wehrenberg, O., et al.: Bedeutung des Epiphysenhormons Melatonin bei Mensch und Tier; *Zentralorgan Chirurgie* **231 (7)**, pp. 381-386,1985.
2.Reiter, R.J., : The Pineal Gland: A Regulator of Regulators; *Progress in Psychobiology and Physiological Psychology* **9**, pp. 323-355, 1980.
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4.Bartsch, C., et al.: Stage-Dependent Depression of Melatonin in Patients with Primary Breast Cancer; In: *CANCER* **Vol. 64, No. 2**, pp. 426-433, 1989.
5.Poeggeler, B., et al.; Melatonin, Hydroxyl Radical-mediated Oxidative Damage, and Aging: A Hypothesis; *Journal of Pineal Research*, pp. 151-168, 1993.
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7. Brown, et. al.: Light, melatonin and the sleep wake cycle; *J. Psychiatr. Neurosci.*, **Vol. 19, No. 5**, 345-353, 1994.
8. Webb, et. al.: Role of melatonin in health and disease; *Clinical Endocrinology*, **v. 42**, 221-234, 1995.
9. Reiter, R.J.: Functional diversity of the pineal hormone melatonin: Its role as an antioxidant; *Exp. Clin. Endocrinol.*, **v. 104**, 10-16, 1996.

4.Precautions

– For research use only.
– Some reagents contain sodium azide as preservative. Avoid skin contact.
– Wear disposable gloves when handling immunodiagnostic materials.
– Avoid bacterial contamination of reagents.
– The kit components “Control Serum” are made with human Serum. Because no test method can offer complete assurance that infectious agents are absent, this reagent should be handled as **potentially biohazardous material**.

5.Storage and Stability

The Melatonin-ELISA kit is shipped at ambient temperature and should be stored in the dark at 2-8°C. Do not use components beyond the expiration date shown on the kit labels. Do not mix various lots of any kit component within an individual assay.

6.Contents of the Test

6.1 Extraction Columns20 pieces
1 cc/100 mg, ready to use
C18 reversed phase, after elution with methanol use for
extraction of the next samples or store at 4 - 8ºC
protected from dust, re-use up to 4 times.

6.2Assay Buffer, Concentrate1 bottle
50 ml,
phosphate buffer with tween and stabilizer.
Dilute 1:10 with distilled water

6.3Antiserum3 vials
lyophilized, stabilized,
dissolve contents of each
vial in 2 ml distilled water

6.4Microtiter Strips12 strips
each 8 wells, coated with
goat anti-rabbit antibodies

6.5Melatonin-Biotin3 vials
lyophilized, stabilized,
dissolve contents of each vial in 2 ml
of assay buffer.

6.6Standards A-E6 vials
lyophilized, stabilized
dissolve contents of each vial
in 2 ml of distilled water.
Store aliquots at -20°C.

For each lot of standards, refer to vial labels as well as to quality control certificate for exact concentrations.

6.7Control Serum 1 and 22 vials
lyophilized, stabilized,
dissolve contents of each vial
in 2 ml of distilled water. Store
aliquots at -20°C. For concentration

see quality control certificate.

6.8Enzyme Conjugate, Concentrate1 vial
250 µl , concentrate,
anti-biotin-alkaline phosphatase in
tris buffer with stabilizers, dilute
1:81 with Assay Buffer.

6.9PNPP Substrate Tablets9 tablets
p-nitrophenyl phosphate (pNPP)

6.10PNPP Substrate Buffer1 bottle
30 ml, ready for use,
contains diethanolamine.

6.11PNPP Stop Solution1 bottle
10 ml, ready for use,
contains 1 N NaOH with 0.25 M EDTA.

6.12Adhesive foil3 pieces

Materials Required but not Supplied:

– Pipettes 25, 50, 100, 200, 250 µl; Multipette Eppendorf or similar products
– Centrifuge (alternative: vacuum manifold)
– Evaporator centrifuge (speed-vac, alternative: Sample Concentrator by use of nitrogen)
– Rotary mixer
– Microtiter plate mixer
– Polystyrene tubes (12 x 75 mm)
– ELISA reader capable of reading absorbance at 405 nm.
– Distilled water
– Methanol

7.Specimen Collection and Storage

The procedure should be performed with serum or plasma. Do not use hemolytic or lipemic samples. The samples can be stored at 2-8°C for 24 hours. For longer period of storage freeze the samples at -20°C.

Repeated freezing and thawing of samples should be avoided.

For the determination of daytime values blood collection should be performed after 8.00 a.m. due to circadian rhythm.

8.Extraction Procedure

Any sample reading greater than the highest standard should be diluted appropriately with ready for use Assay Buffer prior to extraction.

Note: Filter or centrifuge the samples prior to extraction in order to avoid clogging of the columns.

Each sample including standards and controls have to be extracted.

A. Standard version: Procedure for centrifuge and evaporator centrifuge.

Extraction may be performed in advance. The dried extracts (after evaporation of methanol, see point 8.5) may be stored at 2-8oC or -20oC for up to 24 hours.

This procedure yields approximately 90-100% recovery of added Melatonin in samples.

8.1 Column preparation and conditioning

– Place one extraction column for each sample to be extracted into polystyrene or glass tubes (not less than 12 x 75 mm).
– Add 2 x 1 ml of methanol (undiluted) to columns, centrifuge for 1 minute at **200 x g***
– Add 2 x 1 ml of distilled water to columns, centrifuge for 1 minute at 200 x g.
– Proceed with sample application without delay in order to avoid the columns getting dry.

***Note**: g (relative centrifugal force) is not equivalent to rounds per minute (rpm). The rpm value has to be calculated depending on the radius of the centrifuge. **Example**: At a radius of 142 mm from the rotation centre of the centrifuge, 200 x g are equivalent to approximately 1100 rpm.

8.2 Sample application

– Place each column into correspondingly marked tubes.

– Add 0.5 ml of standards, controls and samples to columns, centrifuge for 1 minute at 200 x g.

8.3 Washing

– Add 2 x 1 ml of 10% methanol in distilled water (v/v) to columns, centrifuge for 1 minute at 500 x g.

8.4 Elution of extract

– Place the columns into clean correspondingly marked polystyrene tubes.
– Add 1 ml of methanol (undiluted) to columns, centrifuge for 1 minute at 200 x g.
– Remove columns from tubes, avoiding drops to be left at the columns.

Use columns for extracting next samples (re-use up to 4 times) or store columns at 4 - 8ºC protected from dust.

8.5 Evaporation and reconstitution of extract

– Evaporate the methanol to dryness and reconstitute the samples with 0.15 ml of distilled water.
– Vortex for at least 1 minute and assay immediately.

B. Alternative extraction procedure: Using a vacuum manifold instead of a centrifuge

For the extraction scheme, please follow points 8.1 to 8.5 accordingly. The volumes remain unchanged.

– Let the solvent pass through the column using vacuum and a flow rate of not more than 5 ml/minute.
– For the samples and extracts use a flow rate of not more than 2 ml/min.
– The evaporation of the solvent may be performed by using an evaporator centrifuge or by nitrogen.

9.Preparation of Reagents

The contents of the kit can be divided into three separate runs. The volumes stated below are for one test procedure with 4 strips (32 determinations). If a larger number of strips is to be used, the volumes have to be changed accordingly.

9.1Standards
Dissolve contents of each vial in 2 ml of distilled water and wait for 15 min. Mix gently. Aliquots should be stored at -20°C.

9.2Control Sera
Dissolve contents of each vial in 2 ml of distilled water and wait for 15 min. Mix gently. Aliquots should be stored at -20°C.

9.3Assay Buffer
15 ml of the concentrate have to be diluted 1:10 with distilled water up to 150 ml . This gives the ready for use Assay Buffer. Store at 2-8°C.

9.4Melatonin-Biotin
Add 2 ml of the Assay Buffer to one vial of lyophilized Melatonin-Biotin and wait for 15 min. Mix gently and use only once. If a larger volume of Melatonin-Biotin is required, vials can be pooled. Do not store reconstituted.

9.5Enzyme Conjugate
Dilute 70 µl of Enzyme Conjugate (concentrated) in 5.6 ml of the Assay Buffer. Prepare freshly for each test run. Use only once. Do not store diluted.

9.6Antiserum
Dissolve in 2 ml distilled water and wait 15 min. Mix gently. If a larger volume of Antiserum is required, vials can be pooled. Do not store reconstituted.

9.7PNPP Substrate Solution (should be prepared during the test procedure)
Dissolve 3 Substrate Tablets in 8 ml of Substrate Buffer. Important: Prepare the substrate solution 10 min. prior to its use and use only once.

10. Test Procedure

10.1 Pipette **50 µl** each of extracted **standards, controls** and **patient samples** into the appropriate wells.

10.2 Pipette **50 µl Melatonin-Biotin** and subsequently **50 ml** of **Antiserum** into each well and shake the plate carefully.

10.3 Seal the plate with the adhesive foil and incubate **overnight (14-20h)** at 2-8°C.

10.4 Wash each well **three times** with **Assay Buffer** (the use of a washer is recommended). Remove the Assay Buffer carefully. Invert plate and remove any remaining liquid by tapping on clean blotting paper.

Note: Sensitivity and precision of this assay is markedly influenced by the correct performance of the washing

procedure.

10.5 Pipette **150 µl** of **Enzyme Conjugate** into the wells.

10.6 Seal with the adhesive foil and incubate for **120 min. at room temperature** on an orbital shaker (500 U/min).

Prepare the PNPP substrate solution 10 min. before end of incubation period!

10.7 Wash each well **3 x** with **Assay Buffer** (see above).

10.8 Pipette **200 µl PNPP Substrate Solution** into each well.

10.9 Incubate at **room temperature** for **20-40 min.** on an orbital shaker (500 U/min).

10.10 Stop the substrate reaction by adding **50 µl Stop Solution** into each well, briefly mix contents by gently shaking the plate.

10.11 Read the optical density at **405 nm** (reference wave length 600-650 nm) with a microtiter plate reader within 60 min. after stopping.

11.Calculation of results

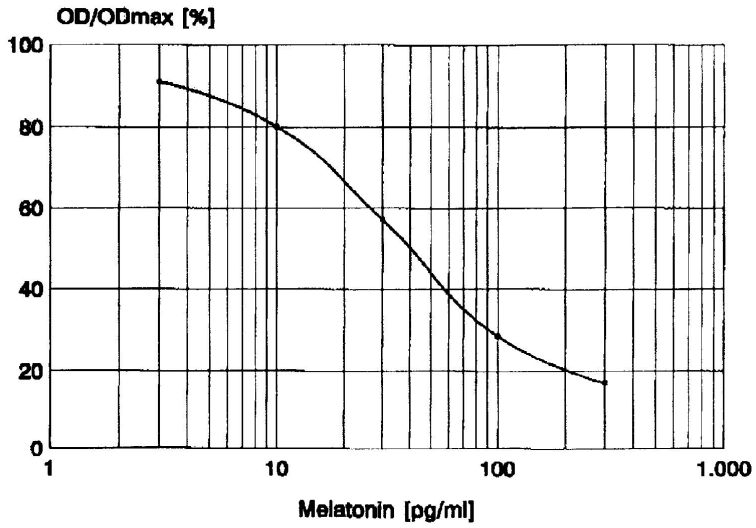
The concentrations of the standards (abscissa, logarithmic) are plotted against their corresponding optical density (ordinate, linear) on a semilogarithmic graph paper.

The concentration of the samples can be read directly from this standard curve by using their average optical density. Any sample reading greater than the highest standard should be diluted appropriately with Assay Buffer prior to extraction and reassaying.

A typical example of a standard curve with the Melatonin-ELISA is listed below.

Concentration (pg/ml)	OD1	OD2	Mean Value OD	OD/ODmax (%)	CV (%)
0.0	1.489	1.545	1.517	100.0	2.6
3.0	1.389	1.376	1.383	91.1	0.7
10	1.222	1.207	1.214	80.1	0.9
30	0.872	0.861	0.867	57.1	0.9
100	0.442	0.426	0.434	28.6	2.6
300	0.263	0.257	0.260	17.1	1.6

Typical standard curve



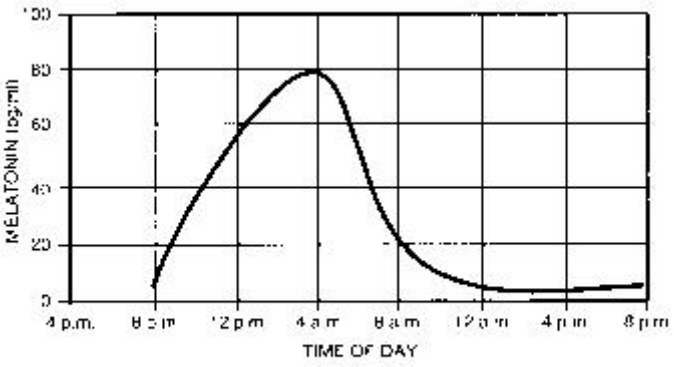
12. Assay Characteristics

A. Expected values

It is recommended that each laboratory establishes its own range of normal melatonin values.

The melatonin levels in humans show a marked circadian rhythm characterized by very low levels during daytime and high levels during night time, and show a considerable interindividual variation. Furthermore, the melatonin concentration is age dependent..

The figure below shows the circadian rhythm of melatonin in serum in a group of 6 healthy persons. The mean value reaches a minimum of about 4.6 pg/ml during day time at 4 p.m. and a maximum of about 77.5 pg/ml during night time at 4 a.m.



B. Specificity

The cross-reactivity of the antiserum has been measured against various compounds.

Compound	Cross-Reactivity (%)
Melatonin	100.0
5-Methoxytryptophol	0.5
N-Acetyl-Serotonin	0.4
5-Methoxytryptamine	0.1
5-Hydroxy-L-Tryptophan	0.01
6-Methoxytryptamine	<0.01
5-Methoxyindole-3-Acetic Acid	<0.01
Serotonin	<0.01
DL-Tryptophan	<0.01
DL-5-Methoxytryptophan	<0.01

C. Sensitivity

The lowest detectable level is 3.0 pg/ml.

D. Precision

INTRA-assay variation (Enzyme Immunoassay)

(all concentrations in pg/ml)
MeanStandardCV (%)n
Deviation
6.80.59 8.812
19.22.0 10.512
48.91.6 3.312

INTER-assay variation (Enzyme Immunoassay)
(all concentrations in pg/ml)
MeanStandardCV (%)n
Deviation
9.21.515.712
745.0 6.912

INTER-assay variation (Extraction and Immunoassay)
(all concentrations in pg/ml)
MeanStandardCV (%)n
Deviation
5.20.9919.010
343.09.010

E. Linearity

Unknown patient samples were diluted with diluent buffer before sample preparation and assayed. The calculated results are shown in pg/ml.

Dilution	**undiluted**	**1/2**	**1/4**	**1/8**	**1/16**
Sample 1	300 (100%)	312 (104%)	324 (108%)	318 (106%)	328 (109%)
Sample 2	233 (100%)	225 (97%)	233 (100%)	290 (124%)	224 (96%)
Sample 3	114 (100%)	129 (113%)	133 (116%)	115 (101%)	99 (87%)
Sample 4	173 (100%)	174 (100%)	202 (117%)	163 (94%)	139 (81%)

F. Recovery

Human serum was enriched with increasing amounts of melatonin (concentrations in pg/ml).

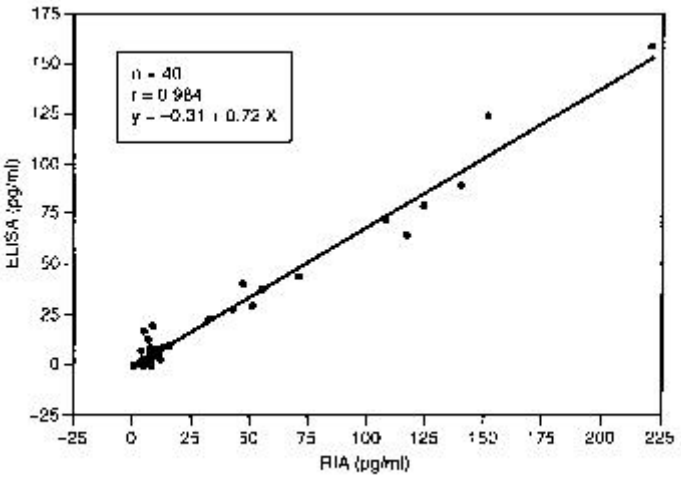
ActualAmountAmountAmountRecovery
conc.addedrecoveredexpected%
Sample 17.7513.312.7105
7.71017.217.797
7.72026.227.795
7.74051.347.7108
7.78085.487.797
7.7160158.4167.794

Sample 27.5512.912.5103
7.51018.517.5 106
7.52028.027.5102
7.54047.847.5101
7.58087.687.5100
7.5 160138.0167.582

Sample 36.6511.111.6100
6.61016.516.6115
6.62025.426.6139
6.64049.886.6131
6.68082.086.695
6.6160161.6166.697

Sample 48.3512.112.398
8.31017.118.393
8.32027.828.398
8.34048.648.3101
8.38088.088.3100
8.3160163.2168.397

G. Method comparison ELISA - RIA



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13.Warranty

The single components of each kit are carefully matched. In case of exchange or mixture of any components from different lots, the manufacturer does not guarantee reliable results.