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Short communication

# Highly sensitive method for the determination of melatonin by normal-phase high-performance liquid chromatography with fluorometric detection

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**Abstract**

In the present study a new chromatographic method was developed to quantify melatonin in rat pineal that can be extended to other tissues. Melatonin was extracted from an acid homogenate with ethyl acetate to avoid amine interference. HPLC was performed with silica normal-phase column and fluorescence detection. This method is sensitive enough for detecting melatonin in a single pineal gland with a detection limit of 3 pg/mg tissue.

*Keywords:* Melatonin

## 1. Introduction

Since its discovery nearly forty years ago [1] melatonin (N-acetyl-5-methoxytryptamine) has been widely investigated with regard to the factors that control its synthesis and in reference to its endocrine consequences. Increasing evidence suggests that melatonin may somehow delay the aging process and/or the progression of age-related disease processes, perhaps owing to its ability to scavenge free radicals [2–6]. Melatonin is also involved in the physiology of circadian rhythms [7], the controls of certain reproductive functions [8,9] and possible genomic actions [10].

Besides its presence in the pineal gland, melatonin has been found in a variety of other tissues [11]. Many analytical methods have been developed for the quantitation of melatonin in several tissues and body fluids, including bioassay [12], fluorometry [13], gas chromatography with electron-capture detection [14], liquid chromatography with amperometric [15,16] or both, amperometric and fluorometric detection [17] and radioimmunoassay (RIA) [18,19].

High-performance liquid chromatography (HPLC) has been used for the determination of pineal indoles because of the simplicity of sample preparation. Several authors have reported its potential use for melatonin determination by means of ultraviolet–

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visible [20], fluorometric [21–24] or electrochemical detection [25–27] but under experimental conditions with higher detection limits (30–50 pg/ml) than the method cited herein. A better detection limit (0.5 pg/ml) is obtained for determination of melatonin in plasma by gas chromatography–negative-ion chemical ionization mass spectrometry methodology [28]. However, because daytime melatonin levels are sometimes below sensitivity limits in HPLC assays reported up to now, RIA techniques were the most widely accepted in animal experiments.

In this paper, we describe a simple, rapid and sensitive method for melatonin assay by HPLC with fluorometric detection, with the same detection limit (3 pg/ml) as that of RIA (4 pg/ml).

## 2. Experimental

### 2.1. HPLC system and conditions

HPLC separation and peak detection were carried out on a Porasil (10 $\mu$m particle size; 250×4.6 mm I.D.) normal-phase silica column purchased from Waters (Milford, MA, USA). The HPLC system consisted of a LKB 2249 (Bromma, Sweden) solvent-delivery pump with Rheodyne injector with a 10-$\mu$l loop, and fluorometric detector Chrompack (Middelburg, Netherlands), 18 nm slit width, 1.5 s time constant, coupled to a LKB 2221 integrator. The mobile phase was pure HPLC-grade ethyl acetate purchased from Aldrich (Milwaukee, WI, USA) at a flow-rate of 0.8 ml/min (isocratic conditions). Excitation and emission wavelengths were set at 285 and 345 nm, respectively, gain 1000, attenuation 4.

### 2.2. Preparation of standards

Melatonin (Sigma, St. Louis, MO, USA) was dissolved in ethyl acetate to yield different concentrations in the range of 3 to 100 pg/ml for the calibration curve. These standard solutions were kept in darkness at −18°C until use, they are stable for 2 h (on the basis of reproducibility test), or 8 h if they are kept at −70°C. The use of freshly prepared melatonin standard solutions is strongly recommended.

### 2.3. Sample preparation

Male Sprague–Dawley rats (200–300 g) were maintained under a cycle of light and darkness for 12 h (lights on at 7:00 a.m.). The animals were given access to food and water ad libitum. The rats were killed by decapitation, and their pineals were removed rapidly and stored at −18°C until assayed (maximum 2 h, or 8 h if they were stored at −70°C). It is recommended that samples are processed as soon as possible to prevent melatonin decomposition during storage.

Each frozen pineal (ca. 0.5 mg) was mashed with neutral alumina (50 mg) and 500 $\mu$l of 0.1 *M* HCl. The suspension was homogenised in a sonicator bath for 5 min, and centrifuged at 1500 *g* for 10 min at 0°C. Then the aqueous phase was extracted twice with 250 $\mu$l of ethyl acetate (HPLC grade), the upper layer (ethyl acetate) was removed and further dried over anhydrous sodium sulfate. Melatonin was determined immediately by injection of 10 $\mu$l of this layer.

## 3. Results and discussion

The usual RIA methodology has in many cases been replaced by HPLC with electrochemical and fluorescence detection [21–27]. However, when only melatonin is the target molecule to be quantified [20–23], the HPLC techniques reported have amines like serotonin and tryptamine among others as interferences. These can disturb the chromatographic separations due to their occurrence in higher concentrations than those of the melatonin itself. For example, the serotonin/melatonin ratio is higher than 100 in rat pineal.

The fact that melatonin occurs in glands in such small quantities (usually pg/gland) makes it necessary to use a very sensitive detection system, such as electrochemical or fluorescence detectors. Since reversed-phase HPLC is usually used with aqueous mobile phases, the limit of detection is higher than in non-polar solvents, and consequently the detection range is restricted and decreased.

In order to overcome these difficulties, a methodology is described in this paper including a very simple prior clean-up followed by isocratic HPLC in normal-phase (silica) with ethyl acetate as eluent.

Under these conditions, melatonin is extracted from biogenic amines occurring in the pineal gland.

The use of an organic non-polar mobile phase like ethyl acetate provides a wide range of detection because of its very low fluorescence quenching properties. For this reason, these conditions allow determinations of melatonin in even a single pineal. The calibration curve showed linearity from 3 to 100 pg (five points, $r$=0.9997). Standards were determined, within-day coefficient of variation was 3–8%, with 5% mean value being typical. Within-day coefficient of variation for tissue samples ($n$=5) was ca. 12%. When known amounts of melatonin were added to the pineal homogenate 95% recovery was obtained. The endogenous melatonin was evaluated from a control (unspiked) sample. Fig. 1 shows three chromatograms: (A) pure melatonin (100 pg/ml), (B) a pineal gland extract (20 pg/ml, equivalent to 20 pg/mg of pineal), and (C) the pineal gland extract coinjected with pure melatonin.

The general values of melatonin are in agreement with those previously reported in the literature (between 10 pg/mg and 1000 pg/mg of rat pineal) [1–6].

The simplicity of this method makes it available for the measure of melatonin in different tissues and suitable for operators without special equipment or training.

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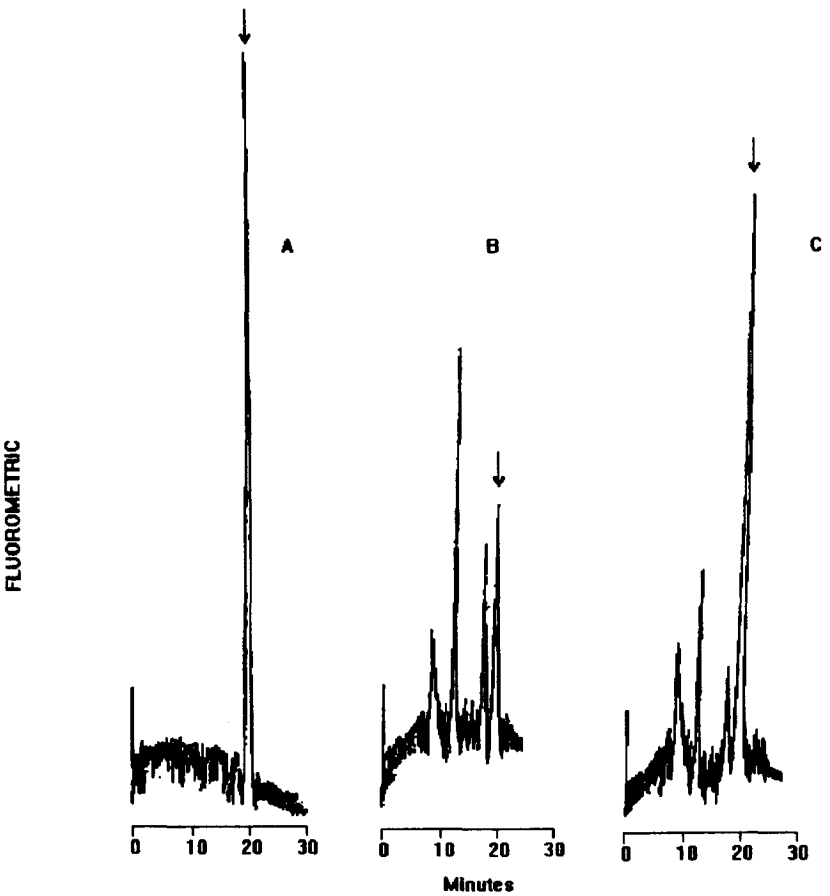


Fig. 1. HPLC of melatonin. (A) Pure melatonin (100 pg/ml, $t_R$ 19.46 min); (B) extract of rat pineal (20 pg/ml); (C) extract of pineal rat coinjected with pure melatonin.

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