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Isolation of melatonin by immunoaffinity chromatography

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Abstract

A single-step, highly specific and easy-to-use method was developed for isolation and purification of melatonin from complex biological matrices. Polyclonal antibodies highly specific against melatonin (with cross-reactivities with related compounds below 0.02%, except for 6-hydroxymelatonin) were raised, characterised by enzyme-linked immunosorbent assay (ELISA) and used for preparation of immunoaffinity gel. Melatonin recovery by the immunoaffinity method was ~95%, allowing single-step processing of samples prior to electrospray HPLC–MS analysis (with detection limit 10 fmol). The method was successfully used for determining melatonin in human serum and turned out to be better than the non-specific solid-phase extraction published earlier. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) is a neurohormone discovered by Lerner [1] to be secreted by the pineal gland. It was found to play an important role in circadian rhythmicity [2] and photoperiodic responses not only among various members of the animal kingdom [3] but also in plants [4]. The role of melatonin as radical scavenger [5] and the relationship between health and well-being on the one hand and melatonin level in human blood [6] on the other has been the subject of many investigations.

Determination of melatonin may be based on antibodies (radioimmunoassay [7], enzyme immunoassay [8]) or instrumental techniques, e.g. reversed-phase HPLC using electrochemical [9] or fluorescence detection. Both native fluorescence [10] and fluorescence of derivatised melatonin [11] may be exploited in the assay. Another technique, a combination of gas chromatography and negative ion chemical ionisation [12] or electron impact mass spectrometry (for review see Ref. [13]), requires prior derivatisation by, for example pentafluoropropionic anhydride [14]. The use of HPLC coupled to positive electrospray-tandem mass spectrometry has also been described [4], with multiple reactant monitoring of m/z 233 $[M]^+$ and m/z 174. Here, prior extraction or a purification step is again generally necessary, depending on the complexity of the matrix. For instance, determination of melatonin in human plasma by HPLC with fluorescence detection has been preceded by chloroform extraction [15] or C_{18} solid-phase extraction [16]. On the other hand,

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Bechgaard et al. [17] subjected the plasma to HPLC with fluorescence detection with no prior extraction or purification, achieving a detection limit of 1 ng per ml of human plasma (4 pmol/ml). Melatonin from the rat pineal gland was readily extracted with methanol [11] while the purification of melatonin from plant material is more complicated [4]. Since plant tissue contains significantly higher amounts of radicals than animal tissue, it is necessary to work under special preservative conditions [18].

To make the purification of melatonin faster and easier, we raised highly specific rabbit polyclonal anti-melatonin antibodies and characterised them by enzyme-linked immunosorbent assay (ELISA). In this paper, we also describe the use of the antibodies for preparing and performing immunoaffinity chromatography. To demonstrate the potential of the reported chromatographic method, we isolated melatonin from human blood serum and analysed it by HPLC/single-quadrupole mass spectrometry. We showed immunoaffinity purification (IAC) to be preferable to solid-phase extraction (SPE), yielding an extract with a lower amount of ballast compounds.

2. Experimental

2.1. Materials

Melatonin was obtained from Monelli (Olomouc, Czech Republic). The other indole compounds, bovine serum albumin and rabbit IgG were purchased from Sigma (St. Louis, MO, USA). Alkaline phosphatase for enzyme immunoassay (2500 U/mg) and *p*-nitrophenylphosphate were from Boehringer (Mannheim, Germany). [*O*-methyl-$^3H_3$]Melatonin with a specific activity of 3.07 TBq/mmol was obtained from Amersham Pharmacia Biotech (Little Chalfont, UK), [*O*-methyl-$^2H_3$]melatonin was synthesised by Dr Jan Hanuš (Isotope Laboratory, Institute of Experimental Botany, ASCR, Prague, Czech Republic; the synthesis will be described later). HPLC-grade methanol was purchased from Riedel-de Haën (Seelze, Germany). Water was purified by the Simplicity 185 water purification system (Millipore, Bedford, USA). Other solvents and reagents were of reagent grade, provided by either Sigma (St. Louis, MO, USA) or Lachema (Brno, Czech Republic).

2.2. Preparation of antibodies and enzyme-linked immunosorbent assay (ELISA)

The methylene-bridged melatonin–bovine serum albumin conjugate was prepared by Mannich synthesis as described earlier [19] in a hapten:protein molar ratio of 19. The same reaction was used for the synthesis of melatonin–alkaline phosphatase conjugate. The immunisation schedule used and purification of antibodies were done following the procedures described by Strnad et al. [20,21].

Anti-melatonin antibodies isolated from the rabbit serum by ammonium sulphate precipitation were used in ELISA as follows.

A 96-well microtitre plate (Sarstedt, Nümbrecht, Germany) was coated with 150 μl per well of rabbit anti-melatonin antibodies (1.5 μg/ml) in 50 m*M* $NaHCO_3$ (pH 9.6) and incubated at 4 °C overnight. The wells were washed with distilled water, filled with 200 μl of bovine serum albumin solution (0.2 g/l) in Tris-buffered saline (TBS; 50 m*M* Tris, 10 m*M* NaCl, 1 m*M* $MgCl_2$, 0.1% NaN_3, pH 7.5), and the plate was incubated at 25 °C for 1 h. The wells were then washed twice with distilled water and subsequently filled with 50 μl of TBS, 50 μl of standard or tested compound in TBS and 50 μl of melatonin–alkaline phosphatase tracer (diluted in TBS-solution of bovine serum albumin, 0.2 g/l). After 1-min shaking and 1-h incubation (25 °C), the plate was decanted with distilled water (twice) and TBS (twice) and filled with 150 μl per well of *p*-nitrophenylphosphate solution (1 mg/ml) in 50 m*M* $NaHCO_3$ (pH 9.6). It was then incubated for 1 h at 25 °C. The reaction was stopped by adding 50 μl per well of 0.5 *M* NaOH and absorbance was measured at 405 nm using the Labsystem Multiscan®PLUS (Labsystems, Finland). The resulting data were processed by a Wia-Calc computer program (LKB, Bromma, Sweden).

2.3. Preparation and use of immunoaffinity columns

The total IgG fraction (including the anti-melatonin antibodies) was isolated from the rabbit

serum using protein A bead column and concentrated by ammonium sulphate precipitation. Subsequently, the antibodies were dialysed against 0.1 MOPS (pH 8.0) and coupled to an *N*-hydroxysuccinimide-ester activated agarose (Affi-Gel 10, Bio-Rad, Hercules, USA; 25 mg/g). The immunoaffinity gel so prepared was dispensed into plastic cartridges (0.5 ml per unit) and stored in phosphate-buffered saline (PBS; 50 m*M* NaH_2PO_4, 15 m*M* NaCl, 0.1% NaN_3, pH 7.2) at 4 °C. Capacity of the columns was estimated using [*O*-methyl-$^3H_3$]melatonin and the recovery of different amounts of melatonin was estimated by using deuterium-labelled melatonin. The following procedure was developed for using the columns: a thrice repeated application of the melatonin sample in PBS (0.5–3 ml): washing with PBS (0.5 ml); washing with double distilled water (9 ml); elution of retained melatonin with MeOH (3 ml, −20 °C); washing with double distilled water (6 ml); and washing with PBS (9 ml). Before each sample purification, the immunocolumns were preconditioned with 9 ml PBS, 9 ml H_2O, 6 ml MeOH (−20 °C), 6 ml H_2O, and 9 ml PBS.

To eliminate compounds unselectively binding to the immunoreactive gel, the samples were first passed through precolumns containing 0.5 ml gel with immobilised rabbit globulins. The gel was prepared by coupling rabbit IgG to the Affi-Gel 10 (25 mg/g) and stored under the same conditions as the immunoaffinity matrix.

2.4. HPLC–MS analyses

Isocratic HPLC was performed using a Waters 2690 Separations Module (Waters, Milford, USA) equipped with 100-μl sample loop. The samples were injected on a C_8 reversed-phase column (Symmetry C8; 5 μm; 150×2.1 mm I.D.; Waters, Milford, USA) kept at 30 °C and eluted with a mobile phase containing 40% methanol and 10 m*M* ammonium formate (pH 3.6), flow-rate 250 μl/min. Using post-column split of 1/1, the effluent was introduced into a ZMD single-quadrupole mass spectrometer (Micromass, Manchester, UK) and Waters 996 PDA detector (Waters, Milford, USA).

The mass spectrometer was equipped with an electrospray interface (capillary voltage +4.5 kV). The source block temperature was 150 °C, the desolvation gas flow-rate was 550 l/h, the cone gas flow-rate was 60 l/h, and the cone voltage was 52 V. The mass spectrometer was tuned with a melatonin solution ($5 \cdot 10^{-5}$ *M*) in mobile phase, flow-rate 20 μl/min. For quantification, selected ion recording mode of m/z 174 (as described in Ref. [4]) and 177 was used for unlabelled and deuterium-labelled melatonin, respectively (dwell time 1.45 s, inter-channel delay 0.02 s, span 0.1 μm). The data were processed by the MassLynx software (Micromass, Manchester, UK).

The PDA detector was used in the scanning range of 240–320 nm, with 1.2-nm resolution.

2.5. Estimation of melatonin in human serum

The blood of five Caucasian males aged 27–34 years was used for estimation of melatonin in biological materials. The men stayed in a 17-h light/7-h dark cycle for 7 days before the experiment. Blood collection was carried out 1 h before the end of the dark period into test tubes with no special treatment. For measurement of recovery, the blood of a 28-year-old man from the above group was taken 7 h after the end of the dark period to obtain ‘‘zero’’ serum with nearly zero level of melatonin.

The blood was left at 25 °C for 1 h, centrifuged (600 *g*, 10 min, 4 °C) and the serum dispensed into silanized plastic microtubes (1 ml per microtube). The serum was either frozen at −20 °C or processed at once.

Immunoaffinity chromatography was done by subsequent application of the sample on precolumn and immunoaffinity column. A 1-ml sample of serum was diluted with 1 ml of phosphate-buffered saline (PBS; 50 m*M* NaH_2PO_4, 15 m*M* NaCl, pH 7.2) and 200 fmol of [*O*-methyl-$^2H_3$]melatonin was added as an internal tracer. The mixture was gently mixed and passed through a 0.22-μm Optex filter (Millipore, Bedford, USA). The filtrate was passed through the precolumn. The precolumn was washed with two 0.5-ml portions of PBS, the fractions taken together and applied on the preconditioned anti-melatonin immunoaffinity column. The solution was collected and its application repeated three times. The column was rinsed with PBS (0.5 ml) and double distilled water (9 ml). Melatonin retained on the column was

eluted with MeOH (3 ml, −20 °C, HPLC grade) into a silanized glass tube. After evaporation of MeOH, the residue was reconstituted in 25 μl of MeOH and 100 μl of ammonium formate (10 m*M*, pH 3.6) was added. A volume of 50 μl of the mixture was injected for HPLC–MS quantification of melatonin.

A comparative purification using solid-phase extraction was done as follows [16]: 1 ml of the serum was passed through C_{18} sorbent (BondElut® C18, 1 g per cartridge, Varian, Harbor City, USA), the column was washed with 10% methanol (1 ml) and eluted with two 300-μl portions of methanol. The eluate was dried and analysed by HPLC–MS as described above.

3. Results and discussion

3.1. Anti-melatonin antibody characteristics and immunoaffinity chromatography

The anti-melatonin antibodies were characterised by enzyme-linked immunosorbent assay (ELISA). Table 1 summarises some of the parameters of ELISA based on the antibodies. The ELISA was used for estimating the cross-reactivities of various melatonin-related compounds with the antibodies (Table 2).

As already mentioned by Brown and Grota [22], specific antibodies are reliably obtained if the melatonin methylene-bridged conjugate (melatonin coupled at position 1 or 2 of the indole nucleus) is used to produce the antisera. The conjugation at or near the indole nitrogen may therefore be recommended for preparing specific antibodies against indolealkylamines. Table 2 documents that, except for 6-hydroxymelatonin showing cross-reactivity of 2.6%, none of the tested compounds exceeded the value 0.02%. This makes the antibodies ideal for use in immunoaffinity chromatography to perform compound-specific purification and estimation of melatonin.

Table 1
Parameters of enzyme immunoassay based on polyclonal anti-melatonin antibodies

Parameter	
Amount of tracer/assay	11 ng
Detection limit	$1.0 \cdot 10^{-8}$ *M* (0.5 pmol)
Linear average of logit/log plot	$(1.0 \cdot 10^{-8}–3.1 \cdot 10^{-5})$ *M* (0.5–1500) pmol
Midrange (50% binding)	$1.8 \cdot 10^{-7}$ *M* (9 pmol)
Unspecific binding	1.2%
Intraassay variance[a]	1.9%
Interassay variance[b]	5.4%

[a] Eight replicates.
[b] Ten replicates.

Table 2
Molar cross-reactivities of various melatonin-related compounds with anti-melatonin antibody

	Cross-reactivity (%)
Melatonin	100
6-Hydroxymelatonin	2.6
Indole	<0.02
Indole-3-acetaldehyde	<0.02
Indole-3-acetamide	<0.02
Indole-3-acetic acid	<0.02
Indole-3-acetic acid ethylester	<0.02
Indole-3-acetone	<0.02
3-β-Indoleacrylic acid	<0.02
Indole-3-butyric acid	<0.02
Indole-3-carbinol	<0.02
Indole-3-carboxylic acid	<0.02
Indole-3-ethanol	<0.02
DL-Indole-3-lactic acid	<0.02
Indole-3-propionic acid	<0.02
Indole-3-pyruvic acid	<0.02
5-Hydroxyindole-3-acetic acid	<0.02
L-Tryptophan	<0.02
N-Acetyl-DL-tryptophan	<0.02
5-Methyl-DL-tryptophan	<0.02
Tryptamine	<0.02
5-Hydroxy-tryptamine (serotonin)	<0.02
N-Acetylserotonin	<0.02

The values are expressed as a per cent ratio of molar concentrations of melatonin and competitor at 50% binding.

The immunoaffinity chromatography was done by subsequent application of the sample on precolumn and immunoaffinity column, each containing 0.5 ml of the preimmune or immunoaffinity gel, respectively. Measurement of melatonin retention at various applied concentrations of melatonin with tritium tracer (data not shown) showed that the capacity of the immunoaffinity columns was ~750 pmol per cartridge, while the retention on precolumns was practically zero.

To estimate the recovery of the immunoaffinity chromatography, we applied various mixtures of authentic and deuterium-labelled melatonin (both in

3 ml of PBS and in a mixture of 2 ml of PBS and 1 ml of "zero" serum obtained from blood collected 7 h after the end of the dark period when almost zero level of melatonin might be expected) and subsequently determined its contents in the eluates by HPLC–MS (data not shown). According to the expected levels of melatonin in human blood serum, we analysed the recovery at the following amounts of authentic melatonin: 50, 100, 200, and 400 fmol. Each of the applied solutions contained 200 fmol of deuterium-labelled melatonin. In all cases, the recovery varied from 92 to 98%, i.e. a value comparable with that obtained with SPE [16]. However, since immunoaffinity purification is based on specific interactions between antibody and antigen, it has the advantage of a lower amount of impurities in the eluate as compared with SPE.

We also measured the recovery and capacity of the immunoaffinity columns after 20 and 40 cycles. The recovery remained practically unchanged while the capacity slightly decreased after 40 runs (~700 fmol per cartridge).

3.2. HPLC–MS

High performance liquid chromatography coupled to positive electrospray-quadrupole mass spectrometry was developed for quantitative analyses of melatonin. The calibration curve was measured in the range from 10 fmol to 10 pmol using 10^{-8}, 10^{-7}, and 10^{-6} *M* melatonin solutions. The detection limit of melatonin was 10 fmol ($S/N=3$).

Quantification of melatonin was done by using deuterium-labelled melatonin. To different concentrations of authentic melatonin a constant amount of deuterium-labelled melatonin was added to form seven solutions containing 10^{-8} *M* of deuterated tracer (concentration$_{dx}$) and authentic melatonin in concentrations 1, 2, $5\cdot10^{-9}$ M; 1, 2, $5\cdot10^{-8}$ M; and $1\cdot10^{-7}$ *M* (concentration$_x$). Concentration$_{dx}$ was chosen in accordance with the mean concentration of melatonin in human blood serum [23]. Corresponding area$_{dx}$ and area$_x$ were measured at SIR of 177 for labelled and 174 for authentic melatonin, respectively. The calibration curve obtained after log transformation shows a linear fit with slope 1 and intercept 0:

$$x=(0.9947\pm0.0054)y+(-0.0160\pm0.0037)$$

where $y=\log(\text{area}_x/\text{area}_{dx})$ and $x=\log(\text{concentration}_x/\text{concentration}_{dx})$. Fig. 1(a) shows a chromatogram of the 1:1 mixture of authentic (200 fmol) and labelled (200 fmol) melatonin measured at $m/z=174$ and 177, respectively.

3.3. Determination of melatonin in human serum

Our IAC procedure was used for isolation of melatonin from human blood serum with subsequent HPLC–MS analysis. The melatonin level of 200 fmol per ml of sera (40 pg/ml) or higher might be expected [23] under the chosen conditions (five

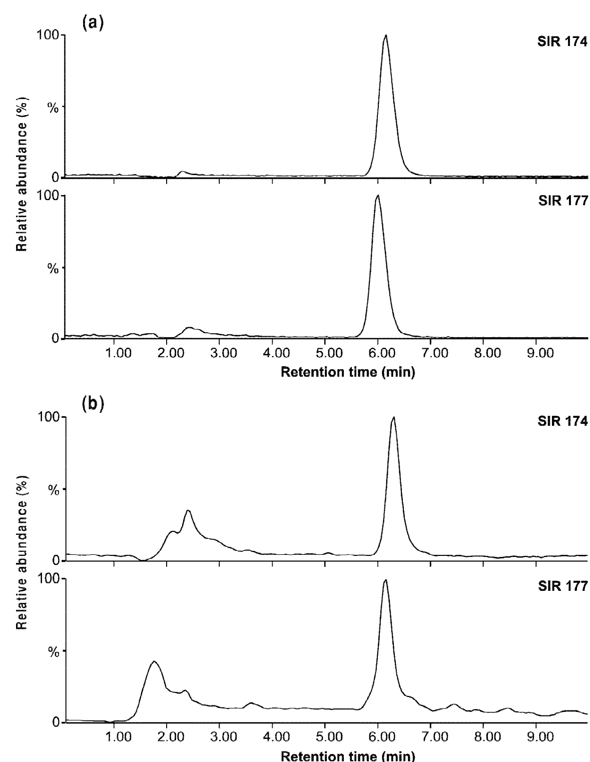

Fig. 1. LC–MS analysis of a 1:1 mixture of authentic (200 fmol) and deuterium labeled (200 fmol) melatonin standards (a) and LC–MS analysis of the real sample (81 fmol of authentic and 76 fmol of deuterium labeled melatonin injected) after immunopurification (b), both measured at $m/z=174$ and 177 for authentic and labeled melatonin, respectively. The sample processing and measurements were performed as described in the Experimental section.

Table 3
Melatonin levels in sera of five male volunteers

Volunteer number	Age of volunteer	Estimated level of melatonin[a] (fmol/ml)
1	27	227±9
2	28	181±16
3	29	305±22
4	31	136±12
5	34	204±19

The sera were collected after a periodic light/dark cycle 1 week in duration, 1 h before the end of the dark period. A 1-ml sample of serum was purified by immunoaffinity chromatography and the level of melatonin was estimated by HPLC-(ES)+MS.
[a] The samples were processed in triplicates.

males aged 27–34 years exposed to a periodic light/dark cycle for 1 week before the experiment, blood collected 1 h before the end of the dark period). The amount of 200 fmol of deuterium-labelled melatonin was therefore added to 1 ml of individual serum samples. The samples were processed in triplicates. The mass chromatogram of one of the samples at $m/z=174$ and 177 is displayed in Fig. 1(b). The estimated concentrations of melatonin ranged from 136±12 fmol (31.6±2.8 pg) to 305±22 fmol (70.8±5.1 pg) per ml of serum (Table 3).

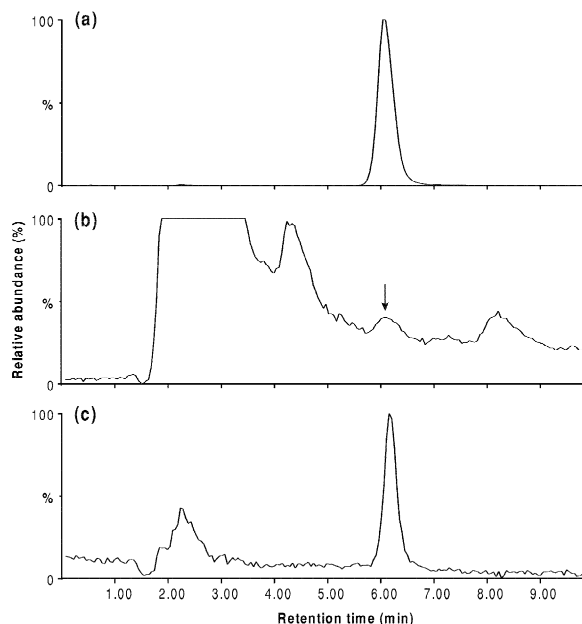

Fig. 2. Comparison of LC–MS analyses of human serum sample processed by solid-phase extraction (b, the melatonin peak indicated by an arrow) and by immunoaffinity purification (c) and LC–MS analysis of melatonin standard (a). Respective amounts of injected melatonin were 500 fmol (a) and 68 fmol (c). The ion chromatograms were obtained by measurement at $m/z=174$.

To compare the method with solid-phase extraction, we processed the serum of one of the males by a method described in Ref. [16] which consists of retention of plasma melatonin on C_{18} cartridge, its elution with methanol and subsequent analysis by HPLC with fluorescence detection. However, we used HPLC coupled to ES+ mass spectrometry instead. Fig. 2 shows a comparison of chromatograms obtained by analyses (at $m/z=174$) of the sample processed by the SPE method and by immunoaffinity purification, respectively. Immunoaffinity purification (IAC) appears to be preferable to solid-phase extraction (SPE) since it yields an extract with a lower amount of ballast compounds.

4. Conclusion

Highly specific anti-melatonin antibodies were raised and used for development of a highly efficient immunoaffinity chromatographic method. The immunoaffinity columns so prepared were successfully used for single-step purification of melatonin from human serum. With its recovery of more than 90%, the method is applicable to the isolation of melatonin prior to HPLC–MS analyses. The high specificity of the presented method is useful especially if compared with solid-phase extraction, which is commonly used for isolation of melatonin from biological materials. Possible utilisation of the method for analyses of melatonin in more complicated matrices (e.g. plant material) is under investigation.

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