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Spectrophotometric Determination of Melatonin Drug in its Pure and Dosage Forms Using Potassium Ferricyanide-Fe(III) System

Marwa El-Badry Mohamed[1,✉], Maher M. Hamed[1], Nadia G. Zaki[2], Mohamed M. Abdou[2], Gehad G. Mohamed[1] and Abanoub M. Abdallah[2]

Asian Journal of Organic & Medicinal Chemistry

Volume: 2 Year: 2017
Issue: 1 Month: January–March
pp: 37-41
DOI: https://doi.org/10.14233/ajomc.2017.AJOMC-P64

Received: 15 February 2017
Accepted: 15 March 2017
Published: 30 March 2017

ABSTRACT

A simple, fast and accurate spectrophotometric method for determination of melatonin drug in its pure and dosage forms was developed using potassium ferricyanide–Fe(III) detection system. This method was based on the reduction of Fe(III) to Fe(II) by melatonin, then *in situ* formed Fe(II) reacted with potassium ferricyanide ($K_3[Fe^{III}(CN)_6]$) to form the soluble prussian blue product, $KFe^{III}[Fe^{II}(CN)_6]$. The absorbance of the soluble prussian blue was measured at λ_{max} of 705 nm. The optimum conditions for this method were studied accurately. The absorbance was found to be linear to melatonin concentration in the range of 1.60-32.00 µg mL^{-1}. The different analytical parameters were discussed. The method has been successfully applied to determine melatonin drug in its pharmaceutical forms and the results obtained were in a good accord with results obtained by the official one as indicated by the percent recovery values.

KEYWORDS

Spectrophotometry, Melatonin, Potassium ferricyanide, Soluble prussian blue.

Author affiliations:

[1]Chemistry Department, Faculty of Science, Cairo University, Giza 12613, Egypt
[2]Narcotic Research Department, National Center for Social and Criminological Research (NCSCR), Giza 11561, Egypt

✉To whom correspondence to be addressed:

E-mail: mar.elbadry@gmail.com

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INTRODUCTION

Melatonin (N-[2-(5-methoxy-1*H*-indol-3-yl)ethyl]acetamide), which is an indole derivative with the chemical structure shown in Fig. 1 [1], is synthesized in the pineal gland of vertebrates from tryptophan *via* 5-hydroxytryptophan, serotonin and N-acetylserotonin [2]. Its biological activity involves changes in certain neurons in the central nervous system [3]. It was isolated for the first time from bovine pineal glands and chemically identified by Lerner and co-workers in 1959 [4].

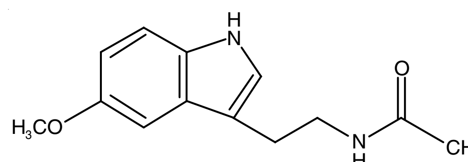


Fig. 1. Chemical structure of melatonin drug

The neurohormone melatonin is vital for synchronizing subjects suffering from old age, blindness, night work, jet lag,

and shift work. It has many pharmacological effects, such as sedative, regenerative, antidepressant, anxiolytic, antitoxic, antioxidant, anticonvulsant and analgesic effects. Therapeutically, it is used in Alzheimer's disease treatment, cancer therapy, and many neurodegenerative disorders [5].

Drug addiction has been considered as a main reason of morbidity, death and lost productivity. Recently, it was evidenced that melatonin reverses the effects of many drugs of abuse [6]. For example, it was shown that melatonin reversed the development of physical dependence and tolerance to morphine in mice and also inhibited its withdrawal syndromes. It was also found melatonin weakened the withdrawal contraction caused by naloxone in an isolated guinea pig ileum. In addition, melatonin had a reversal effect on the influence of morphine-induced rewarding effect, and this effect may be mediated through the activation of the receptor subtype melatonin MT2 within the tissues of the central nervous system in mice [6]. Furthermore, melatonin inhibited the behavioral sensitization induced by cocaine in mice [7].

Melatonin has a broad action on breast cancer due to its inhibition effect on tumor metabolism, genomic instability, and signaling, being an efficient direct scavenger of reactive oxygen species (ROS), lack of toxicity, synergism with other cancer therapeutic agents, minimal cost and wide availability [8].

The first report for quantitative determination of melatonin was its bioassay using tadpole skin [2], thereafter, several methods have been developed and improved for determination of melatonin included gas chromatography [9], High performance liquid chromatography (HPLC) method with fluorescent detection [10] or with mass spectrometer as a detector [11], radioimmunoassay [12], electrochemical methods [13-16], enzyme-linked immunosorbent assay (ELISA) [17], spectrophotometric [18] and spectrofluorimetric methods [19,20].

Spectrophotometry has been widely used in biomedical and pharmaceutical analysis for quantitative purposes and also for the characterization of drugs, metabolites, impurities and related substances [21].

The present work aims principally to study the oxidation-reduction reaction between melatonin drug and Fe(III) and also to utilize this reaction to present a simple, fast and accurate spectrophotometric method for determination of melatonin drug in its pure form and in some of its pharmaceutical dosages by using potassium ferricyanide $K_3[Fe^{III}(CN)_6]$ complex. The different experimental conditions, stoichiometry and mechanism of the reaction were discussed. Beer's law was valid in a satisfactory concentration range and the results obtained by this proposed method were compared with results obtained by the official one.

EXPERIMENTAL

All the chemicals and reagents used throughout this study were of analytical reagent grade and used without additional purification. Melatonin (purity > 99 %) (ML; $C_{13}H_{16}N_2O_2$; 232.28 g mol^{-1}) was purchased from Sigma-Aldrich Chemical Company (USA), ferric chloride ($FeCl_3{\cdot}6H_2O$, 270.32 g mol^{-1}) was purchased from Merck, Germany and potassium ferricyanide ($K_3[Fe(CN)_6]$; 329.26 g mol^{-1}) was purchased from Riedel-de Haën AG, Germany. The dosage form of melatonin (10 mg per capsule) was purchased from Puritan's Pride, Inc. NY, USA.

All the solutions were freshly prepared daily, standard solution of melatonin at a concentration of 1.722×10^{-3} mol L^{-1} was prepared by dissolving the accurately weighed amount of 0.04 g of melatonin in the least amount of acetonitrile, subsequently diluted with bi-distilled water to 100 mL (0.4 mg mL^{-1}). Afterward, the solution was stored at 4 °C in the dark. Ferric chloride solution at concentration of 1.5×10^{-2} mol L^{-1} was prepared by dissolving the accurately weighed amount of 0.4055 g of $FeCl_3$ in bi-distilled water, and 0.5 mL of HCl (2.00 mol L^{-1}) was added to the solution to prevent hydrolysis, subsequently diluted to 100 mL (4.055 mg mL^{-1}). The solution of 1.5×10^{-2} mol L^{-1} potassium ferricyanide was obtained by dissolving the accurately weighed amount of 0.4938 g of $K_3[Fe(CN)_6]$ in 100 mL standard flask with bi-distilled water (4.938 mg mL^{-1}).

A Shimadzu UVmini-1240 UV-visible spectrophotometer, Japan, with matched quartz cells of 1 cm optical path length was used for all the spectrophotometric measurements.

A manual Unico 1200 spectrophotometer with wavelength range of 325-1000 nm (United Products and Instruments Inc.) was used to investigate the ruggedness and robustness of the proposed method.

HPLC measurements for determination of melatonin drug by the official method [22] were performed at the Microanalytical Center, Cairo University, using YL9100 HPLC instrument (Korea).

Aliquots containing melatonin drug in the working concentration range of 1.6-32 µg mL^{-1} were transferred into 5 mL volumetric flask. 0.3 mL of Fe(III) solution and 1 mL of $K_3[Fe(CN)_6]$ were added. The solutions were completed to the mark by using bi-distilled water and mixed well (pH = 4.0) and then were left for 0.5 h at 27 ± 1 °C before the absorbance was measured at λ = 705 nm against a blank solution prepared in the same way without melatonin drug. The melatonin drug concentration was calculated from the standard calibration graph prepared under the same identical optimum conditions.

For the pharmaceutical samples, the contents of twenty capsules of melatonin were mixed carefully and weighed. An accurately weighed amount of this powder was dissolved in the least amount of acetonitrile, filtered by using a Whatman filter paper No. 2 and washed with acetonitrile solvent. The filtrate was diluted with bi-distilled water till bring the concentration to 0.4 mg mL^{-1} in a calibrated measuring flask, mixed well and preserved without light at 4 °C. An aliquot was used to determine melatonin concentration according to the procedure mentioned above.

RESULTS AND DISCUSSION

Spectrophotometric properties of the soluble prussian blue product ($KFe^{III}[Fe^{II}(CN)_6]$) and also the different parameters affecting the color development were extensively studied to determine the optimal conditions for the assay procedure. The reaction was studied as a function of the volume of all reagent solutions, temperature, time of reaction, effect of different acids and stoichiometry of the reaction components.

Absorption spectra, stoichiometry and reaction mechanism: Fig. 2 represents the absorption spectrum of the soluble prussian blue product ($KFe^{III}[Fe^{II}(CN)_6]$) which absorbed maximally at λ = 705 nm with a high molar absorptivity value (ε = 1.71 × 10^4 L mol^{-1} cm^{-1}).

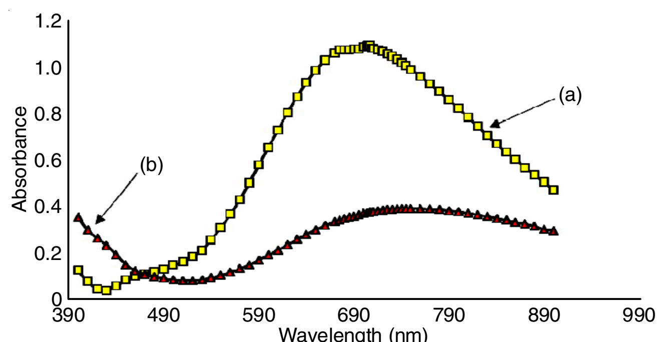
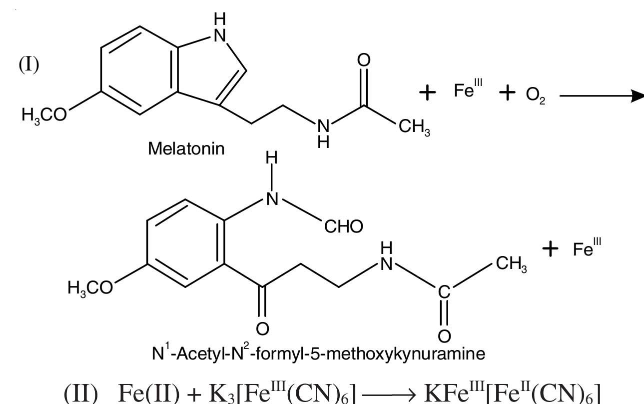


Fig. 2. Absorption spectra of (a) Fe(III)-$K_3[Fe(CN)_6]$-melatonin drug against reagent blank; (b) Fe(III)-$K_3[Fe(CN)_6]$ (reagent blank) against water; [ML], 8.0 µg mL^{-1} (0.1 mL); [Fe(III)], 81.1 µg mL^{-1} (0.1 mL); [$K_3[Fe(CN)_6]$], 98.78 µg mL^{-1} (0.1 mL); t, 5 min; T, 27 °C

The proposed method was based on the oxidation-reduction reaction between melatonin and Fe(III) as shown in **Scheme-I**. Fe(III) was reduced to Fe(II) by melatonin drug [23] at pH 4.0 and then the *in situ* formed Fe(II) reacted with potassium ferricyanide ($K_3[Fe^{III}(CN)_6]$) to form a soluble prussian blue product, ($KFe^{III}[Fe^{II}(CN)_6]$) [24].



(II) $Fe(II) + K_3[Fe^{III}(CN)_6] \longrightarrow KFe^{III}[Fe^{II}(CN)_6]$

Scheme-I: Reaction mechanism (I) Fe(III) was reduced to Fe(II) by melatonin, the reaction stoichiometric ratio was 1:1 and melatonin was oxidized to N^1-acetyl-N^2-formyl-5-methoxykynuramine [23]; (II) The *in situ* formed Fe(II) reacted with $K_3[Fe(CN)_6$ to form soluble prussian blue

Both of Job's continuous variation [25] and molar ratio [26] methods were applied in order to specify the stoichiometry of melatonin-Fe(III) reaction. The results showed that the reaction stoichiometric ratio was 1:1.

Influence of time and temperature: The optimum time for the complete reaction between melatonin drug and Fe(III) was determined by following the color development of the soluble prussian blue ($KFe^{III}[Fe^{II}(CN)_6]$) spectrophotometrically at room temperature and at λ_{max} = 705 nm. It was found that complete color development was attained after 0.5 h.

Absorbance was also determined at different temperatures with keeping time fixed at 0.5 h, the absorbance attained its maximum at room temperature (27 ± 1 °C).

Influence of ferric(III) and potassium ferricyanide concentrations: The influence of the concentration of ferric chloride on absorbance was studied. It was found that 0.3 mL (243.29 µg mL^{-1}) of Fe(III) was sufficient for the absorbance to reach its maximum and absorbance almost didn't affect by any more addition of Fe(III). This clearly indicated that melatonin was completely oxidized by Fe(III) and the concentration of both the produced Fe(II) and the formed soluble prussian blue reached their maximum.

The influence of the concentration of potassium ferricyanide on the absorbance was also studied, the maximum absorbance was reached when the amount of potassium ferricyanide was 1.0 mL (987.76 µg mL^{-1}).

Effect of different acids: Keeping other conditions fixed, the effect of different acids (2 mol L^{-1}) on the absorbance was examined as shown in Fig. 3. It is obvious from the figure that the absorbance declined of a different degree by increasing the amount of the different acids, this is due to that Fe(III)'s oxidation potential reduces along, by increasing the acidity of the solution which makes the reducing ability of melatonin decreases [24]. It was shown from the given results that H_3PO_4 has a great effect on the absorbance; when the volume of H_3PO_4 was more than 0.30 mL, the absorbance decreased obviously from 0.730 (0.00 mL) to 0.443 (0.50 mL). This was due to the formation of the steady colorless complex $Fe(HPO_4)^+$ which made the oxidation potential of Fe(III) reduced and so the absorbance decreased consequently [24].

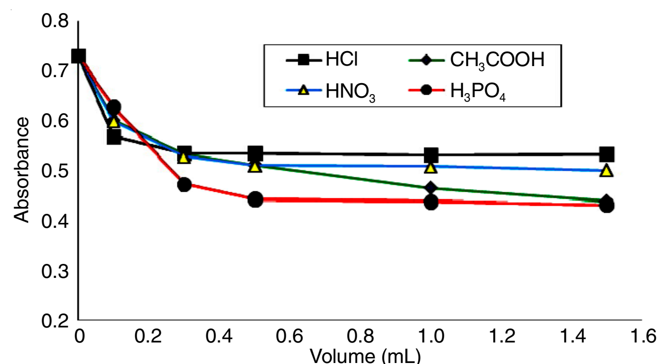


Fig. 3. Influence of different acids on the absorbance. [ML], 8.0 µg mL^{-1} (0.1 mL); [Fe(III)], 243.29 µg mL^{-1} (0.3 mL); [$K_3[Fe(CN)_6]$], 987.76 µg mL^{-1} (1.0 mL); t, 30 min; T, 27 °C; λ_{max}, 705 nm

Validity of Beer's law and method validation: Under the optimum reaction conditions described above, calibration graph was constructed to define the concentration limits of melatonin drug at which the reaction is quantitative. Consequently, this spectrophotometric method can be easily applied for quantitative determination of melatonin in its pharmaceutical formulations *via* the redox reaction with Fe(III) and by using $K_3[Fe(CN)_6]$.

The different analytical parameters obtained for this method are given in Table-1, which shows that Beer's law was valid over the concentration range of 1.60-32.00 µg mL^{-1} and the correlation coefficient of the data obtained is 0.9992. The molar absorptivity (ε), Sandell sensitivity (S), limit of detection (LOD), limit of quantification (LOQ) and regression equation are also listed in Table-1. The small values of Sandell sensitivity, LOD, and LOQ indicated the high sensitivity of this method.

TABLE-1
ANALYTICAL PARAMETERS FOR SPECTROPHOTOMETRIC DETERMINATION OF MELATONIN DRUG *via* ITS REDOX REACTION WITH Fe(III)

Parameter	Value
λ_{max} (nm)	705
t (min)	30
Temperature (°C)	27
Concentration range (μg mL^{-1})	1.60-32
ε (L mol^{-1} cm^{-1})	1.7131×10^4
S (μg cm^{-2})	0.0136
Recovery (%)	99.5-101.52
Standard deviation	0.015-0.151
Relative standard deviation (%)	0.45-1.73
Regression equation	y = ax + b
Slope (a)	0.0738
Intercept (b)	0.0552
Correlation coefficient (r^2)	0.9992
LOD (μg mL^{-1})	1.2
LOQ (μg mL^{-1})	3.9

Five replicate measurements were performed at different concentration levels and both of standard deviation (SD) and relative standard deviation (% RSD) values were calculated and found to be ranged from 0.015 to 0.151 and from 0.45 to 1.73%, respectively. The small values of SD and % RSD also indicated the high accuracy and high precision of this proposed method.

The accuracy of an analytical method is an indication about to what extent the found value is close to the value which is accepted either as an accepted reference value or a conventional true value [27].

Precision was performed at two different levels; repeatability (intra-day) and reproducibility (inter-day). Repeatability (intra-day) indicates how it is easy for an operator in a laboratory to get the same result for the same batch of material at different times using the same method and the same instrument and reagents while reproducibility (inter-day) results from variations such as different instrument, analysts and days [27,28].

In order to demonstrate the validity and applicability of this proposed method and to what extent the results obtained were reproducible, five replicate experiments at three different concentrations of melatonin drug in both of its pure and pharmaceutical forms were carried out.

The values of the intra- and inter- day relative standard deviations for three different concentrations of melatonin obtained over a period of 5 days are shown in Table-2. The small values indicate that the proposed method is highly precise and the values of the percentage recoveries which ranged from 99.50 to 100.5% indicate the accuracy of this proposed method.

LOD is the lowest concentration of melatonin drug in a sample that can be detected, not quantified, while LOQ expresses the lowest concentration that can be determined with good precision and accuracy under the optimum operational conditions of the method. From Table-1, the LOD and LOQ values were 1.2 and 3.9 μg mL^{-1}, respectively.

The ruggedness of this proposed method was assessed by applying the procedure using two different spectrophotometers and at two different time intervals. It was found that the RSD (%) did not exceed 2% which indicated that none of these variables affected the assay of melatonin as shown in Table-3.

Robustness of procedure was accomplished by estimating the effect of small variation of reaction time on the analytical performance of the method. The small variation did not

TABLE-2
INTER-DAY AND INTRA-DAY PRECISION

Drug taken (μg mL^{-1})	Intra-day				Inter-day			
	Found (μg mL^{-1})	Recovery (%)	SD[a]	RSD[a] (%)	Found (μg mL^{-1})	Recovery (%)	SD[a]	RSD[a] (%)
4.00	3.98	99.50	0.015	0.38	3.99	99.75	0.044	1.11
8.00	8.07	100.8	0.024	0.30	8.01	100.1	0.022	0.28
24.00	23.96	99.83	0.151	0.63	24.09	100.4	0.135	0.56

[a]Means and relative standard deviations for five experiments carried out over 5 different days.

TABLE-3
RUGGEDNESS AND ROBUSTNESS OF THE METHOD

Drug taken (μg mL^{-1})	Effect		Pure form			Capsules		
			Found (μg mL^{-1})	Recovery (%) ± SD	RSD (%)	Found (μg mL^{-1})	Recovery (%) ± SD	RSD (%)
8	Instrument	1	7.97	99.63 ± 0.069	0.87	7.96	99.50 ± 0.037	0.46
		2	8.03	100.4 ± 0.075	0.93	8.01	100.1 ± 0.077	0.96
	Time (min)	25	7.98	99.75 ± 0.031	0.39	7.94	99.25 ± 0.075	0.94
		35	8.01	100.1 ± 0.092	1.15	7.99	99.88 ± 0.081	1.02

TABLE-4
SPECTROPHOTOMETRIC DETERMINATION OF MELATONIN IN ITS PHARMACEUTICAL PREPARATION BY USING THE PROPOSED METHOD *via* POTASSIUM FERRICYANIDE-Fe(III) SYSTEM AND BY USING THE OFFICIAL METHOD[a]

Proposed [Drug (μg mL^{-1})]		Official [Drug (μg mL^{-1})]		Recovery (%)		SD[b]	SD[c]	F-test	t-test
Taken	Found	Taken	Found	Proposed	Official				
4.00	3.97	4.00	3.94	99.25	98.50	0.042	0.023	3.33	1.60
8.00	8.02	8.00	7.96	100.3	99.50	0.067	0.042	2.54	2.00
24.00	23.99	24.00	23.96	99.96	99.83	0.032	0.029	1.22	2.10

[a]Number of replicates (n), 5. Standard F-values at 95% confidence level, 5.050. Standard t-values at 95% confidence level, 2.571 [Ref. 28].
[b]Standard deviation values using proposed method. [c]Standard deviation values using official method.

significantly affect the results (Table-3), so this indicated the reliability of this proposed method for further routine work.

Comparison between our proposed method and the official one [22]: The proposed method was successfully applied for the determination of melatonin in its pharmaceutical preparation and the results obtained are given in Table-4. According to the calculated t- and F-values, it is clear that the results obtained by the proposed method are in a good accord with those obtained by the applied standard one. The proposed method was accurate, with high recoveries amounting to 99.25-100.4%.

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