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Voltammetric and flow amperometric methods for the determination of melatonin in pharmaceuticals

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Abstract

Melatonin can be sensitively detected in pharmaceuticals by two different and simple electrochemical methods: cyclic voltammetry (CV) and amperometric detection in a flow injection analysis system (FIA-ED). An adequate pre-treatment of the carbon paste electrode in the first case and the employ of a high flow rate in the second one were the key for obtaining a very good reproducibility (R.S.D. values of 1.5 ($n = 10$) and 1.3% ($n = 20$), respectively). Low limits of detection were achieved and with the coupling of a flow system a linear dynamic range of three orders of magnitude (from 10^{-8} to 10^{-5} M) was obtained. Both methods were applied to the determination of melatonin in pharmaceuticals. In order to best validate these methodologies a fluorescent procedure was developed to contrast the results. As no interferences from the matrix were found the employ of a separation technique is not necessary. In this way the procedure is fastened and simplified. Moreover, the low price, ease of handling, possibility of automation and high sample throughput are important advantages that convert the flow methodology in an attractive alternative for quality control of pharmaceuticals.

Keywords: Melatonin; Pharmaceuticals; Voltammetry; Amperometric detection; Flow injection analysis; Carbon paste electrodes

1. Introduction

Melatonin, *N*-acetyl-5-methoxytryptamine, is a hormone mainly synthesised in the pineal gland, which concentration depends on the cyrcadian rhythms. Most of melatonin is produced at night [1]. This hormone has influence on a variety of physiological and behavioural processes including neurological, psychiatric [2] and reproductive [3]. Disorders such as anxiety and seasonal depression are related to it [4]. It even may delay the aging process due to its ability to scavenge free radicals [5] and can also affect some genomic actions [6].

So, due to its importance in mammalian life, several analytical methods were developed for the determination of melatonin [7] in different matrices mainly pineal, plasma and pharmaceuticals. Most of them, due to the complexity of the matrix

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or the simultaneous determination of several compounds include a separation method. Although gas chromatography has been employed coupled to electron impact mass spectrometry [8], high performance liquid chromatography is the most common. In the last case the detection is fluorometric [9–12] or electrochemical [13–18]. A serial flouorimetric–electrochemical detection was employed as a convenient way of increasing confidence in compound identity and purity of six indolic compounds, including melatonin [19]. In this case, detection limits for this hormone were 60 and 135 pg per gland for fluorometric and electrochemical detection, respectively. Both methods of detection were compared for the determination of the serotonin *N*-acetyltransferase activity [20], a key regulatory enzyme in the melatonin biosynthetic pathway. A major advantage of the electrochemical detection method, relative to the fluorometric, was the lower limit of detection. The sensitivity of this principle of detection has extended its use coupling it also to high performance capillary electrophoresis [21,22]. However, a simple and cheap flow electrochemical methodology has never been reported in the bibliography for melatonin determination although many examples of its use in pharmaceutical analysis can be found [23–25]**.** Taking into account that separation is not always necessary, the price and maintenance of chromatographic systems could be avoided. Moreover, analysis time can be greatly reduced due to the high sample throughput of flow systems. Possibility of automation is also important when dealing with pharmaceutical analysis.

Graphite electrodes maintained at +0.85 or + 0.90 V (vs. Ag/AgCl) were commonly used in electrochemical detection. In the case of capillary electrophoresis the diameter of the carbon electrode is reduced to 500 [21] or 33 μm [22]. The feasibility of analysing this hormone in vivo was tested voltammetrically employing 12 μm carbon fibre microelectrodes [26]. Voltammetric determination of drugs in pharmaceutical products is by far the most common electroanalytical technique. Sample preparation usually consists in dissolving the active component from a particular formulation in a suitable solvent and performing a direct analysis on an aliquot of this solution. The selectivity of this method is normally excellent because the analyte can be readily identified by its voltammetric peak potential.

Carbon paste electrodes, introduced by Adams in 1958 [27] and composed by a mixture of graphite powder and a non-conductive organic liquid, have been widely used for the determination of analytes that are susceptible of suffering oxidation processes [23,24,28,29]. These electrodes have been employed for the pre-concentration of indolic compounds and their determination in the concentration range 1–8 μM [30]. Melatonin has been determined in capsules [25], the methodology having a limit of detection of 2.3×10^{-6} M and a relative standard deviation (R.S.D.) of 3.5% for five repetitive measurements. The use of a pasting liquid with more adsorptive properties allowed a decrease in the limit of detection (10^{-9} M) and a similar R.S.D. (3.8%) was obtained [31]. The linear dynamic range was comprised between 5×10^{-8} and 10^{-7} M and the method was applied to serum samples.

In this paper, an adequate pretreatment of the carbon paste electrode has allowed an excellent reproducibility of voltammetric measurements, the methodology being applied to pharmaceutical samples. Moreover, a simple flow methodology for the determination of this hormone is described and discussed. Lower detection limit, wider linear dynamic range (three orders of magnitude) and a similar reproducibility without any pretreatment of the electrode were obtained. Its price and ease of handling together with high sample throughput makes this methodology greatly interesting for the analysis of melatonin in matrices such as pharmaceuticals.

2. Experimental

2.1. Reagents

Stock solutions of melatonin (Sigma, St. Louis, MO, 63178) were prepared monthly in 0.1 M perchloric acid (Merck, D-6100 Darmstadt, Germany). Before being transferred to a volumetric flask the initial solution is heated at 50 °C for 45 min in order to favour the solubility. Once

prepared, is stored at 4 °C protected from light. Dilutions were daily made in 0.1 M perchloric acid.

In case of fluorescent measurements melatonin standards were freshly prepared in ethyl acetate (Merck) and kept in darkness at less than 0 °C before use.

Britton–Robinson buffers, prepared from an acid mixture (acetic (Merck), phosphoric (Merck) and boric (Panreac, E-08110 Barcelona, Spain) acids) and sodium hydroxide (Merck), covering the pH range 2–11 were used for pH dependence studies as well as 0.1 M perchloric acid and 0.1 M sodium hydroxide.

Carbon paste was monthly prepared by intimately mixing 0.36 ml of paraffin oil (Uvasol, Merck) with 1 g of spectroscopic grade graphite powder (Ultracarbon, Dicoex, Bilbao). The paste was stored at 4 °C protected from light.

Water was purified by distillation and deionization in Milli-R and Milli-Q (Millipore, Bedford, MA 01730) systems, respectively. All other reagents were of analytical reagent grade.

2.2. Instruments

Cyclic voltammetry (CV) was performed with an Autolab PGSTAT 10 (Eco Chemie) potentiostat interfaced to an ADL Pentium 120 computer system and controlled by Autolab GPES software version 4.6 for WINDOWS 98.

All the measurements were carried out at room temperature in a 10 ml Metrohm cell with a three-electrode configuration. A working carbon paste electrode, with a surface diameter of 3 mm, was made of a hollow PTFE tube in which an inserted stainless steel rod acted as the electric contact between the carbon paste and the potentiostat. At the same time, it acts as a piston that allows renewing the surface what it is made manually by smoothing it on a clean paper before use. Commonly, the carbon paste of the electrode is daily renewed.

A platinum wire served as auxiliary electrode and a Ag/AgCl/saturated KCl electrode was used as reference. An Asincro magnetic stirrer was used in some steps of the procedure.

The FIA apparatus consisted of a 16-cylinder Perimax Spetec peristaltic pump, with tubing obtained from Elkay Eireann (Galway, Ireland) and made from PVC (1.148 mm i.d.), a six-port rotary valve purchased from Omnifit (Cambridge, UK) equipped with a 100 μl loop and a home-made thin layer flow cell constituted by two methacrylate blocks fixed together with four strews. A carbon paste electrode of 7.1 mm^2 of geometric area was included. A coupled downstream compartment contained the reference electrode (Ag/AgCl/saturated KCl) and a stainless steel waste tube acting as auxiliary electrode. Connectors and tubing (0.8 mm i.d.) were obtained from Omnifit.

A Metrohm VA-641 potentiostat was used for the detection and its output signals were recorded on a L6012B Linseis recorder.

Fluorescence was measured using a Perkin–Elmer (LS-50B) luminiscence spectrometer (Buckinghamshire, England), which has a Xenon discharge excitation source. Instrumental parameters and processing data were controlled by the FLUORESCENCE DATA MANAGER software.

A Micro-pH 2001 Crison pH-meter was also employed.

2.3. Procedures

2.3.1. Electrode activation

In cyclic voltammetric experiments the electrode was kept in a stirred solution (500 rpm in 0.1 M $HClO_4$) for 30 s with electrolysis at +1.60 V. Potential was then changed to +0.65 V and maintained for 30 s under stirring. Afterwards, stirring was switched off and the solution was allowed to quiesce for 15 s before the potential scan was started.

2.3.2. Sample preparation

2.3.2.1. Electrochemical method. A tablet was pulverised with the help of a mortar and dissolved in 0.1 M perchloric acid by heating (50 °C) for 45 min. After filtering through an Albet 145 filter paper ($\phi = 7$–11 μm) the filtrate was quantitatively transferred to a flask and volume was made up to

500 ml with 0.1 M perchloric acid. The solution was analysed the same day of preparation.

2.3.2.2. Fluorometric method. A tablet was pulverised with the help of a mortar, dissolved in ethyl acetate and stirred at 700 rpm for 2 min. After filtering through an Albet 145 filter paper ($\phi = 7$–11 μm) the filtrate was quantitatively transferred to a flask and volume was made up to 100 ml with ethyl acetate. The solution was freshly prepared before use. Dilutions were also made in ethyl acetate, kept away from light and rapidly processed to prevent melatonin decomposition.

2.3.3. Voltammetric measurement

After carrying out the electrode pretreatment the potential was scanned at 50 mV s^{-1} between +0.65 and +0.9 V. Solution was then stirred at 500 rpm for carrying out the electrode pretreatment and next measurement. The height at the maximum of the peak constitutes the analytical signal.

2.3.4. Amperometric measurement

Once the carrier flowed adequatly at 3 ml min^{-1} the electrochemical cell was connected and the potential of the working electrode was maintained at +0.85 V. When the baseline was stable, the loop of the valve ('load' position) was loaded with a solution of melatonin with the help of a syringe. By changing the valve position to 'inject' the content of the loop was inserted into the system. The height at the maximum of the fiagram constitutes the analytical signal. Next injection was made when the baseline attains the initial value.

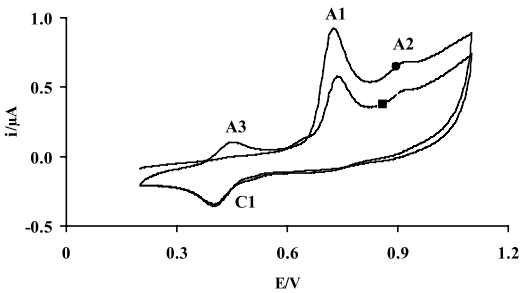

Fig. 1. Cyclic voltammograms recorded in a 10^{-5} M solution of melatonin (●) first and (■) second scan ($v = 100$ mV s^{-1}).

2.3.5. Fluorometric measurement

Once the sample was ready and after standard solutions preparation in ethyl acetate, quantitation is achieved by the standard addition method, in such a way that final concentrations are included between 10 and 100 ng ml^{-1}. The procedure was similar to a previous one found in the literature [32]. In our case the excitation wavelength was set at 285 nm and the height at the maximum in the emission spectrum at 336 nm constituted the analytical signal. The slit width was 5 nm.

3. Results and discussion

3.1. Cyclic voltammetry

Fig. 1 shows two consecutive cyclic voltammograms obtained in a 10^{-5} M solution of melatonin in 0.1 M $HClO_4$. Two well-defined oxidation processes at +0.76 (A_1) and +0.93 V (A_2) were obtained in the first scan together with a reduction process at +0.38V (C_1). Last process together with an anodic peak that appeared in the second scan at +0.42 V (A_3) forms part of a reversible pair. This was observed whenever the first anodic scan achieved the potential of the A_1 process. Attaining the A_2 potential was not necessary. This suggested that the oxidation product of the melatonin obtained at +0.76 V was responsible of the pair. This was in accordance to the experiments carried out for postulating an oxidation mechanism [30]. A first one-electron abstraction process generates a radical cation that is further oxidised by the loss of a second electron and proton to a quinoneimine, which is susceptible to nucleophilic attack. The attack by water would yield a 4,5-dihydroxy indolic derivative, which is in equilibrium with its oxidised form (quinone).

The behaviour of melatonin was examined in a 10^{-6} M solution with varying pH over a wide range of values from acidic (0.1 M $HClO_4$) to alkaline (0.1 M NaOH). For intermediate values Britton–Robinson buffers with pH comprised between 2 and 11 were employed. Several conclu-

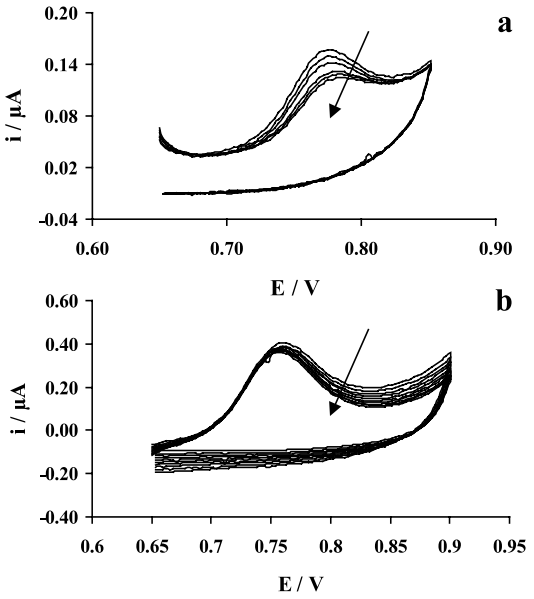

Fig. 2. (a) Six successive cyclic voltammograms obtained without electrode activation ($v = 100$ mV s^{-1}). (b) Ten successive cyclic voltammograms obtained after following an electrode pre-treatment ($v = 50$ mV s^{-1}). In both cases they were recorded in a 10^{-6} M solution of melatonin in 0.1 M $HClO_4$.

sions were obtained: after pH 3 A_1 and A_2 processes overlapped moving its potential to less positive values with increasing pH showing a linear relationship (E_p vs. pH) with a slope of −48 mV per pH unit. On the other hand, peak current decreased progressively with pH. Concerning the C_1–A_3 redox pair, it was only observable at pH values less than 4. As in the case of the other processes and since a loss of protons occurs the increase of pH favours the oxidation showing a decrease in potentials (slope of the linear equation: −65 mV per pH unit).

$HClO_4$ (0.1 M) was chosen as background electrolyte for the remainder of the work since best definition and higher intensity of the process A_1, the one selected for monitoring melatonin, were obtained.

The previously commented pH study was made renewing the carbon paste of the electrode for each solution. Repetitive scans recorded without renewing the carbon paste in a 10^{-6} M solution of melatonin showed a decrease in the peak current (Fig. 2a) due to the adsorption of products on the electrode surface. A 31% of decrease in the peak intensity was obtained from the first to the sixth scan. This phenomenon that was explained in earlier works [24,29] could be overcome by activating the electrode at high anodic potentials. The adsorbed organic layer can be then displaced by oxidative changes produced in the graphite surface. Potentials ranging from +1.1 to +1.6 V were applied from 30 to 60 s to the electrode under stirring. Best results were obtained following the procedure described under Section 2 (+1.60 V, 30 s). As can be seen in the voltammograms of the Fig. 2b peak currents were highly reproducible. The R.S.D. was 1.5% for $n = 10$. The use of this procedure meant that it is not necessary to renew the electrode surface every time a measurement was made thus decreasing analysis time and increasing precision. This supposed an important advance on previous papers where an intermittent renewal of the electrode had to be made, what constituted a major drawback to the procedure [25] or the electrode had to be transferred to a blank electrolyte solution where a series of cyclic scans were carried out until a voltammogram corresponding to the residual current was obtained [31]. The R.S.D. reported for five repetitive measurements were 3.5 and 3.8%, respectively.

The effect of scan rate on the peak current was studied in order to elucidate the character of the process. When scan rate was varied from 10 to 100 mV s^{-1} in a 10^{-6} M solution of melatonin, a linear dependence of the peak intensity upon the square root of the scan rate was found, demonstrating a diffusional behaviour. The equation that relates both variables is:

$$i_p\ (\text{nA}) = 24.66 v^{1/2}\ (\text{mV s}^{-1})^{1/2} - 60.03,$$
$$r = 0.998, \quad n = 10$$

The existence of an intercept could means the presence of an adsorptive component in the process of melatonin. In order to confirm this, the linearity between log i_p and log v was studied. The equation obtained is:

$$\log(i_p)\ (\text{nA}) = 0.893 \log(v)\ (\text{mV s}^{-1}) + 0.516$$
$$r = 0.997, \quad n = 10$$

The slope deviation of the value predicted by the theory [33] (0.50) for irreversible and diffusional in nature processes, confirm the existence of this adsorptive component.

The relationship between the peak current and the concentration was linear from 6.0×10^{-8} to 8.0×10^{-7} M. The study was carried out using a scan rate of 50 mV s^{-1}. Linearity was expressed by the following equation:

$$i_p\ (\text{nA}) = 40.9C \times 10^{-7}\ (\text{M}) - 2, \quad r = 0.999, \quad n = 6$$

here, S.D. of slope = 0.9; S.D. of intercept = 4.1.

Limits of detection and quantitation, calculated as the concentration corresponding to a signal that is three and ten times, respectively, the standard deviation (S.D.) of the intercept estimate, were found to be 3.0×10^{-8} and 1.1×10^{-7} M.

3.2. Flow injection analysis with amperometric detection

The possibilities of employing a flow injection analysis for improving the analytical characteristics of the methodology were studied. The flow system employed was specified under Section 2. The background electrolyte chosen for cyclic voltammetric studies, 0.1 M $HClO_4$ was now the carrier stream. With the aim of fixing the working potential a hydrodynamic voltammogram was constructed varying it from +0.6 to +1 V. Injections of 100 μl of a 10^{-6} M solution of melatonin were made. For each condition, measurements were taken by triplicate. Since at +0.85 V the maximum velocity of mass transfer was attained and the intensity was not affected by background electrolysis this potential was chosen for further studies.

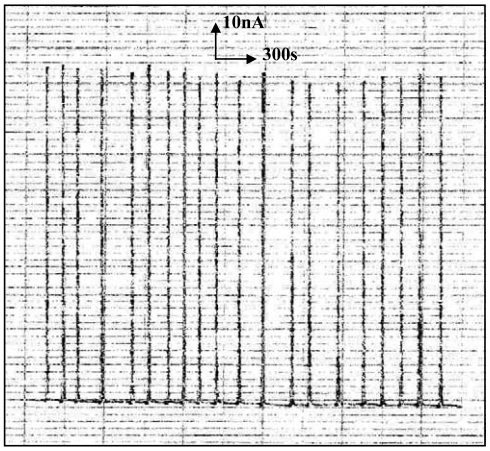

Fig. 3. Twenty successive fiagrams corresponding to a 10^{-6} M solution of melatonin (carrier stream: 0.1 M $HClO_4$, $E = +0.85$ V, $v = 3$ ml min^{-1}, $V_l = 100$ μl).

A parameter that has to be optimised in a flow system is the flow rate. This was varied from 0.5 to 4.5 ml min^{-1} obtaining higher intensities with higher flow rates until 2.5 ml min^{-1} where a plateau is attained. Three ml min^{-1} was selected as the most appropriate since high flow rates produced short analysis time and improved the cleaning of the electrode surface.

An important drawback of solid electrodes as commented in CV is the lack of a renewable electrode surface, influenced by adsorption processes. In the case of flow systems, the continuous passage of the carrier stream could produce an effective cleaning being not necessary an activation of the electrode [23,34,35]. This, in turn, saves time because electrode treatment stages can be omitted. The flow rate and the composition of the carrier stream could help to this cleaning. For melatonin, the passage of the 0.1 M $HClO_4$ stream at 3 ml min^{-1} was enough for ensuring an adequate repetitivity of the signals. No treatment of the electrode was necessary. This is shown in the fiagrams of the Fig. 3, corresponding to successive injections of a 10^{-6} M solution of melatonin recorded under the same conditions. The R.S.D. was 1.3% for $n = 20$.

In order to know the dynamic linear range of the methodology, solutions with increasing concentrations of melatonin were injected. A linear range as wide as three orders of magnitude, from 10^{-8} to 10^{-5} M was obtained. The equation of the calibration curve is reported below:

$$i_p\ (\text{nA}) = 9.79C \times 10^{-7}\ (\text{M}) + 5, \quad r = 0.9997, \quad n = 10$$

here, S.D. of slope = 0.007; S.D. of intercept = 2.7.

Table 1
Results obtained for the voltammetric and amperometric determination of melatonin in pharmaceuticals

Capsule	CV (mg)	FIA-ED (mg)
1	0.992±0.003	1.00±0.02
2	1.00±0.02	0.99±0.02
3	1.00±0.03	1.00±0.02

Limits of detection and quantitation calculated as in voltammetric experiments were 8×10^{-9} and 3.6×10^{-8} M, respectively.

3.3. Analytical application

The cyclic voltammetric response of the melatonin and the amperometric in the flow system were used for its determination in pharmaceuticals. The last one provides a more rapid and sensitive methodology but taking into account that pharmaceutical laboratories can be supplied with only one of both, the determination was carried out by the two principles. Melatonin capsules (Schiff® Products, Inc., Salt Lake City, UT 84104) with a nominal value of 1 mg and also containing 2 mg of vitamin B_6 together with calcium carbonate, maltodextrin, cellulose, croscarmellose sodium and magnesium stearate as other ingredients were treated as stated under Section 2. When a cyclic voltammogram is recorded in a sample solution no more processes different to that of melatonin appear, what indicates the absence of interferences from the sample matrix. Therefore, the flow methodology results adequate since the signal obtained at +0.85 V comes from melatonin and so a fast, simple and cheap procedure can be applied.

Three capsules were analysed by CV, each one by triplicate. With this aim, 75 μl of the sample were added to the cell containing 10 ml of 0.1 M $HClO_4$. Quantitation of the hormone was achieved by the standard addition method. In the case of the FIA-ED three 1:5 dilutions with 0.1 M $HClO_4$ were prepared for each capsule. They were injected in the system recording the signal by triplicate and referring the value to a calibration curve.

The results obtained following both methodologies are given in Table 1. In Fig. 4 voltammograms and fiagrams corresponding to melatonin determinations in capsules are shown. A *t*-test was carried out to evaluate the absence of systematic errors comparing both values and taking the nominal value as the true one. Considering a level of confidence of 98% and 2 degrees of freedom it can be concluded that there are no systematic errors in this methodology.

In order to validate the proposed methodologies a simple melatonin determination based on fluorescence measurements was performed [32]. For this reason five tablets were treated as previously

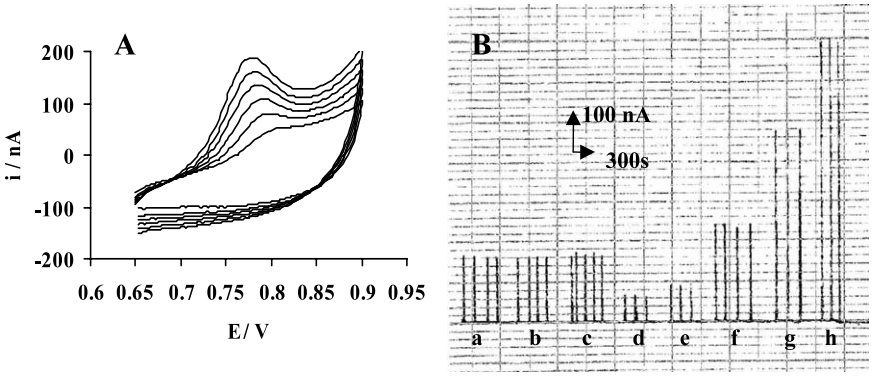

Fig. 4. (A) Cyclic voltammograms corresponding to melatonin determination in an aliquot of a capsule following the standard addition method (additions of 6×10^{-8} M, $v = 50$ mV s^{-1}). (B) Fiagrams corresponding to melatonin determination in three aliquots (a, b, c) of a capsule employing a calibration curve (d, 0.75×10^{-6}; e, 1×10^{-6}; f, 2.5×10^{-6}; g, 5×10^{-6}; h, 7.5×10^{-6} M; carrier stream: 0.1 M $HClO_4$, $E = +0.85$ V, $v = 3$ ml min^{-1}, $V_1 = 100$ μl).

Table 2
Analytical characteristics for melatonin determination with different electrochemical techniques

Electrochemical technique	R.S.D. (%)	Sensitivity (μA M^{-1})	Limit of detection (M)	Linear dynamic range (M)
CV	1.5, $n = 10$	$\mathbf{4.09 \times 10^5}$	$\mathbf{3 \times 10^{-8}}$	6×10^{-8}–8×10^{-7}
FIA-ED	**1.3, $n = 20$**	9.80×10^4	8×10^{-9}	$\mathbf{1 \times 10^{-8}}$–$\mathbf{1 \times 10^{-5}}$
CV[a]	3.5, $n = 5$	–	2×10^{-6}	3×10^{-6}–5.5×10^{-4}
DPV[b]	3.8, $n = 5$	1.66×10^5	$\mathbf{1 \times 10^{-9}}$	5×10^{-8}–1×10^{-7}
CE-ED[c]	2.8, $n = 7$	1.11×10^2	1×10^{-6}	2.5×10^{-6}–1×10^{-3}

[a] [25].
[b] [31].
[c] [22].

described and the hormone quantitation was achieved by the standard addition method. The value obtained for these five capsules was 0.99 ± 0.15 mg. To evaluate the absence of systematic errors this value and the nominal one (1 mg) were again compared. The t-test considering a level of confidence of 98% and 4 degrees of freedom revealed that no systematic errors were present in this methodology.

Finally electrochemical and fluorometric results were statistically compared following another t-test. This revealed that there was no significative difference between the average values obtained with both procedures.

4. Conclusions

Two simple, fast and cheap different electrochemical methodologies that employ carbon paste electrodes have been applied to the determination of melatonin in capsules. CV and flow injection analysis with amperometric detection have proved to be adequate for carrying out quality control of pharmaceuticals. In Table 2 analytical characteristics of the methodologies here proposed and other reported in the bibliography employing different electrochemical techniques (using a carbon paste or carbon fibre electrode as working electrode) were compared. In summary, the widest linear ranges and best reproducibility were achieved by amperometry in a flow injection system. However, lower limit of detection and higher sensitivity were obtained when differential pulse and CV (with electrode pretreatment) was employed, respectively. The reproducibility of the current signal was excellent in the methodologies proposed which is based on an adequate pretreatment of the carbon paste electrode and the use of high flow rates for the first and second methods, respectively. Since no interferences coming from the sample matix have been found the employ of a flow system with amperometric detection results extremely adequate in order to reduce price, time and complexity of the common procedures. To best validate the results obtained a fluorescent procedure was also employed.

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