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Determination of melatonin in galenic preparations by LC and voltammetry

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Abstract

The amount of melatonin in galenic tablets was determined by means of two feasible and accurate analytical methods. The high performance liquid chromatography (HPLC) with ultraviolet detection at 223 nm used a C_{18} reversed-phase column; the linear scan voltammetric procedure (LSV) employs a pH 3.0 phosphate buffer as the supporting electrolyte for the oxidation process of melatonin, which has a current intensity maximum at + 850 mV. The two methods gave satisfactory results in terms of precision and accuracy. In fact, the data of repeatability and intermediate precision expressed as percentage relative standard deviations (RSD%) were ≤ 2.8 and the accuracy values, resulting from recovery experiments, were between 99.0 and 101.3%. Both methods are suitable for quality control of melatonin in galenic preparations; the LSV procedure is more rapid, the HPLC method is more sensitive and more precise.

Keywords: Melatonin; Liquid chromatography; Linear scan voltammetry; Galenic tablets; Quality control

1. Introduction

Melatonin (*N* - acetyl - 5 - methoxytryptamine) (Fig. 1) is a hormone of the pineal parenchymal cells, synthesized from serotonin by *N*-acetylation and *O*-methylation, and secreted by them into the blood and the cerebrospinal fluid [1]. Melatonin was discovered by Lerner in 1958, and this name is due to its blanching effect on melanophores [2]. Melatonin synthesis and secretion are increased during hours of darkness and may affect the sleeping pattern [1]. The biochemical role of melatonin is important in many physiological processes, but some of these have yet to be clarified, like the melatonin role in neurological and psychiatric disorders [3,4]. The employment of melatonin as a drug has been proved in the alleviation of jet-lag (5 mg daily) [5] and other disorders resulting from the delay of sleep [6,7]. Oral administrations of melatonin have a positive effect in some forms of insomnia [8], and depressive disorders [9]. Moreover, the melatonin/steroid combination has a contraceptive activity [10] and this use of melatonin may be associated with a reduced risk of breast cancer [11]. Melatonin in large doses is also used in association with inter-

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leukin-2 for the therapy of malignant neoplasms [12] or as chemioprevention of different kinds of tumors when combined with other drugs [13,14]. In a recent paper, it has been reported that melatonin inhibits the activation of poly(ADP-ribose) synthetase, and that it has pharmacological effects on shock, inflammation and ischemia injury [15]. The relationship between aging and melatonin had been studied in the past, and some authors considered it as a 'wonder drug', because of its antioxidant effects [16].

The discovery of these therapeutical properties of melatonin led to a great increase in the pharmaceutical preparation, expecially galenic preparats, containing this drug, and consenquently the need to have analytical methods at disposal for the quality control was even greater.

Several methods are reported in literature for the analysis of melatonin. The determination of melatonin at very low levels in biological samples was carried out by high performance liquid chromatography (HPLC) with electrochemical detection in rat plasma [17] or HPLC with fluorescence detection after derivatization in pineal gland or fish plasma [18,19]. Furthermore, a method based on capillary electrophoresis with amperometric detection [20] was also used for the analysis of melatonin in rat pineal gland and tablets; and with UV detection [21] only in tablets. Melatonin concentrations were determined in biological fluids or in pharmaceutical formulations by gas chromatography–mass spectrometry [22–24] and also in plasma samples by radioimmunoassays [25].

An electrochemical method using cyclic voltammetry for the determination of melatonin was carried out in a perchloric acid medium at a carbon paste electrode [26], with the objective of delucidating the oxidation mechanism of melatonin and briefly for assay of melatonin tablets. Spectrofluorimetric and colorimetric methods [27] have been reported for the determination of melatonin in tablets and serum, with laborious derivatization procedures: by means of a reaction of *p*-dimethylaminobenzaldehyde in hydrochloric acid (van Urk reagent)–ferric chloride in sulfuric acid (Salkowski reagent) mixture for the colorimetric method, and a reaction with *o*-phtaldehyde in acid medium for the spectrofluorimetric method.

The purpose of this paper is to develop simple and reliable analytical methods for the determination of melatonin in galenic formulations. A linear scan voltammetric method (LCV) and HPLC method with UV detection were developed and used for the quality control of melatonin tablets.

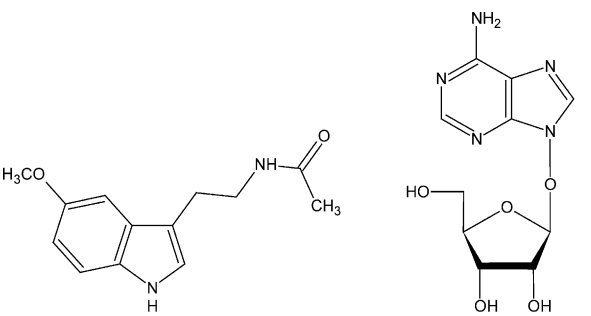
2. Experimental

2.1. Chemicals

Melatonin (*N*-acetyl-5-methoxytryptamine) and adenosine (9-β-D-ribofuranosyladenine) analytical grade were purchased from A.C.E.F. (Fiorenzuola d'Arda, PC, Italy). Methanol (MeOH) and acetonitrile (MeCN), HPLC grade, potassium dihydrogen phosphate (KH_2PO_4), 85% (m/m) orthophosphoric acid, sodium hydroxide and potassium hydroxide, analytical grade, were from Carlo Erba (Milan, Italy). Triethylamine (99%, for analysis) was purchased from Fluka (Bucks, Switzerland).

The declared composition of the galenic preparation, available as tablets, obtained from a pharmacy of Bologna (Italy), was 2 mg of melatonin and excipients up to 150 mg.

Ultrapure water (18.2 MΩ cm) was obtained by means of a Millipore (Bedford, MA, USA) Milli Q apparatus.



Melatonin **Adenosine**

Fig. 1. Chemical structures of melatonin and adenosine (IS).

2.2. Apparatus and experimental conditions

2.2.1. High performance liquid chromatography

The chromatographic apparatus consisted of a JASCO PU-980 isocratic pump, equipped with a JASCO UV-975 spectrophotometric detector set at 223 nm. The separation was achieved on a Phenomenex (Torrance, CA, USA) Hypersil ODS reversed phase column (C18, 250 × 4.6 mm, 5 μm), connected to a Phenomenex Hypersil ODS reversed phase precolumn (C18, 50 × 4.6 mm, 5 μm). The mobile phase was a (15 mM; pH 2.43) phosphate buffer, MeOH and MeCN (67:19:14, v/v/v) mixture, containing 0.25% (v/v) triethylamine (pH 3.0). Prior to use, the HPLC eluent was filtered through a Millipore membrane filter, (nylon 47 mm diameter, 0.2 μm pore size) and degassed by sonication (Transsonic T-310 apparatus from Elma GmbH, Singen, Germany). The apparatus was maintained at room temperature; the injection loop was 20 μl and the flow rate was 1 ml min^{-1}.

2.2.2. Linear scan voltammetry

The voltammetric assays were carried out using an AMEL (Milan, Italy) Model 433 voltammeter (working electrode: glassy carbon; reference electrode: Ag/AgCl; auxiliary electrode: stainless steel). Voltammograms were obtained by means of LSV, with the voltage increasing from 0 to 1000 mV at the constant rate of 5 mV s^{-1} using a phosphate buffer (50 mM, pH 3) as the supporting electrolyte. The assays were carried out while thermostatting the solutions at 25 °C, measuring the current intensity at the potential value of + 850 mV. Every morning, before use the electrodes were washed with ultrapure water, then 10 cycles of cyclic scan voltammetry (0–1000 mV, 200 mV s^{-1}) with water and one LSV run with the supporting electrolyte were carried out. Between two consecutive analytical runs an LSV run with the supporting electrolyte was carried out.

2.2.3. Spectrophotometry

The spectrophotometric assays were carried out using a JASCO (Tokio, Japan) Uvidec-610 double-beam spectrophotometer. Quartz cuvettes with an optical path of 1 cm were used.

2.3. Solutions and extraction procedure

The stock solutions (1 mg ml^{-1}) of melatonin were prepared by dissolving 10 mg of compound in 10 ml of MeOH. The different standard solutions were prepared by diluting suitable amounts of the standard solution with pH 3.0, 50 mM phosphate buffer in order to obtain working solutions of concentration ranging from 0.125 to 1.0 μg ml^{-1} for HPLC analysis and 6.25 to 50 μg ml^{-1} for voltammetric analysis. The stock solutions of the analyte in MeOH were kept in freezer (− 20 °C) for at least 1 month, while the standard solutions were prepared daily from the stock solutions, in order to avoid any alteration of the drug.

Melatonin was extracted from the galenic oral tablets using the following procedure:

At first, 20 tablets were accurately weighed, finely ground to a powder and thoroughly mixed. A weighed portion of the tablet powder, equal to 100 mg was transferred into a test tube with 100 ml of pH 3.0, 50 mM phosphate buffer and after agitation in an ultrasonic bath for 10 min, followed by centrifugation for 10 min at 3000 rpm. Finally the supernatant was filtered through a cellulose acetate syringe filter (0.20 μm, Albet-Jacs). This solution has a melatonin nominal concentration of 13.3 μg ml^{-1}.

2.4. Analytical procedures

2.4.1. HPLC method

Stock solutions of melatonin were in MeOH (1 mg ml^{-1}). Dilutions were made using pH 3.0, 50 mM phosphate buffer.

Adenosine was used as the internal standard for the control of retention times only, and was injected into HPLC at the concentration of 0.300 μg ml^{-1} after dilution with pH 3.0, 50 mM phosphate buffer from the solution stock in MeOH (1 mg ml^{-1}).

A 10-point calibration curve was set up by plotting area values against melatonin standard solutions concentrations, in the 0.125–1.0 μg ml^{-1} range. The melatonin galenic tablets solu-

tion with nominal concentration of 13.3 μg ml^{-1} was diluted in order to obtain a solution of 0.665 μg ml^{-1} that was injected into the HPLC.

2.4.2. Voltammetric method

The working solutions were prepared from stock solutions of melatonin in MeOH (1 mg ml^{-1}), by dilution with phosphate buffer (pH 3.0, 50 mM). The calibration curve was set up analysing standard solutions at different concentrations (in the 6.25–50 μg ml^{-1} range) and plotting the value of the current intensity at + 850 mV against the melatonin concentration. The melatonin galenic tablets solution with nominal concentration of 13.3 μg ml^{-1}, obtained dissolving 100 mg of an accurate weighed portion of the tablet powder with pH 3.0, 50 mM phosphate buffer. The extracted solution, after agitation, centrifugation and filtration, was subsequently analysed.

For the two methods, the values resulting from the analysis of diluted melatonin galenic tablets solutions were interpolated on the respective calibration curves and the percentage of melatonin found related to the declared amount was expressed as follows: (concentration found/concentration declared) × 100.

2.4.3. Precision assays

Melatonin standard and galenic tablets solutions were prepared and analysed six times within the same day to obtain the repeatability, and six times over different days to obtain the intermediate precision, according to USP 24 requirements [28].

Each assay was carried out on a different extraction of melatonin from the galenic tablets. The percentage relative standard deviations (RSD%) of the data obtained were calculated.

2.4.4. Accuracy

The accuracy of the methods was evaluated by means of recovery determinations, adding a known quantity of the reference powder to a certain amount of the galenic formulation, in order to obtain three different levels of addition. The samples were analysed and the mean recovery, as well as the repeatability was calculated on six assays for each concentration added.

3. Results and discussion

The ultraviolet spectrum of melatonin standard solutions in MeOH show two absorbance bands with maxima at 223 and 275 nm. Preliminary spectrophotometric assays on melatonin standard solutions in MeOH were carried out in order to monitor the stability of the standard solutions. The assays carried out indicated that melatonin stock solutions (1 mg ml^{-1}) in MeOH are stable for at least 6 months when stored at − 20 °C; these results were confirmed by the data obtained by means of HPLC–UV method, which did not show any interference, even when injecting solutions prepared from a 6-month-old melatonin stock solution.

3.1. HPLC method

The analysis of melatonin was carried out using a liquid chromatographic apparatus with a UV detector set at 223 nm. Preliminary assays were performed using the same chromatographic conditions of one of our previous works [29], where melatonin was tested only to verify its possible interference in the determination of other CNS drugs.

A reversed phase column (C18, 250 × 4.6 mm, 5 μm) was used, and as mobile phase a mixture of MeCN, MeOH and 10.4 mM pH 1.9 phosphate buffer (17.5/20/62.5, v/v/v), containing 0.25% (v/v) triethylamine. Adenosine was selected as the internal standard, which was used for the control of retention times only. In order to improve the separation of the adenosine from the injection peak, some minor changes were made to this mobile phase, in fact the percentage of MeOH was reduced to 19%, and also the percentage of MeCN was decreased to 14%. Moreover, to reduce the peak tailing and to obtain a better symmetry of the peaks, the salt concentration was increased; in fact a phosphate buffer with a concentration of 15 mM was used. The peak corresponding to melatonin has a retention time of 6.0 min and is well separated from the peak of adenosine with a retention time of 2.2 min.

The calibration curve was obtained by plotting area peaks values of melatonin standard solutions

against the melatonin concentration. Linearity was observed between 0.125 and 1.0 μg ml^{-1} melatonin concentration range. The equation of the calibration line, obtained by the least-square regression was: $y = -9.137 + 21.18x$, where x is the melatonin concentration, expressed as ng ml^{-1}, and y is the peak area value of melatonin. The linearity, expressed by the linear correlation coefficient, r, was 0.9995. The LOQ (quantitation limit) value was 0.125 μg ml^{-1} and the LOD (detection limit) value was 0.065 μg ml^{-1}, calculated according to USP [28]. The precision assays gave RSD% values of 0.9 for repeatability and 1.3 for the intemediate precision on 0.500 μg ml^{-1} melatonin standard solution ($n = 6$).

The method was applied to the analysis of melatonin contained in galenic tablets. The chromatogram of an extract having a nominal concentration of 665 ng ml^{-1} of melatonin, is shown in Fig. 2. It is apparent that the peak of melatonin is very neat, with a retention time of 6 min and well separated from that of adenosine (0.300 μg ml^{-1}), used as IS, which has a retention time of 2.2 min. The overall morphology of the chromatogram is nearly identical to that of a standard solution at the same concentration.

The extraction procedure of the drug from the tablets is very simple and feasible being based only on one step treatment of the powder obtained from the tablets with a pH 3.0 phosphate buffer. This method assures a good efficiency and selectivity. In fact, it can be seen that no interference from excipients was revealed.

The percentage of label claim found, reported in Table 1, is near to 100%; this indicates that the galenic tablets were accurately prepared. In effect, the amount of drug found is in accordance with the claimed value, and within the limit prescribed by USP 24 [28].

Precision assays were carried out analysing extracts of the galenic tablets in order to evaluate the RSD% data of intra- and interday assays. These were both satisfactory as reported in Table 1. In fact, the RSD values were 1.5 and 2.4% for the repeatability and intermediate precision, respectively.

The accuracy of the method was evaluated by means of recovery studies by adding to a known amount of the galenic formulation known quantities of pure melatonin at three different concentrations (0.125–0.225–0.325 μg ml^{-1}). The results are reported in Table 2. The high recovery values (the mean recovery is 100.6%) report a quantitative recovery of the analytes indicating the fine accuracy of the proposed HPLC method.

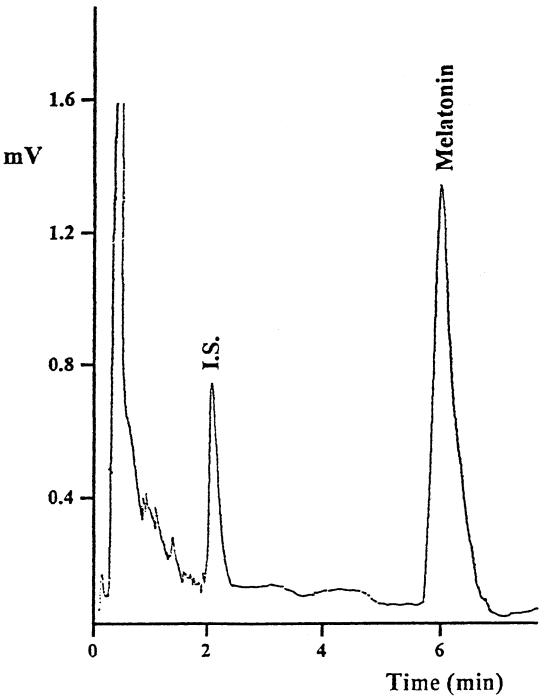


Fig. 2. Chromatogram of a 0.665 μg ml^{-1} (melatonin nominal concentration) galenic tablets extract containing 0.300 μg ml^{-1} of IS (adenosine). Conditions: RP C18 column, 250 length, 4.6 mm i.d., 5 μm particle size. Mobile phase: (15 mM; pH 2.43) phosphate buffer, MeOH and MeCN (67:19:14, v/v/v), containing 0.25% (v/v) triethylamine (pH 3.0) mixture. Flow rate 1 ml min^{-1}. Detection at 223 nm.

Table 1
Quality control of galenic tablets by the two methods

Parameter	HPLC	LSV
% Drug found of declared	99.0	99.1
Repeatability (RSD%)	1.5	1.9
Intermediate precision (RSD%)	2.4	2.8
Concentration (μg ml^{-1})	0.665	13.3

Each value is the result of six independent assays.

Table 2
Recovery assays

Parameter	HPLC	LSV
Low concentration (μg ml^{-1})	0.125	6.5
Recovery %	101.3	101.0
Repeatability (RSD%)	1.8	2.8
Middle concentration (μg ml^{-1})	0.225	13
Recovery %	101.0	100.0
Repeatability (RSD%)	1.6	2.8
High concentration (μg ml^{-1})	0.325	25
Recovery %	99.6	99.0
Repeatability (RSD%)	1.1	2.6

Each value is the result of six independent assays.

The precision of the recovery assays (repeating the procedure six times) was also satisfactory; in fact, the values of RSD% intraday, calculated on six trials, varied between 1.1 and 1.8.

3.2. LSV method

Melatonin is an electroactive substance that can be easily oxidized and is, thus, suitable for analyses with electrochemical techniques, such as LSV. A pH 3.0 phosphate buffer (50 mM) was used as the supporting electrolyte; the oxidation of melatonin was carried out by means of LSV at a constant rate of 5 mV s^{-1}, at a temperature of 25 °C.

Several experiments were carried out to evaluate the effect of the pH on the oxidation of melatonin. The pH values of the supporting electrolyte varied between 2.5 and 5.0. The voltammograms showed that the intensity of the oxidation current was higher at lower pH values. These results are in agreement with the data of literature [26] obtained by cyclic voltammetry, in fact melatonin is oxidized at higher pH values producing a shift of the voltammetric wave to less positive potentials with a decrease in peak current. For this reason a pH 3.0 phosphate buffer was selected.

Fig. 3 shows the voltammograms obtained with: (a) the supporting electrolyte; (b) an extract from galenic tablets having a nominal concentration of 13.3 μg ml^{-1}; and (c) a melatonin standard solution of 15 μg ml^{-1}.

The calibration curve, set up in the 6.25–50 μg ml^{-1} range of melatonin concentration has a good linearity. The regression equation (obtained by means of the least-square method) was $y = 22.57 + 51.29x$, where y is the height of the oxidation wave, expressed as nA, and x is the concentration of melatonin, expressed as μg ml^{-1}. The linear correlation coefficient was $r = 0.9990$. The LOQ was 5 μg ml^{-1} and the LOD value was 2.5 μg ml^{-1}. These values were calculated according to USP [28].

The precision was assessed for 15 μg ml^{-1} melatonin standard solutions, and the RSD% values resulted to be satisfactory being 1.8% for the repeatability assay and 2.6% for the intermediate precision ($n = 6$).

The quality control assays of melatonin in galenic tablets, expressed as percentage of the label claim, gave results that were near to 99%. As one can see from Table 1, the quantity of the drug found is in accordance with that obtained by HPLC and with the value claimed. The precision is also satisfactory. The RSD values obtained (Table 1) were 1.9 for repeatability (RSD% intraday) and 2.8% for the intermediate precision (RSD% interday).

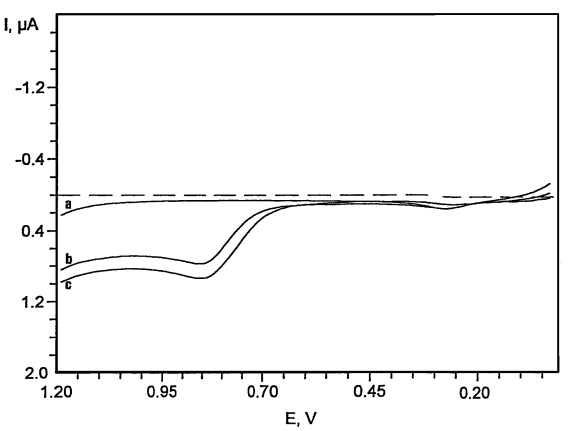


Fig. 3. Potential linear scan voltammograms of the supporting electrolyte (a) a 13.3 μg ml^{-1} (melatonin nominal concentration) galenic tablets extract (b) and a 15 μg ml^{-1} melatonin standard solution (c). Conditions: phosphate buffer (50 mM; pH 3.0) as the supporting electrolyte, constant rate of 5 mV s^{-1}, and temperature 25 °C.

The accuracy of the method was evaluated by means of recovery assays. A suitable amount of melatonin was added to a known amount of the powdered galenic tablets. The spiked samples were subjected to the procedure described in Section 2.3 and analysed by means of potential LSV. All data are summarized in Table 2. The results show that the mean recovery value was 100%, having thus an excellent accuracy. The precision of the recovery assays was also assessed and a mean repeatability value (RSD%) of 2.7% was obtained.

4. Conclusion

The two proposed methods are suitable for the quality control of melatonin in galenic tablets. The sample pretreatment is very rapid, consisting of a simple one-step extraction, filtration and dilution. The selectivity of the methods was sufficient, as no apparent interference from excipients was present in any assay. It should be noted that both methods gave favourable results with respect to repeatability, intermediate precision and accuracy. The RSD% values are all lower than 2.8%, and the recovery as a measure of the accuracy is close to 100% in all cases. The HPLC method in particular is more sensitive and selective than the voltammetric method, which is nevertheless much cheaper and more rapid.

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