

# Highly Sensitive and Specific Assay of Plasma Melatonin Using High-Performance Liquid Chromatography with Fluorescence Detection Preceded by Solid-Phase Extraction

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## Abstract

This paper describes a new, highly sensitive, rapid method for the determination of low levels of endogenous melatonin, the pineal and retinal hormone, in fish plasma samples. The combination of solid-phase extraction and high-performance liquid chromatography with fluorescence detection significantly improves the separation and increases the sensitivity of the assay. The proposed method may be a useful alternative to previously reported procedures. The assay should be applicable to plasma and tissue samples from other animals, including humans, with only minor modifications.

## Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine), the principal hormone of the pineal gland and retina of vertebrates, is implicated in physiological processes that are controlled by photoperiod: circadian rhythms, reproduction, and behavior. Circulating melatonin concentrations increase in response to melatonin production during darkness. Light suppresses or inhibits melatonin production.

High-performance liquid chromatography (HPLC) has been used for the determination of pineal indoles, especially melatonin (1–8). Compared with radioimmunoassay (RIA), the principal advantages of these methods are that sample preparation is relatively simple, radioisotopes are unnecessary, and the assay may be more specific due to potential cross-reactivity of antibodies in RIAs. The most difficult part of many chromatographic analyses of biological samples is the elimination of interfering matrix components. If the analytes are only present at trace levels, the selective removal of the large excess of interfering substances without impairment of the recovery of the desired ana-

lyte is a striking challenge. In addition, increasing the analyte concentration per volume unit of solvent is often required when the sample solution is too dilute for direct injection. Solid-phase extraction (SPE), a well-established sample preparation technique, is often superior to the traditional and widely used organic extraction methods for sample purification and concentration and can improve the reliability and sensitivity of HPLC assays of small amounts of analytes (9,10). This paper describes a new, highly-sensitive, rapid method for the determination of low levels of endogenous melatonin in fish plasma samples, employing HPLC with fluorescence detection preceded by SPE.

## Experimental

### Chemicals

HPLC-grade water and methanol were purchased from J.T. Baker (Deventer, The Netherlands). Synthetic melatonin was obtained from Sigma (St. Louis, MO). Stock solutions of melatonin (1 mg/mL) were prepared by dissolving in HPLC-grade methanol immediately before use. Working standards were prepared by diluting a portion of these stock solutions with HPLC-grade water to give a final concentration range of 3–600 pg/mL. These standard solutions were kept in darkness at –20°C until use (but not longer than 1 h). It is strongly recommended that samples and standards are processed as soon as possible to avoid melatonin decomposition during storage.

### Animals and blood sampling

Flounder (*Platichthys flesus*) (300–550 g) of mixed sex were kept in tanks at the University of Manchester at 7–10°C. Fish were exposed to continuous darkness (DD) or were maintained under the natural photoperiod (8L:16D). Blood samples were collected from the caudal artery of cannulated, unanesthetized fish, centrifuged at 12,000g for 4 min, and stored at –70°C prior to analysis.

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### Sample preparation with SPE

The Baker SPE 12G Column Processor (J.T. Baker, Phillipsburg, NJ), a specially designed vacuum manifold capable of simultaneously processing up to 12 SPE columns, was used. Melatonin was extracted from plasma by reversed-phase chromatography using a C<sub>18</sub> Bakerbond SPE cartridge (1 mL, 60-Å pore size, 40-µm particle diameter). The columns were conditioned with two 1-mL portions of methanol followed by two 1-mL portions of HPLC-grade water. The plasma sample was aspirated through the column, then washed with 1 mL of 10% methanol in HPLC-grade water (1:9). The sample was eluted with two 300-µL portions of methanol. The eluate was collected, dried under air, and held at -20°C prior to analysis. Before assay, each sample was reconstituted with 60% methanol (in water) to 100 µL and mixed well. The 20-µL samples were injected into HPLC for analysis.

### HPLC

HPLC was performed with a Beckman modular system (Beckman Instruments, San Ramon, CA), consisting of two Model 110B solvent delivery modules, a system organizer with a Model 210A sample injector valve, and a column heater connected in series with a Shimadzu (Columbia, MD) spectrofluorometric detector RF-551. Data were digitized by a Beckman 406 analog interface and processed by Beckman analytical series System Gold data acquisition software on an IBM-compatible computer.

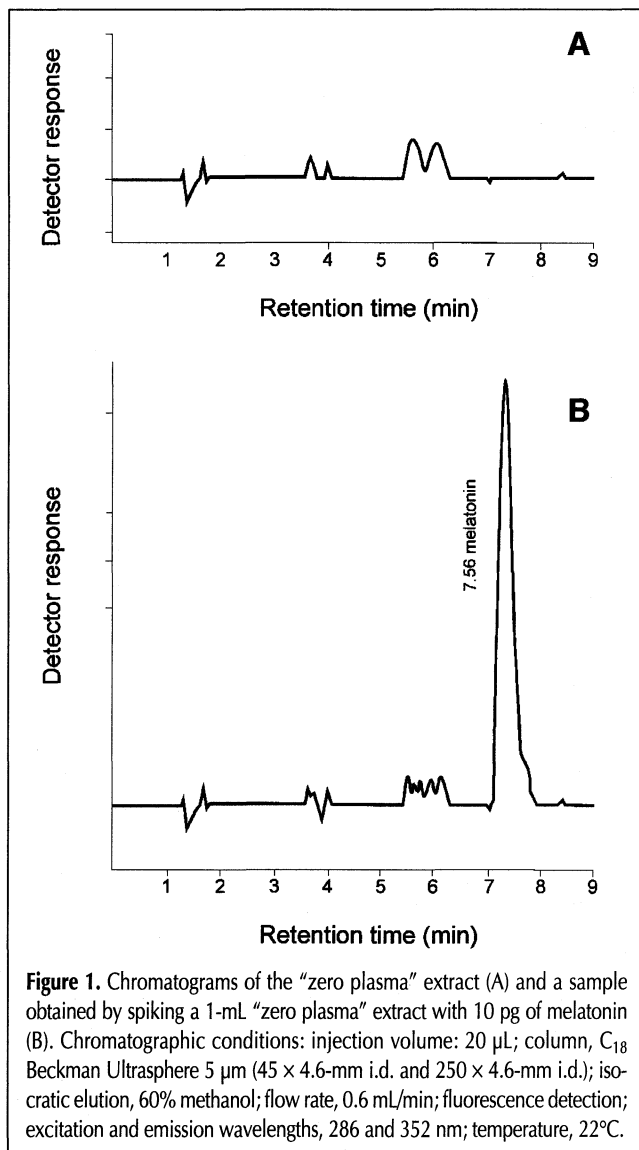
Chromatographic separations were carried out on an Ultrasphere C<sub>18</sub> column (250 × 4.6-mm i.d., 5-µm particle diameter, 80-Å pore size) connected to a guard column (45 × 4.6-mm i.d.) filled with the same material. Both columns were obtained from Beckman Instruments. The column temperature was maintained at 22°C and a flow rate of 0.6 mL/min. Excitation and emission wavelengths were set at 286 and 352 nm, respectively. An isocratic elution system was prepared. The mobile phase was 60% HPLC-grade methanol saturated with helium to remove the air bubbles and dissolved air.

### Results and Discussion

The preliminary extraction efficiency (recovery percentage) was obtained by comparing standard solutions analyzed by HPLC before and after subjection to SPE. Recovery was 96%. In order to establish the recovery of melatonin from plasma, the "zero plasma" extract was used (i.e., the plasma extract obtained following aspiration through a C<sub>18</sub> SPE cartridge). The known amounts of synthetic melatonin added to 1-mL aliquots of "zero plasma" extract were mixed well for 20 min, subjected to SPE, and analyzed as described in the Experimental section. The recovery for various amounts of melatonin from plasma ranged from 90 to 94%. The recovery was found to be optimal when the sample was applied to the cartridge and then eluted at very low flow rates (less than 1 mL/min). This result is in accordance with the flow rates studied previously by Higa and Desiderio (11). The chromatogram of the "zero plasma" extract showed no peak corresponding to endogenous melatonin (Figure 1A).

The retention time of synthetic melatonin was 7.5 min. Endogenous melatonin was identified on the basis of its retention time. Quantitative determination of melatonin in plasma was performed on the basis of a standard curve. The linearity of signal responses was observed in the range of 3–600 pg/mL (coefficient of determination [*r*], 0.984). Figure 1B shows the chromatogram that resulted when 10 pg of melatonin was added to a "zero plasma" extract after SPE followed by HPLC separation. In this study, the detection limit for melatonin was 3 pg per milliliter of plasma. To assess the precision of the assay, the retention time of plasma melatonin and synthetic melatonin at different mobile phase flow rates and column temperatures was studied (1 and 1.5 mL/min and 15 and 35°C). Samples were assayed three times in the same set of experiments and in three different series. The inter- and intra-assay coefficients of variation were 14 and 10%, respectively.

The same plasma samples were assayed by the new method and by a previously published method (8) employing organic extraction and HPLC with electrochemical detection, as outlined below. Melatonin from each 1-mL plasma sample was extracted into 5 mL of chloroform. After centrifugation at 28,000*g* for 15



**Figure 1.** Chromatograms of the "zero plasma" extract (A) and a sample obtained by spiking a 1-mL "zero plasma" extract with 10 pg of melatonin (B). Chromatographic conditions: injection volume: 20 µL; column, C<sub>18</sub> Beckman Ultrasphere 5 µm (45 × 4.6-mm i.d. and 250 × 4.6-mm i.d.); isocratic elution, 60% methanol; flow rate, 0.6 mL/min; fluorescence detection; excitation and emission wavelengths, 286 and 352 nm; temperature, 22°C.

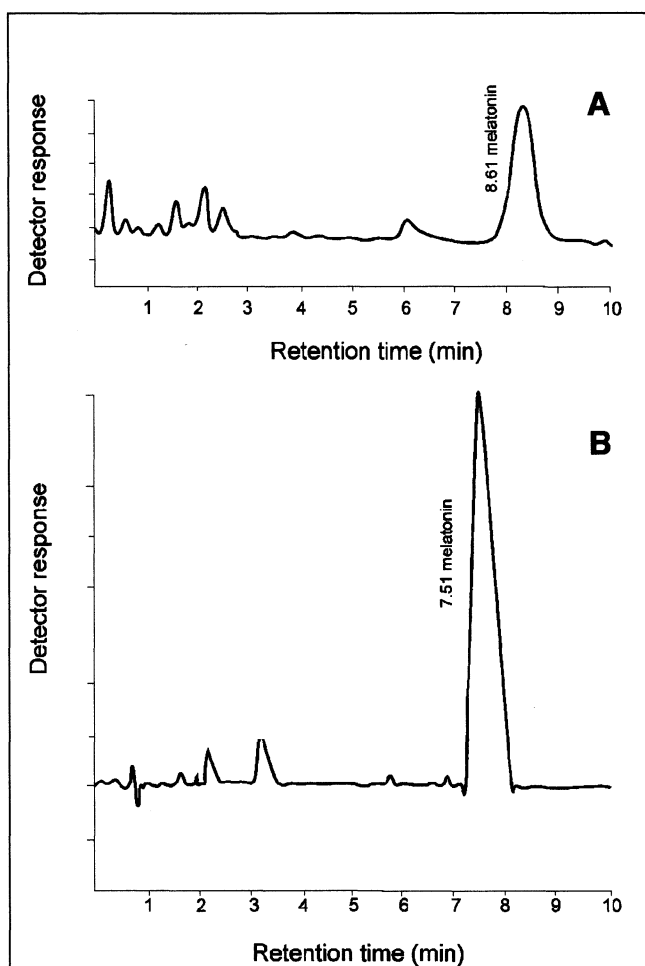
min at 4°C, the chloroform phase was dried under argon in darkness. Then it was redissolved in 200  $\mu$ L of HPLC mobile phase consisting of 100mM phosphoric acid and 24% methanol adjusted to pH 4.7 with NaOH. The aliquots (100  $\mu$ L) were subjected to HPLC on a Brownlee RP-18 Newguard column (15  $\times$  3.2-mm i.d.) and a Whatman PartiSphere C<sub>18</sub> 5- $\mu$ m reversed-phase analytical column (110  $\times$  4.7-mm i.d.) with the above-mentioned mobile phase at a flow rate of 1.5 mL/min. Melatonin was detected with a glassy carbon electrode and EG&G Princeton Applied Research electrochemical detector (model 400) with an applied voltage of 0.88 V relative to an Ag/AgCl electrode.

Figure 2 illustrates representative chromatograms from fish plasma samples obtained by the two above-mentioned methods. The results of both methods are compared in Figure 3. Samples were obtained during the night (23:00 h) from fish kept under the natural photoperiod or came from fish maintained in continuous darkness (DD) for 3 days and sampled at 23:00 h. Plasma

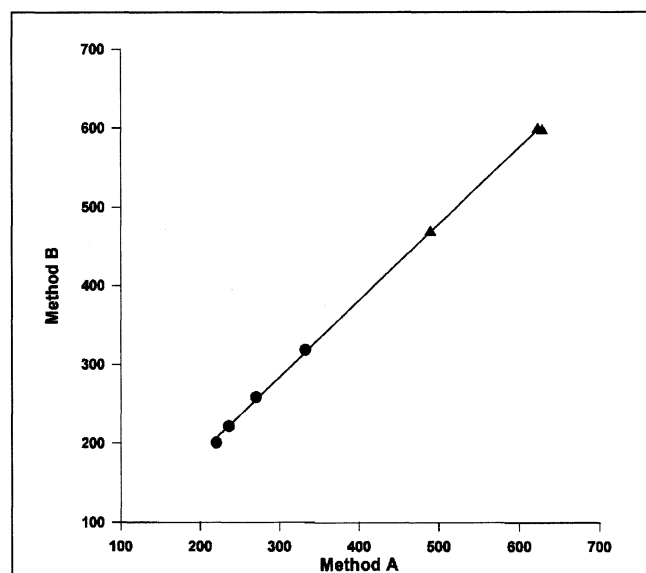
melatonin concentrations measured in flounder by both methods (Figure 3) were within the range seen in other fish: carp, rainbow trout, goldfish, brook trout, and Atlantic salmon (12–18). Higher nocturnal plasma levels of melatonin were observed in flounder kept under the natural photoperiod than in those maintained in continuous darkness. The differences probably reflect the disappearance of the melatonin rhythm known to occur under DD conditions in some species of fish (16). Figure 3 illustrates the good agreement between melatonin values obtained with SPE–fluorescence detection and those observed with the organic extraction–electrochemical detection assays; the values obtained by the two methods were highly correlated ( $r^2 = 0.999$ ,  $p < 0.001$ ). However, the values obtained with SPE–fluorescence (mean, 382 pg/mL) were slightly lower than those obtained with organic extraction–electrochemical detection (mean, 400 pg/mL;  $p < 0.01$ , paired  $t$ -test). This may reflect a small improvement in selectivity with SPE extraction of plasma samples or inter-assay variability.

## Conclusion

In conclusion, the method described here is a useful alternative to previously reported procedures for melatonin. It is rapid and reproducible. The detection limit of this method is comparable with that of RIA. An advantage of fluorescence HPLC assays compared with RIA is that the chromatographic profile is always available to check peak purity after HPLC, whereas with RIA it is hard to know the extent of cross-reactivity occurring. The advan-



**Figure 2.** Chromatograms for a typical fish plasma sample. (A) Chromatographic conditions: injection volume: 100  $\mu$ L; columns, Brownlee RP-18 Newguard (15  $\times$  3.2-mm i.d.) and Whatman PartiSphere C<sub>18</sub> 5  $\mu$ m (110  $\times$  4.7-mm i.d.); isocratic elution, 100mM phosphoric acid and 24% methanol, pH 4.7; flow rate, 1.5 mL/min; electrochemical detection. (B) Chromatographic conditions: injection volume, 20  $\mu$ L; column, C<sub>18</sub> Beckman Ultrasphere 5  $\mu$ m (45  $\times$  4.6-mm i.d. and 250  $\times$  4.6-mm i.d.); isocratic elution, 60% methanol; flow rate, 0.6 mL/min; fluorescence detection; excitation and emission wavelengths, 286 and 352 nm; temperature, 22°C.



**Figure 3.** Comparison of melatonin levels in the same plasma samples measured by two different HPLC methods. Method A: organic extraction followed by HPLC with electrochemical detection. Method B: SPE with fluorescence detection. Data are expressed as picograms of melatonin per milliliters of plasma. Assays of the plasma samples by the two methods correlated significantly ( $r^2 = 0.999$ ,  $p < 0.001$ ). Plasma samples were taken at 23:00 from fish kept either under the natural photoperiod (black triangles) or in continuous darkness (black circles).

tages of the new SPE-HPLC method over previous HPLC-based melatonin methods are that the assay is technically easier, less time-consuming, and more sensitive. Moreover, SPE seemed to improve the specificity of the method. The assay should be applicable to plasma and tissue samples from other animals, including humans, with only minor modifications.

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