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High-performance liquid chromatographic analysis of melatonin in human plasma and rabbit serum with on-line column enrichment

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Abstract

This paper describes the development of a simple and sensitive analytical method for the quantification of melatonin in human plasma and rabbit serum, using standard analytical equipment and on-line column enrichment without prior extraction, clean-up or derivatization. The analytical procedure was found to be accurate, precise and linear. For human plasma, the accuracy was 101% (range 89–106%), and the mean precision was 5% (range 2–9%) for all concentrations (0, 2, 10, 50 and 200 ng/ml) tested ($n=6$). The accuracy in rabbit serum was 101% (range 90–112%), and the mean precision was 13% (range 8–19%) for all concentrations (0, 2, 10, 50, 200 and 500 ng/ml) tested ($n=6$). The retention time of melatonin was about 8 min and the total recoveries were found to be approximately 65 and 85%, respectively, for human plasma and rabbit serum. The limit of detection was found to be lower than 1 ng/ml for human plasma and around 2 ng/ml for rabbit serum. The method is, therefore, found to be suitable for melatonin bioavailability studies in rabbits and presumably also in humans.

Keywords: Melatonin

1. Introduction

Melatonin is a hormone secreted by the pineal gland, where it is synthesized from serotonin (5-HT). It is anticipated to induce sleep in humans [1,2]. The in vivo plasma level of naturally occurring melatonin follows the circadian rhythm. It is low during the daytime (<10 pg/ml) and starts to rise in the late evening and is maintained at 25–120 pg/ml during the night (over 8 h) until it returns to the daytime baseline level [3]. Melatonin has a range of therapeutic indications, such as jet-lag [4] and sleep-quality improvement [5].

Generally, clinical doses of hormones are very low. Therefore, the analytical procedures have to be more sensitive than for conventional drugs, typically in the nanogram to picogram range. The biological matrix contains numerous substances (e.g. hormones and peptides) that may interfere with the analytical procedure. Therefore, the development of a sensitive and specific method for the analysis of plasma or serum is a great challenge [6].

The current knowledge of melatonin pharmacokinetics in humans is limited [7–9], although various plasma methods have been developed, e.g. radioimmunoassay (RIA) and HPLC [10]. These methods are generally developed for measurement of the physiological daily rhythm of human plasma melatonin. RIA is a very sensitive and specific method, and it correlates well with HPLC–MS [10].

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HPLC with electrochemical detection has been applied for the measurement of melatonin in plasma, with a sensitivity of about 20 ng/ml [11]. The sensitivity of HPLC with fluorescence detection is also very sensitive, e.g. about 3 pg/ml for an 8-ml plasma sample [12]. Additionally, the correlation of this method to RIA was very good. This method, however, uses solid and liquid extraction prior to gradient elution HPLC and, consequently, only one sample can be injected per hour.

The purpose of this work was to develop a simple and sensitive isocratic HPLC method with fluorescence detection for melatonin in a small volume (50 μl) of human plasma or rabbit serum using standard analytical equipment and on-line column enrichment with no prior extraction, clean-up or derivatization. The method should be sufficiently sensitive for use in bioavailability studies.

2. Experimental

2.1. Equipment

The HPLC system was from Merck (Darmstadt, Germany) and consisted of an Hitachi 655A-11 pump and an Hitachi LaChrom L-7480 fluorescence detector connected to an Hitachi D-2500 chromato-integrator (plot attenuation=4). The injector was a Rheodyne model 7125 (Berkeley, CA, USA), equipped with a 14×3.9 mm I.D. on-line enrichment column in place of the injection loop. The enrichment column was packed in a ratio 2:1 with Sep-Pak C_{18} (55–105 μm) from Waters and Bondesil C_{18} (40 μm) from Analytical Instruments (Værløse, Denmark). In pilot studies, this combination has been found to be optimal with respect to maximum recovery and reduced blank values and blocking. The coarser particles (Sep-Pak C_{18}) were placed closest to the injection port, primarily to reduce blocking.

2.2. Chromatography

The column used was a 259×4 mm I.D. LiChrosorb RP-18, and the guard column was a LiChroCART 4-4, both from Merck. The mobile phase (pH 4.5) consisted of an aqueous solution of 50 m*M* sodium phosphate and 40% (v/v) methanol. The mobile phase was filtered through a Millipore 0.45 μm filter and degassed ultrasonically. The flow-rate was 1.0 ml/min. The excitation wavelength was 286 nm and the emission wavelength was 380 nm.

2.3. Reagents and glassware

The melatonin was obtained from Bachem (Bubendorf, Switzerland). Demineralized water was used throughout the test. Methanol was purchased from Romil Chemicals (Leicester, UK). Sodium phosphate was of analytical grade and was obtained from Merck. Pooled human plasma ($n=2$), prepared with citrate phosphate and dextrose, was obtained from Rigshospitalet (Copenhagen, Denmark). Rabbit blood was collected in microcentrifuge tubes (TEN-15), and serum was stored at −20°C in microcentrifuge tubes (TEN-15), which were purchased from Bie and Berntsen (Copenhagen, Denmark).

2.4. Procedure

Prior to injection, the plasma and serum were diluted 1:1 (v/v) with water. Before each injection, the enrichment column was washed with 2×1 ml of 95% (v/v) methanol in water followed by 4×1 ml of water. Next, a 100-μl sample was applied followed by 2×1 ml of water and 1 ml of 20% (v/v) methanol in water. The sample and the subsequent water were injected at rates of 0.6 and 1.2 ml/min, respectively, as described by Bechgaard et al. [6]. Fig. 1 shows typical chromatograms of blank human plasma and human plasma spiked with 10 ng/ml melatonin. Fig. 2 shows typical chromatograms of blank rabbit serum, rabbit serum spiked with 5 ng/ml, and of rabbit serum 60 min after intranasal application of 4 mg of melatonin.

2.5. Testing of the analytical procedure

The accuracy, precision and linearity of the method were determined using human plasma ($n=6$) at 0, 2, 10, 50 and 200 ng/ml and spiked rabbit serum ($n=6$) at 0, 2, 10, 50, 200 and 500 ng/ml. The sample concentrations in human plasma and rabbit serum were calculated on the basis of the peak height of melatonin, by reference to the standard curve

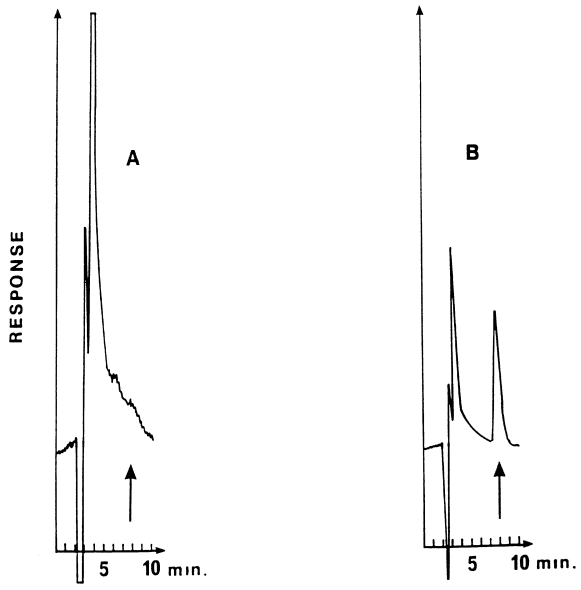

Fig. 1. Typical chromatogram of: (A) Blank from human plasma at recording range 10 and (B) human plasma spiked with 10 ng/ml melatonin at recording range 50. The arrows indicate the retention time of melatonin.

(blank and 2, 10 and 100 ng/ml) obtained by linear regression.

The total recovery of melatonin was determined as the response of analyzed sample relative to the response of 50 μl of an aqueous solution of melatonin (10 ng/ml) that was injected directly, without the subsequent washing with 2×1 ml of water and 1 ml of 20% (v/v) methanol in water.

3. Results and discussion

The analytical procedure for melatonin in human plasma and rabbit serum was found to be accurate, precise and linear. The overall accuracy for human plasma was 101% (range 98–106%), calculated as the percentage found on the basis of the plasma standards (Table 1). The mean precision, expressed as the coefficient of variance (C.V. %), was found to be 5% (range 2–6%) for all concentrations. At the lowest concentration level tested (2 ng/ml), the precision was 9%.

For rabbit serum, the overall accuracy was 101%

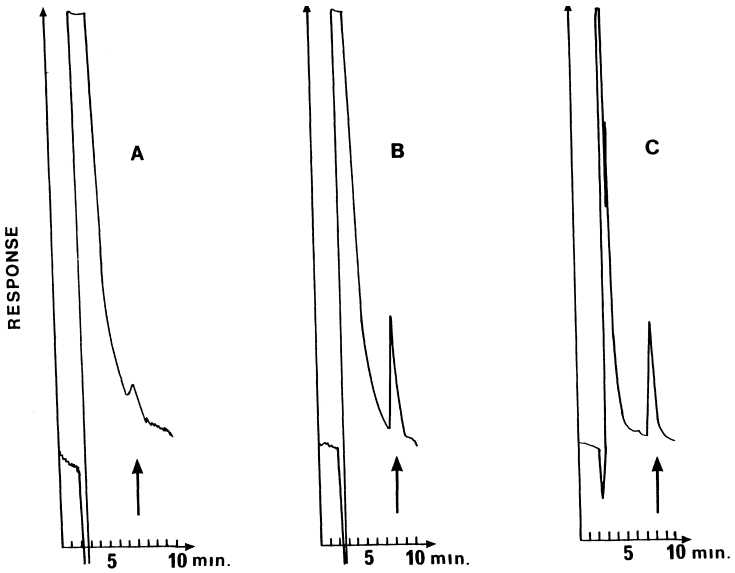

Fig. 2. Typical chromatogram of: (A) Blank from rabbit serum at recording range 10, (B) rabbit serum spiked with 5 ng/ml melatonin at recording range 20 and (C) rabbit serum 60 min after intranasal application of 1.5 mg of melatonin at recording range 50. The arrows indicate the retention time of melatonin.

Table 1
Accuracy and precision of the analytical procedure for melatonin ($n=6$) in human plasma

Added (ng/ml)	Found (ng/ml)	Accuracy (found %)	Precision (C.V. %)
2	2	100	9
10	11	106	2
50	49	98	3
200	200	100	4
Mean		101	5

(range 90–112%), calculated as the percentage found on the basis of the serum standards (Table 2). The mean precision (C.V. %) was found to be 13% (range 8–19%) for all concentrations. At the lowest concentration level tested (2 ng/ml), the precision was 9%.

The analytical procedure was linear within the concentration range studied, since the deviation of accuracy from 100% was about 10%. The ''blank'' values for human plasma ($n=2$) were estimated to contain less than 0.3 ng/ml melatonin, and the blank values for the rabbit serum tested ($n=6$) were determined to be about 1 ng/ml, within a range from 0.2–1.8 ng/ml.

Based on this observation, the limit of detection was estimated to be less than 1 ng/ml for human plasma and about 2 ng/ml for rabbit serum. The sensitivity of this method may be improved by injection of a larger sample volume, especially for human plasma, where no blank was observed. Setting the emission wavelength to 352 nm increased the detector sensitivity by a factor of about two, but this wavelength caused high blank values in in-vitro studies with glucose–Ringer solution containing albumin. The total recoveries were found to be about 65 and 85% for human plasma and rabbit serum, respectively, which were similar to the 70% obtained by pre-chromatographic solid and liquid extraction [12]. The sensitivity of the two HPLC systems was also similar, with less than 10 pg of melatonin injected onto the column.

The choice of injecting a small sample volume (50 μl) may be one of the reasons why an isocratic HPLC system could be used at maximum sensitivity. Still, the 2 ng/ml limit of detection obtained appears to be sufficient for bioavailability studies, as seen from Fig. 3. The retention time for melatonin was about 8 min (Fig. 1), which resulted in a capacity of about six samples per hour. The specificity of the method is anticipated to be sufficient for bioavailability studies, as the main metabolite of melatonin is the polar 6-hydroxymelatonin sulphate [10].

As seen from Fig. 3, the bioavailability after nasal application of 1.5 mg of melatonin is about 60%, and C_{max}, t_{max} and $t_{1/2}$ are about 160 ng/ml, 5 min and 10 min, respectively. It is concluded that the described HPLC/fluorescence system, with on-line column extraction is a simple, accurate, precise and relatively sensitive method for the determination of melatonin in human plasma and rabbit serum. The

Table 2
Accuracy and precision of the analytical procedure for melatonin ($n=6$) in rabbit serum

Added (ng/ml)	Found (ng/ml)	Accuracy (found %)	Precision (C.V. %)
2	2	100	9
10	10	102	19
50	56	112	15
200	200	100	8
500	450	90	13
Mean		101	13

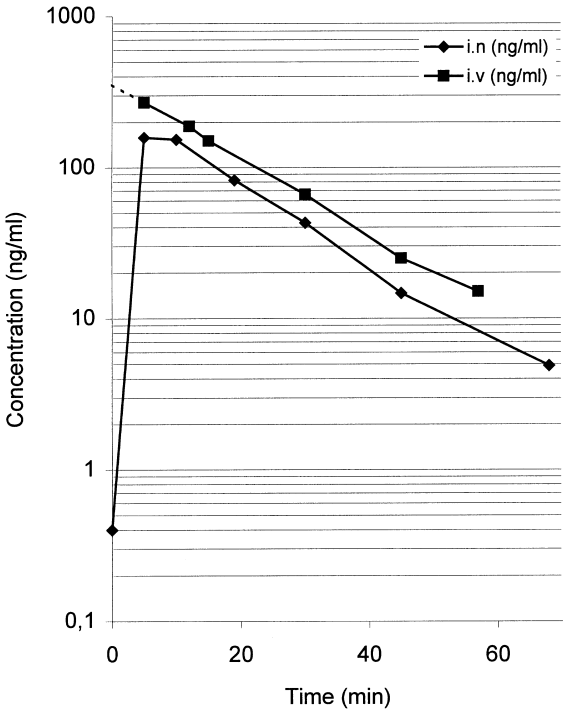

Fig. 3. Example of semilogarithmic serum concentration profiles following intravenous and intranasal dosage of 1.5 mg melatonin, respectively, to rabbits.

method is, therefore, found to be suitable for melatonin bioavailability studies in rabbits and presumably also in humans.

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