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ARTICLES

Dispersive Liquid–Liquid Microextraction for the Determnation of Salivary Melatonin as a Biomarker of Circadian Rhythm[1]

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Abstract– Melatonin (N-acetyl-methoxytryptamine) is secreted from a part of the brain called suprachiasmatic nuclei. The biological clock of the body or the circadian rhythm is regulated through the secretion of hormones such as melatonin and cortisol. The dispersive liquid–liquid microextraction (**DLLME**) was optimized for the determination of human salivary melatonin. Different variables including the nature of extracting and dispersing solvent, their volumes, sample pH, ionic strength, extraction and centrifugation times were screened using One Factor At a Time design and then, the significant variables were selected as optimum values. Accuracy and precision of the optimized method were evaluated at concentrations of 50, 100, 250 pg/mL by achieving CV% for day-to-day and within-day reproducibility. Considering the appropriate results of the study, DLLME procedure can be used for the determination of melatonin hormone in saliva samples.

Keywords: melatonin, circadian rhythm, dispersive liquid–liquid microextraction, one factor at a time, saliva, HPLC

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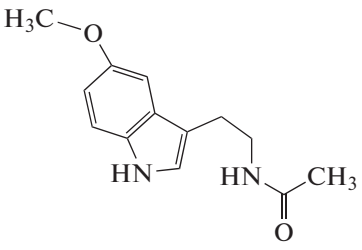
Melatonin (N-acetyl-methoxytryptamine) is secreted from a part of the brain called suprachiasmatic nuclei. This hormone has the highest level of secretion at night and the lowest during the day [1]. The biological clock of the body, or the circadian rhythm, is regulated through the secretion of hormones such as melatonin and cortisol [2]. Disturbance in circadian rhythm is specifically connected with shift working. Melatonin is known as a good biomarker to show circadian rhythm irregularities. So, in most studies it is used to determine the degree of disturbance in circadian rhythm due to shift working [3]. The chemical structure of melatonin is shown below:



The chemical structure of melatonin.

Because of the low concentration of melatonin and also the existence of other compounds in body fluids, measuring the amount of melatonin is a serious challenge for the analysts [4]. This hormone can be measured in the blood [5, 6], urine [7, 8] and saliva [9, 10]. However, saliva sampling is the preferred method because, its collection is non-invasive, non-painful,

[1] The article is published in the original

Table 1. Evaluation of sample size based on the different parameters and processes

Parameter	pH	Extraction solvent	Volume ofextraction solvent	Disperser solvent	Volume ofdisperser solvent	Extraction time	Centrifugation time	Ionic strength (NaCl, %, w/v)
Level	5	4	4	4	4	4	4	4
Number of repetitions	3	3	3	3	3	3	3	3
Trials for each variable	15	12	12	12	12	12	12	12
Sum	99							
Validation	Within day		Calibration curve		Day to day		Sum	
Trials for each	6 × 3 = 18		5 × 6 = 30		6 × 3 = 18		66	
Total	99 + 66 = 165							

and easy [11]. Several studies have found a correlation between the salivary melatonin and its level in plasma [9, 12].

The most important part of many chromatographic analyses of biological samples is the elimination of interferers. If the analyte in the sample is present at trace level, elimination of interferents without disturbance in recovering the analyte is considered a serious problem. In addition, to inject the sample into HPLC, it is necessary to increase the concentration of the analyte [13–17].

Before doing chromatography to measure the melatonin amounts, the sample preparation is unavoidable. In previous studies, liquid–liquid extraction (**LLE**) [18] and solid-phase extraction (**SPE**) [1, 19, 20] were used in order to prepare melatonin in different samples. Although the use of LLE and SPE is prevalent, each of them has some disadvantages. The principle disadvantages of LLE are that it is time-consuming and costly, as well as needing a high volume of the sample and toxic organic solvents. SPE is boring and rather costly, although it uses a small volume of organic solvents. In addition, the high volume of the sample in SPE causes breakthrough [6].

As an alternative, dispersive liquid–liquid microextraction, developed in recent years can be used for preparation of melatonin. In this method, a water-insoluble extraction solvent is injected with pressure into the sample's aquatic environment by a syringe. As a result, the extracting solvent turns into tiny drops. This change causes the analyte to be extracted from the aquatic environment of the sample and transmitted to the organic environment of the extracting solvent. Recently, DLLME has been used to preconcentrate the impurities of metals, organic substances, organometallic compounds, and some non-metals. It is increasingly applied to develop the analytical methods [21, 22]. The main advantages of this method are high speed and simplicity, low cost, and low consumption of solvents [23]. The purpose of the present study was to optimize a new, sensitive, safe, quick and cost-effective DLLME to measure trace amounts of melatonin in saliva.

EXPERIMENTAL

Chemicals. Melatonin (+99%) was purchased from Alfa Aesaer (Karlsruhe, Germany). Stock solution of melatonin with the concentration of 200 mg/L in methanol was prepared. Other chemicals included methanol, ethanol, acetone, acetonitrile, and formic acid (HPLC grade) to determine the optimum dispersing solvent; dichloromethane, chloroform, carbon tetrachloride, 1,2-dichlorobenzene (99%) for determination of the optimum extraction solvent; NaCl (99%) for changing the amount of the salt of the sample solvent, and nitrogen gas for drying the solvents. All reagents were purchased from Merck (Darmstadt, Germany).

Apparatus. An HPLC equipped with a K-1001 single piston pump (Knauer, Germany), C18 column (25 cm × 4.6 mm i.d., 5 μm), fluorescent detector RF_10AXL (Knauer, Germany) were used. The pH of the solvents was measured using the digital pH-meter Metrhom 744 (Metrhom, USA). Materials and reagents were weighed using a Sartorius CP225D balance (Sartorius, Germany).

Samples. The study was conducted based on One Factor At a Time (**OFAT**) method: 99 experiments were done for optimization stage, which involved examining eight factors affecting DLLME method. Of these factors, seven were examined in four levels, and just pH in five levels. All the stages of optimization were repeated three times. Also, in order to validate the optimized method, 66 experiments were conducted as day to day and within day reproducibility evaluation. Table 1 shows evaluation of sample size based on the different parameters and processes.

Sample preparation and analysis. The DLLME method was used as follows: first, a specified volume of the disperser solvent containing a specified volume of the extraction solvent was, under pressure, injected quickly by a syringe into the test tube containing the sample, which caused the solution to become cloudy. Then, to extract melatonin from the sample, the solution was gently shacken for a while. After that, through centrifugation the solution was divided into two

Table 2. Variables, codes, and levels of experimental design

Variable	Codified variables	Level				
		1	2	3	4	5
Disperser solvent	(A)*	Acetonitrile	Methanol	Ethanol	Acetone	–
Volume of disperser solvent, mL	(B)	0.5	1	1.5	2	–
Extraction solvent	(C)	CCl_4	$CHCl_3$	CH_2Cl_2	$C_6H_4Cl_2$	–
Volume of extraction solvent, μL	(D)	50	100	150	200	–
Extraction time, min	(E)	1	3	5	7	–
Centrifugation time, min	(F)	2	4	6	8	–
Ionic strength (NaCl, %, w/v)	(G)	0	2.5	5	7.5	–
Sample pH	(H)	3	5	7	9	11

* "A1" means that the disperser solvent is acetonitrile.

phases, so that the solution containing melatonin got settled at the bottom and the aqueous phase of the sample at the top of the test tube. The phase containing melatonin was separated by a syringe, poured into another test tube, and its solvent evaporated by the gentle flow of N_2. At the end, the remaining melatonin was dissolved in methanol, and then the solution was injected into HPLC to detect melatonin.

To analyze the samples by the use of HPLC, the peak area was considered as the detector response. The extraction recovery (**ER**) was calculated through comparing the peak area in the chromatogram of extracts and that of the standard samples [24] as following:

RESULTS AND DISCUSSION

In order to find the optimum factors affecting the melatonin extraction, eight effective factors were selected: disperser solvent, volume of the disperser solvent, extraction solvent, volume of the extraction solvent, duration of extraction time, duration of centrifugation, ionic strength, and sample pH. In each step, seven factors were constant and one factor was varied at different levels to determine the optimum quantity. Table 2 shows variables, codes, and levels of experimental design, and Table 3 shows OFAT design to show effects of factors on recovery of melatonin. In all experiments, melatonin with concentration of 100 pg/mL was extracted from 10 mL sample solution. Figure 1 shows chromatograms of extracted blank aqueous sample (a) and aqueous standard of melatonin equivalent to 100 pg/mL (b).

Effects of factors on the extraction efficiency. *Disperser solvent.* To determine the optimum disperser solvent, four solvents (acetonitrile, methanol, ethanol and acetone) were examined. For the other seven factors fixed amounts were used. At the end, it was observed that acetonitrile had the highest extracting recovery (ER% = 90.3) compared with the other solvents (Table 3). Therefore, acetonitrile was chosen as the dispersing solvent.

Volume of the disperser solvent. To examine the effect of the volume of the disperser solvent, four volumes (0.5, 1, 1.5, and 2 mL) of acetonitrile, which was selected as the optimum disperser solvent in the first step, were chosen. The highest extracting recovery was obtained with the volume of 2 mL (Table 3). This volume, therefore, was selected as the optimum level for the next steps.

Extraction solvent. To choose the optimum extraction solvent, four solvents (CCl_4, $CHCl_3$, CH_2Cl_2, and $C_6H_6Cl_2$) were selected. The solvent with the highest percentage of extraction (ER% = 91.5) was CCl_4, which was determined as the optimum extraction solvent for the next steps of the study.

Volume of the extraction solvent. To examine the effect of the extraction solvent, four different volumes of 50, 100, 150, and 200 μl were selected of the optimum extraction solvent, that is CCl_4, which was determined in the previous step. The highest level of extraction recovery (ER% = 92.2) was obtained in the volume 200 μl (Table 3). This volume, therefore, was chosen as the optimum volume of the extraction solvent for the next steps.

Extraction time. To examine the effect of the extraction time, the process was done with four different durations (1, 3, 5, and 7 min). The highest ER (92.8%) was obtained with 3 min (Table 3). Therefore, this time was selected as the optimum for the next steps of the study.

Centrifugation time. To find the optimum centrifugation time, the process was done in four different durations (2, 4, 6, and 8 min). The highest ER (94.0%) was related to the 6 min duration (Table 3) which was selected as the optimum centrifugation time.

Ionic strength. To obtain the optimum amount of the salt, different amounts of NaCl (0, 2.5, 5, and 7.5%, w/v) were added to the sample matrix. After extracting and measuring melatonin in each step it was found that the highest ER (95.3%) was obtained with

Table 3. One-factor-at-a-time design to show effects of factors on recovery of melatonin

Step	Constants	Variable/ER (%)				
Selecting the optimum disperser solvent	B4, C1, D4, E2, F1, G1, H3	**A1***	A2	A3	A4	–
		90.3	34.5	26.8	33.2	
Selecting the optimum volume of disperser solvent	**A1***, C1, D4, E2, F1, G1, H3	B1	B2	B3	**B4***	–
		29.8	51.1	47.7	**90.7**	
Selecting the optimum extraction solvent	**A1***, **B4***, D4, E2, F1, G1, H3	**C1***	C2	C3	C4	–
		91.5	20.2	15.6	—	
Selecting the optimum volume of extraction solvent	**A1***, **B4***, **C1***, E2, F1, G1, H3	D1	D2	D3	**D4***	–
		38.1	52.4	77.9	**92.2**	
Selecting the optimum extraction time	**A1***, **B4***, **C1***, **D4***, F1, G1, H3	E1	**E2***	E3	E4	–
		53.3	**92.8**	61.1	80.1	
Selecting the optimum centrifugation time	**A1***, **B4***, **C1***, **D4***, **E2***, G1, H3	F1	F2	**F3***	F4	–
		79.9	79.0	**94.0**	67.0	
Selecting the optimum Ionic strength	**A1***, **B4***, **C1***, **D4***, **E2***, **F3***, H3	G1	G2	**G3***	G4	–
		77.1	88.2	**95.3**	89.6	
Selecting the optimum pH	**A1***, **B4***, **C1***, **D4***, **E2***, **F3***, **G3***	H1	H2	**H3***	H4	H5
		65.3	69.6	**95.8**	89.8	69.1

* The variables that were chosen as the optimum at each step in the next steps were taken as a constant.

5% of the salt (Table 3). This percentage, therefore, was chosen as optimum.

Sample pH. To examine the effect of sample pH, five sample solutions with different pHs (3, 4, 7, 9, and 11) were selected. After the melatonin was extracted and measured, it was found that the highest ER (95.8%) was related to the solution with pH 7 (Table 3). This pH was chosen as the optimum.

Method validation. At first, for the method validation, the calibration curve was plotted. The tested concentration interval was from 0 to 1000 pg/mL. The linear relationship between the peak area and melatonin concentration was obtained ($y = 0.6898x - 6.4767$, $R^2 = 0.999$). Based on the lenear range calibration curve, the limits of detection (**LOD**) and quantitation (**LOQ**) were calculated as 2.7 and 9 pg/mL, respectively. In addition, the relative standard deviation (**RSD**) for five replicate experiments was calculated to be 3.9%.

Table 4. Day-to-day reproducibility (the recovery% values are given) of melatonin spiked in saliva (sample volume, 10 mL)

Day	Melatonin added, pg/mL		
	250	100	50
1	98.8	91.1	109.4
2	100.5	91.6	96.9
3	102.1	95.3	103.0
4	98.6	94.2	103.9
5	99.9	96.6	93.9
6	96.8	94.5	109.3
Mean, %	99.4	93.9	102.8
SD	1.8	2.1	6.4
CV, %	1.8	2.3	6.2

In order that the DLLME method could be used for real samples, the validation of the optimized method was necessary. To do so, day to day reproducibility and within-day reproducibility were evaluated using three different concentrations (50, 100, and 250 pg/mL). The results are shown in Tables 4 and 5.

Discussion of DLLME optimal conditions obtained. The most important part of analyzing environmental and biological samples is sample preparation. In this process, different tasks are solved such as preconcentration of trace analytes, their separation from interfering factors and adapting the analytes for the mechanism of analytical system. The present study was conducted in order to examine and optimize a new, quick, cheap, and reproducible method for sample preparation to measure traces of melatonin hormone in saliva samples.

To determine the optimum amounts affecting DLLME, eight variables including disperser solvent, volume of the disperser solvent, extraction solvent, volume of the extraction solvent, extraction time, centrifugation time, ionic strength and sample pH were examined in different levels for three times. The kind of disperser solvent affected significantly the formation of the cloudy phase in the solution. Dissolving of the disperser solvent in the aqueous phase of the sample solutions (polar) and dissolving of the extraction

solvent (non-polar) in the disperser solvent are important points in selecting the disperser solvent. Therefore, four solvents under the study, i.e. acetone, methanol, ethanol, and acetonitrile were selected based on the molecule structure (having a polar and a non-polar groups). The results indicated that when acetonitrile was used as the disperser solvent, ER% of melatonin hormone from sample solution was higher than with the other solvents.

To determine the optimum volume of the disperser solvent, volumes of 0.5, 1, 1.5, and 2 mL were examined. Volumes less than 0.5 mL and more than 2 mL were not chosen. The former choice was due to the lack of suitable cloudy phase formation, and the latter choice was because of increasing the ratio of the disperser solvent to the extraction solvent and, therefore, preventing deposition of the extraction solvent. According to the obtained results, the highest ER% was achieved when the volume of 2 mL of acetonitrile was used as the disperser solvent.

The chemical structure of melatonin has low polarity, while the aqueous phase of the saliva sample has high polarity. Considering the two facts, the criteria used to select the extraction solvent were non-polarity, being heavier than water and having suitable chromatography function. The reason for using such criteria to select the extraction solvent is that melatonin has a more tendency to be solved in a non-polar solvent than in a polar one. In addition, if the extraction solvent is non-polar and heavy, it will be easily separated and settled through centrifugation. Based on that, four solvents (carbon teterachloride, chloroform, dichloromethane, and 1,2-dichlorobenzene were selected with relative densities of 1.59, 1.48, 1.33, and 1.30 g/mL, respectively. It was found that the highest recovery was obtained when CCl_4 was used as extraction solvent.

The volume of the extraction solvent can affect the amount of ER too. Volumes less than 50 and more than 200 μL were not selected: the former due to lack of producing cloudy phase and the latter due to the increase of the proportion of extraction solvent and the settlement of its excess. The highest percentage of ER was obtained when 200 μL of CCl_4 was used as the extraction solvent. The reason can be due to the higher percentage of the extraction solvent compared to other quantities.

Concerning extraction time, the highest percentage of ER was obtained when the cloudy sample solution was shaken for 3 min. With the increase of extraction time, there was a decrease in the percentage of ER. In the process of the sample preparation by the DLLME, just the quantities of the analyte which have dissolved in the extraction solvent can be analyzed and quantified. Therefore, the reason for decreasing of ER when extraction time is increased is that some of the analyte dissolved in the extraction solvent can be separated from it and dissolved in the disperser solvent.

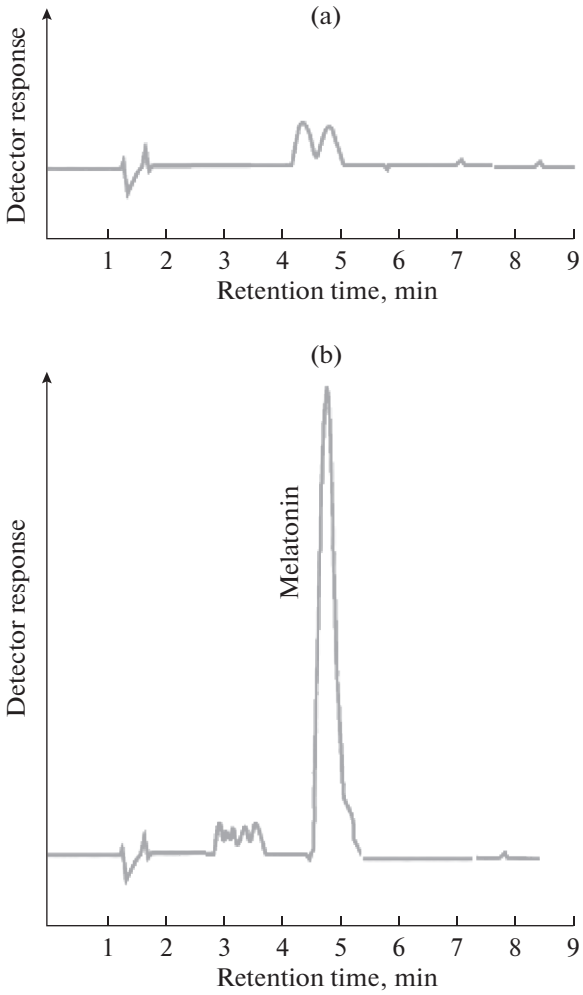


Fig. 1. Chromatograms of extracted blank aqueous sample (a) and aqueous standard of melatonin equivalent to 10 pg/mL (b). Conditions: injection volume, 20 μL; column C18; isocratic mobile phase: methanol–double distilled water (60 : 40); flow rate, 0.7 mL/min; fluorescence detection; excitation and emission wavelengths, 286 and 352 nm; 22°C.

In view of the high solubility of NaCl in water, its adding to the sample solution causes competition with other substances for dissolving in water, resulting in accelerating and strengthening extraction of melatonin from the aqueous phase of the sample and its transfer to the organic phase of the extraction solvent (salting-out effect). For this reason, the effect of adding different amounts of NaCl to the sample solution on the level of melatonin extraction was examined. The highest percentage of the ER was obtained through adding 5% (w/v) of this substance to the sample.

The present study was done on melatonin hormone as a relatively non-polar molecule with a low solubility in water. Since the extracting solution was a non-polar one, to obtain the highest percentage of ER it was nec-

Table 5. Within-day reproducibility (the recovery% values are given) of melatonin spiked in saliva (sample volume: 10 mL)

Experiment	Melatonin added, pg/mL		
	250	100	50
1	97.5	102.6	90.8
2	98.6	103.2	84.2
3	102.5	107.3	92.1
4	99.7	103.4	93.5
5	97.9	101.4	91.9
6	100.1	98.9	96.6
Mean, %	99.4	102.8	91.5
SD	1.8	2.7	4.1
CV, %	1.8	2.6	4.4

essary to find the isoelectric point of the melatonin molecule (a specific pH at which the number of negative and positive charges is equal) [25]. Through changing the pH of the sample and conducting the steps of the experiment, the highest level of extraction recovery was obtained when pH was 7 due to minimization of anions and cations of melatonin in this pH.

In the similar study [6], in which a DLLME method was optimized to extract melatonin from plasma sample, it was found that acetonitrile and carbon tetrachloride are the best disperser solvent and extraction solvent, respectively. The result of the mentioned study matches those of the present one.

The validity of the optimized method was examined day to day and within-day. To measure the amount of melatonin hormone of saliva, the standard curve was drawn for each day (6 days). Saliva samples were spiked with concentrations of 50, 100, and 250 pg/mL. The coefficient of variation (CV%) for these concentrations for day to day was 4.4, 2.6, and 1.8, respectively, and for within-day was 6.2, 2.2, and 1.8, respectively, which indicate the high accuracy of the optimized method.

CONCLUSIONS

Considering the results of the study, DLLME can be used for sample preparation to preconcentrate and separate melatonin hormone from saliva samples. It is evident that optimization of the factors affecting the extraction should be relevant to the given analyte. These factors earlier optimized for similar chemical compounds can be used with a slight modification. Sample preparation using DLLME has many advantages such as simplicity, low volume of the solvents, short time, compatibility with other sample preparation techniques and different methods of analysis, and finally cost efficiency.

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