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Quantification of melatonin in human saliva by liquid chromatography–tandem mass spectrometry using stable isotope dilution

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Abstract

A method for the determination of melatonin in human saliva has been developed using high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS–MS). Saliva was collected in plastic tubes. 7-D-Melatonin was added as internal standard and the samples were cleaned and concentrated by solid-phase extraction. The limit of detection was 1.05 pg ml^{-1} and the limit of quantification was 3.0 pg ml^{-1}. The accuracy of the method was ±14% at 5.60 pg ml^{-1} and ±9% at 19.6 pg ml^{-1}. The precision was ±13% at 6.18 pg ml^{-1} and ±11% at 31.2 pg ml^{-1}, respectively. Our HPLC–MS–MS method shows a high sensitivity and specificity for melatonin and more reliable results compared with a radioimmunoassay. The chromatographic method has been used to determine the circadian rhythm of melatonin among three nurses working the night shift and a patient suffering from an inability to fall asleep at night.

Keywords: Melatonin

1. Introduction

Melatonin (*N*-acetyl-5-methoxytryptamine) is synthesised from the dietary amino acid L-tryptophan in the human pineal gland at night under normal environmental conditions [1]. The endogenous rhythm of secretion is generated by the suprachiasmatic nuclei and entrained by the light–dark cycle [2]. Melatonin has been postulated to be a potent sleep-inducing mediator over the past few years. It has been suggested that shift workers who have been exposed to light on several consecutive nights show a melatonin secretion with a phase delay [3]. In addition, it has been suggested that rotating shift workers may develop significant abnormalities of the entero-insular axis in response to abrupt phase shifts [3]. These may be important in the development of diseases such as coronary heart disease, which may be more frequent in shift workers [4]. In Europe approximately 18% of the work force works at least 25% of the time at night and these problems are most certainly of considerable importance [4].

Fourtillan et al. [5] developed a sensitive assay of melatonin in human plasma by gas chromatography (GC)–negative ion chemical ionisation mass spectrometry (MS). Deuterated melatonin ($^{2}H_{4}$-

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melatonin) was used as internal standard. The substances were converted to the fluorinated derivative with pentafluoropropionic anhydride (PFPA). The limit of quantification (LOQ) was determined to be 0.5 pg ml^{-1}. High-performance liquid chormatography (HPLC) with fluorescence detection permits analysis of melatonin without derivatisation. The LOQ varies between approximately 8 and 10 pg ml^{-1} in human plasma as shown in two recently published methods [6,7]. Härtter et al. [8] presented a method where melatonin and its metabolite 6-hydroxymelatonin could be simultaneously determined in human plasma using HPLC–MS. The limit of detection (LOD) for melatonin was 2 ng ml^{-1}. The LOQs and LODs of the methods were considered acceptable by the authors as relatively large volumes of plasma can be collected and analysed. Laboratory studies have been performed to assess the circadian rhythm of melatonin in humans by analysing blood samples and saliva samples that have been collected simultaneously at regular time intervals during approximately 24 h. The concentration of melatonin in serum or plasma during daytime has in these studies been estimated to 2–5 pg ml^{-1} and 20–170 pg ml^{-1} during the night. The saliva concentrations vary from less than a few pg ml^{-1} during the day to a mean of approximately 20–35 pg ml^{-1} at night [9–11]. The time of onset of melatonin determined in saliva was approximately at 23:00 h in the evening and excretion ended at approximately 08:00 h in the morning correlating significantly with concentrations in plasma measured at corresponding time points [9–11].

To evaluate if prolonged night shift work affects the circadian rhythm of melatonin among employees sampling could be performed hourly during consecutive days and preferably for each worker participating in the study. With such a design of a study, a relatively large number of samples have to be collected. Under these circumstances, blood sampling is an inconvenient method. Collection of saliva offers an excellent alternative. The sampling is easily performed and can be carried out by the individual worker following information of the sampling technique. Sampling of 1 ml of saliva is carried out approximately within 4–5 min. During field studies sampling should preferably interfere as little as possible with the tasks or the work process performed by the individual and sampling of more than 1 ml of saliva is in many cases probably not suitable. In addition to a straightforward method of sampling a sensitive analytical method to determine the melatonin concentration in saliva is thus necessary. Radioimmunoassay (RIA) is the assay most commonly used [12]. The technique is simple and relatively fast but unfortunately, cross-reactivity with structurally similar substances may occur which may interfere with assay. In addition, some nutrients present in saliva may interfere with analysis and saliva left in plastic pipettes or plastic tubes more than 2–3 min may give falsely elevated melatonin levels [13].

Recently Simonin et al. [14] developed a GC–MS method with negative chemical ionisation for the quantification of melatonin in human saliva. The saliva sample was washed with *n*-hexane, liquid–liquid extracted with dichloromethane followed by derivatisation of melatonin and the deuterium labelled melatonin used as internal standard with PFPA. The LOQ was estimated to be 1 pg ml^{-1} which is relatively low, but the work up procedure appears to be rather laborious and time-consuming especially when many samples are to be analysed. HPLC–tandem mass spectrometry (MS–MS) offers a sensitive and selective method for the analysis of underivatised melatonin in different human biological matrixes. In this paper, we introduce an HPLC–MS–MS method with a stable isotope of melatonin labelled with seven deuterium atoms (7-D-melatonin, Fig. 1) as internal standard, which quantifies melatonin at the pg-level in a 1-ml saliva sample from humans. The method was used to determine the concentration of melatonin in saliva collected by three hospital nurses working the night shift and one patient with a sleeping disorder, respectively.

2. Experimental

2.1. Chemicals and materials

Melatonin and 1,4-dithio-DL-threitol were purchased from Fluka (Neu-Ulm, Switzerland), methanol and dichloromethane from J.T. Baker (Deventer, The Netherlands). The deuterium-labelled melatonin (98%) was obtained from Euroiso-top (Gif-sur-Yvet-

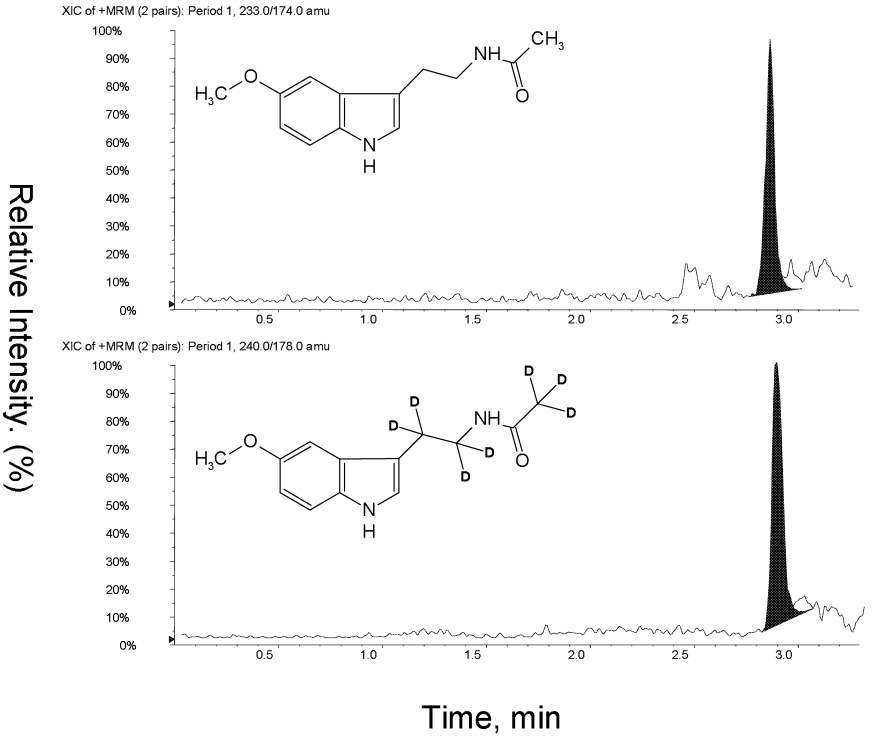

Fig. 1. Analysis of a saliva sample using the HPLC–MS–MS method in the SRM mode for the transition of [M+H] to m/z 174 for melatonin and [M+H] to m/z 178 for 7-D-melatonin, respectively. No interferences could be detected.

te, France). The solid-phase extraction (SPE) column used was Nexus 10 ml, 30 mg from Varian (Harbor City, CA, USA). The sorbent is a highly cross-linked spherical polymer. The particle size is 50–150 μm with a carbon loading of 55.0–75.0% and hydrogen loading of 3.5–10.0%.

2.2. Preparation of stock and standard solutions

Six different standard solutions in the range of 3–300 pg ml^{-1} were prepared from a melatonin stock solution in methanol–water (30:70, v/v). The 7-D-melatonin stock solution was diluted to a concentration of 400 pg ml^{-1} in methanol–water (30:70, v/v).

2.3. Saliva sampling

Batches of 20 ml of saliva from different persons were collected for method evaluation. The individuals did not drink or eat at least 30 min before sampling. The different batches of saliva were divided into 1-ml samples and stored in a freezer at −20 °C until analysis.

Saliva sampling by the nurses and the patient was performed in the following way: the subjects were told not to eat or drink hot beverages 30 min prior to sampling. At least 1 ml of saliva was spit into a 10-ml plastic tube. The saliva samples were stored at −20 °C until sample work up and analysis, which was carried out approximately 1 to 2 weeks later. Melatonin has been shown to be stable in saliva at −20 °C for at least a month [14].

2.4. Sample preparation

Saliva samples that did not contain any endogenous melatonin were spiked with known amounts of the substance in methanol–water and 40 pg of internal standard. To the samples containing endogenous amounts of melatonin and to the samples collected by the nurses and the patient 40 pg of internal

standard was added shortly before the work up procedure.

Saliva is excessively viscous. In order to enhance the passage of saliva through the SPE column 100 μl of a 1,4-dithio-DL-threitol [(±)-threo-1,4-dimercapto-2,3-butanediol] solution ($6.6 \cdot 10^{-2}$ *M*) was added to the sample, and the mix was incubated for 10 min at room temperature. A study recently published has shown that addition of 1,4-dithio-DL-threitol [(±)-threo-1,4-dimercapto-2,3-butanediol] to saliva does not have an effect on the determined concentration of melatonin compared with saliva samples to which 1,4-dithio-DL-threitol has not been added [15]. Following incubation the samples were centrifuged at 5000 rpm (Uniequip, Martinsreid, Germany) for 5 min in order to precipitate particulate matter. After centrifugation, the sample was transferred onto a Nexus SPE column which had been preconditioned with 5 ml of methanol–acetonitrile (50:50) and 1 ml of water. The applied saliva sample was washed with 1 ml of water–methanol (15:85). Melatonin and the internal standard were eluted in 1 ml of dichloromethane. The organic solvent was evaporated under reduced pressure and the residue was reconstituted in 100 μl of methanol–water (30:70, v/v) and this volume was injected onto the HPLC–MS–MS system.

2.5. HPLC–MS–MS

The HPLC–MS–MS system consisted of a PC Sciex 2000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an atmospheric pressure chemical ionisation (APCI) source. The analytical column was a 150 mm×3.9 mm C_8 Symmetry column (Waters, Milford, MA, USA). The eluent was delivered at a flow-rate of 800 μl min^{-1} by two HPLC pumps (Perkin-Elmer series 200 micro pumps). The eluent consisted of methanol and water with 0.1% formic acid. A linear gradient from 40% methanol to 95% methanol was run over 3 min and 95% methanol was then maintained for 2 min. A linear gradient back to 40% methanol was run during 0.5 min followed by an equilibration for 1 min.

The APCI was operated in the positive ion mode with the following mass spectrometric parameters; needle charge 2 kV, declustering potential 20 V, focusing potential 370 V and collision energy 21 V. The mass spectrometer was calibrated using a solution of PPG across the mass range of 50–1700 u. Additional tuning and optimisation of the instrument was performed by continuous post column injection with a syringe pump of a solution of melatonin into the HPLC flow. A single precursor ion–product ion transition was monitored for melatonin—*m*/*z* 233 to *m*/*z* 174—and 7-D-melatonin—*m*/*z* 240 to *m*/*z* 178—respectively, corresponding to the loss of $HN{=}C(OH)CH_3$ and $HN{=}C(OH)CD_3$, respectively, of the protonated molecule [16]. The Analyst software (Applied Biosystems) controlled the HPLC–MS–MS system and the data handling.

2.6. Method validation

2.6.1. Sensitivity

The LODs and the LOQs were determined at $3S_B$ and $10S_B$, respectively, where S_B is the standard deviation of the background noise of a blank sample.

2.6.2. Accuracy of the method

To evaluate the accuracy of the method an experiment was performed as follows. A batch of saliva from three individuals was collected at 15:00 h during 1 day in the winter (February). In February there is daylight between approximately 08:00 and 15:30 h. The endogenous concentration of melatonin in saliva used for this experiment was below the LOD as measured by adding 40 pg to 1 ml of sample. Six samples were spiked with 5.6 pg melatonin and 40 pg of 7-D-melatonin and six samples were spiked with 19.6 pg melatonin and 40 pg of 7-D-melatonin in methanol–water (30:70, v/v).

2.6.3. Precision of the method

The precision of the method was determined by analysing six samples of saliva from each one of two batches. The two batches were collected at 08:00 and 12:00 h, respectively during the same day as sampling for determination of accuracy of the method was carried out. No spiking of external melatonin was needed as the saliva contained quantifiable amounts of endogenous melatonin. To each sample 40 pg of 7-D-melatonin was added. Six samples were analysed at two different occasions, within a time gap of approximately 5 days.

2.6.4. Comparison with RIA

The saliva used in this experiment did not contain any endogenous melatonin. Twelve samples were spiked to a concentration of 11.0 pg ml^{-1} and 12 samples were spiked to a concentration of 25.0 pg ml^{-1}, respectively. Six samples of each concentration were delivered to a commercial laboratory for quantification of melatonin by an RIA (Stockgrand, UK). No 7-D-melatonin was added to these samples. Six samples of each concentration and with internal standard added were analysed by our assay.

2.6.5. Analysis of saliva samples from nurses and a patient

2.6.5.1. Nurses. Three nurses who had been working the night shift for 3 years collected approximately 2 ml of saliva in a plastic tube once every hour from 21:00 until 07:00 h the next morning. Sampling was performed during one night in November with daylight between approximately 09:00–15:00 h. They worked in a medical department at a hospital and they were exposed to light from bulbs and fluorescent tubes during the entire shift as well as during sampling of saliva. The ages of the nurses were 32, 43 and 54 years. The samples were kept at −20 °C until analysis.

2.6.5.2. Patient. An individual (age 43 years) who had worked the night shift (21:00–06:00 h) for 6 years and who suffered from an inability to fall asleep in the evening at the weekend collected saliva once every hour from 18:00 h in the afternoon until 14:00 h the following day. The patient was awake during the sampling period and exposed to light from bulbs. The sample was collected during 1 day in November. The samples were kept at −20 °C for a week until analysis was performed.

3. Results and discussion

3.1. Sample preparation and analysis

When RIA is used to determine the concentration of melatonin in saliva it is recommended that the subject should not eat or drink hot beverages 30 min prior to saliva sampling as impurities may be introduced which might interfere during analysis [8]. In addition, when RIA analysis of melatonin is the method chosen saliva is collected by letting the individual chew on a ‘‘sampler’’ (Salivette) made of cotton or polyester for a few minutes. Following sampling Salivette is centrifuged to release the absorbed saliva. This is a convenient sampling method but unfortunately interferences appeared during analysis using our assay, which caused an overestimation of the amount of melatonin in the saliva collected. We recommend spitting directly into a plastic tube.

The supplier of the LC column informed us that injection of a 100 μl untreated saliva sample onto the column is possible for the determination of the concentration of melatonin. However, chromatographic problems soon occurred probably caused by substances present in the saliva, which were retained on the column. We thus had to get rid of impurities present in the saliva prior to HPLC–MS–MS analysis. Fourtillan et al. [5] noticed irreversible adsorption problems with SPE purification of sub-ng amounts of melatonin and recommended liquid–liquid extraction. We did not experience this kind of problem using the Nexus column if the column was prewashed and equilibrated as previously described.

During MS analysis, both electrospray ionisation (ESI) and APCI were tested. ESI showed an excellent sensitivity when standard solutions of melatonin in methanol–water were analysed. Unfortunately, ESI was found to be extremely sensitive to suppression effects, which made it impossible to analyse saliva samples following SPE (data not shown). APCI offered a relatively stable performance for analysis of melatonin in saliva samples. The MS analysis in the selected reactive monitoring (SRM) mode for the transition of [M+H] to m/z 174 for melatonin and [M+H] to m/z 178 for 7-D-melatonin gave a chromatogram with no endogenous interferences as shown in Fig. 1.

3.2. Method validation

3.2.1. Linear range and limits of detection and quantification

The concentration of melatonin in saliva during the night varies among different individuals but values of 20–35 pg ml^{-1} is normally determined. A few pg ml^{-1} is detected in samples collected during

the day [3,9,10]. The LOD, defined as three times the standard deviation of the blank signal was estimated to be 1.05 pg. When 3.0 pg of melatonin was injected, it showed a signal-to-noise ratio of approximately S/N 15. The detector response was linear in a tested range of melatonin in standard solutions (3–300 pg ml^{-1}), i.e., well within the expected endogenous concentration range in human saliva. The calibration curve was forced through the origo and the regression equation was $y=0.0352x$, with $r=$ 0.9985). These linear range, detection and quantification limit parameters provided a suitable working range for the analysis of melatonin in saliva samples using the HPLC–MS–MS system.

3.2.2. Accuracy of the method

Six samples spiked with 5.60 pg ml^{-1} showed a mean concentration of 4.30 pg ml^{-1} with an RSD of 14% (range 3.8–5.3 pg ml^{-1}.) Six samples spiked with 19.6 pg ml^{-1} of melatonin showed a mean of 20.2 pg ml^{-1} (RSD 9%) and a range of 18.0–21.2 pg ml^{-1}.

3.2.3. Precision of the method

Analysis of samples from the batch collected at 12:00 h showed a mean value of 6.18 pg ml^{-1} with an RSD of 13% and a range of 4.98–6.99 pg ml^{-1}. Samples of the batch collected at 08:00 h demonstrated a mean of 31.2 pg ml^{-1} with an RSD of 11% and a range of 28.0–34.2 pg ml^{-1}.

The accuracy and the precision of the method shows that our assay produces reliable determinations of melatonin in a relatively complex biological matrix.

3.2.4. Comparison between HPLC–MS–MS and RIA analysis

Analysis of six samples with a concentration of 11.0 pg ml^{-1} with our assay showed a mean of 10.3 pg ml^{-1} (RSD 10%, range 9.4–11.6 pg ml^{-1}), while RIA analysis gave a mean of 4.5 pg ml^{-1} with a range of non-detectable ($\leq$2.0 pg ml^{-1}) to 11 pg ml^{-1} with an RSD of 105%. The six samples with a concentration of 25.0 pg ml^{-1} showed a mean of 22.8 pg ml^{-1} (RSD 9%, range 20.2–25.2 pg ml^{-1}) when analysed by HPLC–MS–MS and a mean of 21.0 pg ml^{-1} (range 6–35 pg ml^{-1}, RSD 57%) following RIA analysis. The results are shown in detail in Table 1.

We used the independent t-test to compare the determined means of spiked samples using the two different assays. The test showed that the determined concentrations of melatonin are statistically significantly different ($P<0.03$) at the 11.0 pg ml^{-1} level of melatonin. There is no statistically significantly difference between the means when samples spiked to 25 pg ml^{-1} are analysed with RIA or our HPLC–MS–MS assay. Equal variances between the two sets of analytical results could not be assumed (Levene's test 0.003 and 0.005, respectively, Table 1).

The samples analysed by the commercial laboratory were shipped to them by air. The saliva was kept in plastic tubes made of polypropylene according to the instructions from the laboratory, and with dry ice in the box to keep the samples frozen until

Table 1
Analysis of melatonin in spiked saliva samples with an RIA assay and HPLC–MS–MS, respectively. Six samples of each concentration was analysed by each assay. RSD=relative standard deviation

Spiked concentration of melatonin (pg ml^{-1})	RIA mean (RSD, %) range	HPLC–MS–MS mean (RSD, %) range	Levene's test for equality of variances	Significance (two-tailed) in difference of means***
11.0	4.50 (105) 1.0*–11.0	10.3** (10) 9.4–11.6	0.003	0.03
25.0	21.0 (57) 6.0–35.0	22.8 (9.0) 20.2–25.2	0.005	0.73

* The detection limit of the RIA assay is according to the laboratory 2.0 pg ml^{-1}. Half of the detection limit (1.0 pg ml^{-1}) has been used when calculating the mean of the assay.
** Five samples analysed.
*** Equal variances not assumed.

arrival within 1 day after shipment from our laboratory. Analysis was then performed according to the practice of the laboratory. The results from the six replicates at both levels of melatonin indicate that our method apparently produces more reliable results compared with RIA analysis. The reason for this discrepancy is not clear, but possibly cross-reactivity or additional reactions with other substances in the RIA method may be an explanation.

In addition, our HPLC–MS–MS assay has a cycle time of 6 min and the extraction material is available in a 96-well plate system. This will allow a high through-put preparation as well as analyses of samples which is important for quick answers from field studies, individual tests at patients as well as for economical reasons as the equipment is relatively expensive.

3.2.5. Saliva samples from nurses and a patient

The analysis of saliva samples from three nurses working the night shift showed that the onset of melatonin secretion and the circadian rhythm in saliva could be assessed between two of the participating individuals. The excretion of melatonin in saliva from the first individual demonstrated an onset at approximately midnight with an ending of excretion before seven in the morning (Fig. 2a). The second nurse showed a possible phase delay with

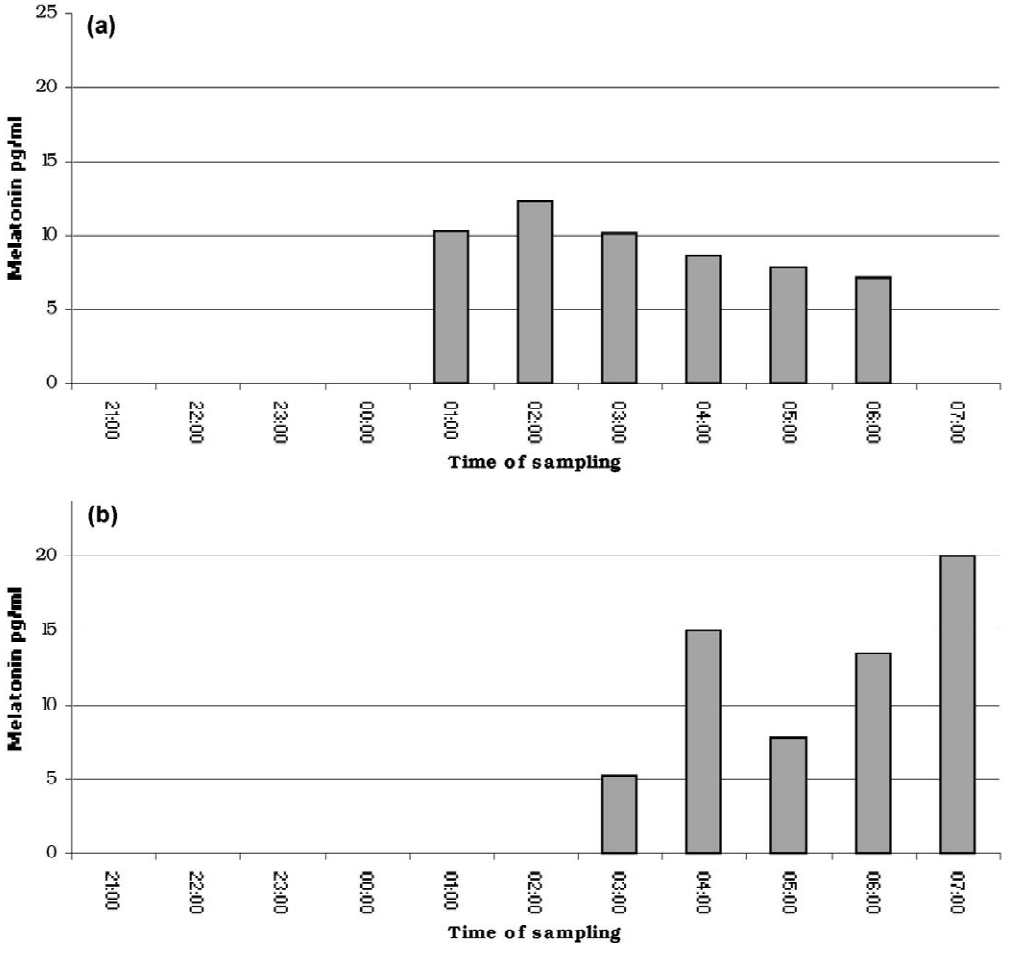

Fig. 2. (a) and (b) Concentration of melatonin in pg ml^{-1} in saliva collected once every hour starting at 21:00 h in the evening and ending at 07:00 h in the morning by two nurses working the night shift.

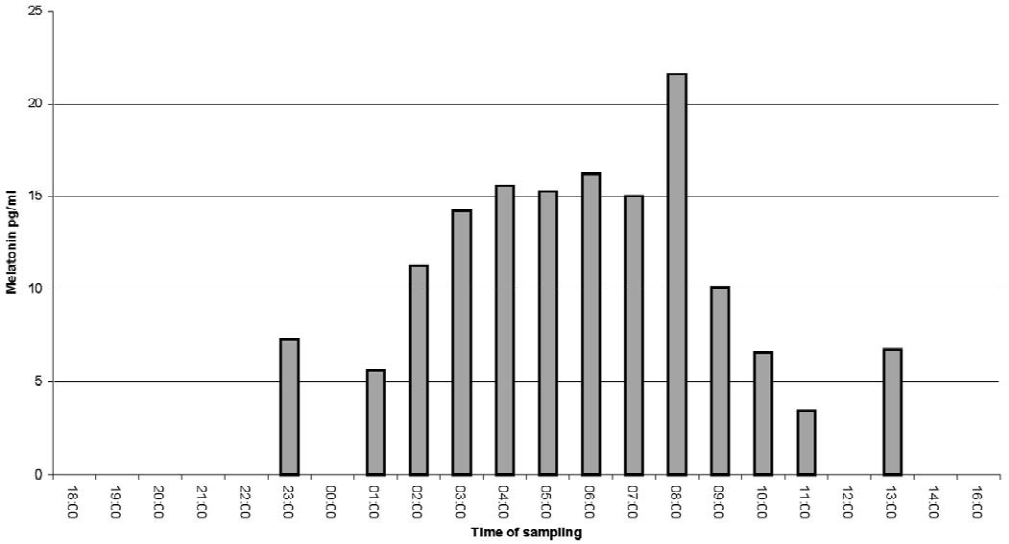

Fig. 3. The circadian rhythm of melatonin as determined in saliva from a patient suffering from an inability to fall asleep at night. Samples were collected once every hour from 18:00 h in the evening until 14:00 h the following day.

onset at approximately 03:00 h and still excreting at 07:00 h (Fig. 2b). Analysis of the saliva from the third woman demonstrated a low excretion of melatonin compared with her colleagues, as the concentration of melatonin in all of the samples collected was below the LOD. This individual may be a low secretor, or the nightshift work may have caused a considerable phase delay, however not detected due to end of sampling.

The result of the analysis of saliva samples from the patient with an inability to fall asleep at night is shown in Fig. 3. The onset of melatonin secretion is approximately at 23:00 h in the evening, increasing until 08:00 h in the morning to a level of 24.1 pg ml^{-1} and then decreasing until 13:00 h to approximately 6.8 pg ml^{-1}. The individual shows a circadian rhythm of melatonin in saliva with a prolonged excretion.

4. Conclusions

HPLC–MS–MS in the APCI mode was successfully applied for determination of melatonin in human saliva following SPE. The analytical results demonstrate that the assay produced more reliable determinations of melatonin in saliva compared with a commercial RIA method. The relatively short cycle time of the method demonstrate the feasibility of the assay as a platform for the rapid sample processing following field studies as well as in clinical studies to determine the circadian rhythm of melatonin in human subjects.

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